MOLECULAR AND BIOCHEMICAL STUDIES ON MISFOLDED PROTEIN IN PREECLAMPSIA

Thesis submitted to
Sri Devaraj Urs Academy of Higher Education and Research

in partial fulfillment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY

in

CELL BIOLOGY AND MOLECULAR GENETICS
under the Faculty of Allied Health and Basic Sciences

by

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March 2020

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ACKNOWLEDGEMENT

It is with profound respect and gratitude I wish to express my gratefulness to **Dr. B. Sharath.,** Associate Professor, Department of Cell Biology and Molecular Genetics, Sri Devaraj Urs Academy of Higher Education, Tamaka, Kolar, for his able guidance, valuable suggestions, and encouragement throughout my work.

I wish to express my gratefulness and thanks to **Dr. S. R. Sheela.,** Professor and Head, Department of Obstetrics and Gynecology, for having allowed me to collect the sample for the study.

I wish to express my sincere thanks to **Mr. S. Ravishankar.,** Assistant Professor, Community Medicine, for his invaluable help in statistical analysis and evaluation of the data.

I record my sincere gratefulness and thanks to **Dr. A.V. Moideen Kutty.,** Pro-Chancellor, for his moral support, keen interest, timely help during my research.

I express my **s**incere gratitude to **Dr. G. Pradeep Kumar.,** Vice-Chancellor, for constant encouragement and support during the course of my study.

I sincerely thank **Dr. K.N.V. Prasad.,** Registrar, for his constant encouragement and help during the period of my research.

I also express my sincere thanks to **Dr. R. Kalyani.,** Professor and Head, Department of Pathology, for allowing me to use ELISA Reader in the Central

Diagnostic Laboratory. My thanks are also due to **Mr. Muniraaju.,** Laboratory Technician, for his valuable help.

I wish to express my sincere thanks to **Dr. P. R. Krishnaswamy.,** Scientific Advisor, for useful suggestions and constant encouragement.

I record my heartfelt thanks to **Dr. Venkateswarlu Raavi.,** Assistant Professor, for his constructive suggestions and valuable help.

I sincerely thank **Dr. Mitesh Shetty.,** Genetic Counselor, who had helped me with his valuable suggestions and **Dr. P. Kiranmayee.,** Assistant Professor**,** for constant support and advice.

I wish to express my sincere thanks to **Dr. M. Jayaram.,** Professor and Head, Department of Speech and Audiology, for his encouragement and support.

I am thankful to **Dr. C.D. Dayanand.,** Professor, Department of Biochemistry, for useful suggestions and support.

I wish to express my sincere thanks to all non-teaching staff of the Department of Cell Biology and Molecular Genetics, for their timely help and co-operation.

I am thankful to all the faculty members of the Department of Obstetrics and Gynaecology, nursing staff and postgraduate students of R. L. Jalappa hospital for extending all the help required. I am deeply grateful to all the participants in this study, who by giving the samples and personal information, have made it possible to complete the study.

My thanks are also due to my fellow research scholars of the Department of Cell Biology and Molecular Genetics for their timely help and co-operation.

I sincerely thank the management of Sri Devaraj Urs Academy of Higher Education and Research and Sri Devaraj Urs Medical College for allowing me to do Ph.D. in this esteemed institution.

Last but not the least, I am grateful to my Father **Dr**. **C.S. Nagarajappa**, for all encouragement and support.

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LIST OF ABBREVIATIONS

A1AT	Alpha-1 Antitrypsin
ACOG	American College of Obstetrics and Gynaecology
BSA	Bovine Serum Albumin
CKD	Chronic Kidney Disease
COPD	Chronic Obstructive Pulmonary Disease
CRD	Congo Red Dot
CRR	Congo Red Retention
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribo Nucleotide Triphosphate
dsDNA	Double Stranded DNA
EDTA	Ethylenediaminetetraacetic acid
ELB	Erythrocyte Lysis Buffer
ELISA	Enzyme Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
HELLP	Hemolysis Elevated Liver Enzymes Low Platelet counts
IgG k	Immunoglobulin G Kappa
IQR	Inter Quartile Range
IUGR	Intrauterine Growth Restriction
$MgCl_2$	Magnesium Chloride
NaCl	Sodium Chloride
NCBI	National Center for Biotechnology Information
PBS	Phosphate Buffer Saline

PCR	Polymerase Chain Reaction
PE	Preeclampsia
PiS	Protease Inhibitor S allele
PiZ	Protease Inhibitor Z allele
PlGF	Placental Growth Factor
RFLP	Restriction Fragment Length Polymorphism
SERPINA1	Serine Proteinase Inhibitor, Clade A, member 1
sFLT-1	Soluble Fms-Like Tyrosine kinase-1
SIFT	Sorting Intolerant from Tolerant
SPSS	Statistical Package for Social Sciences
Taq	*Thermus aquaticus*
TE	Tris-EDTA
UPP	Ubiquitin Proteasome Pathway
UPR	Unfolded Protein Response
UV	Ultra-Violet
VEGF	Vascular Endothelial Growth Factor

Abstract

Abstract

Preeclampsia (PE) is a common complication of pregnancy involving new-onset hypertension and proteinuria. PE is one of the major causes of perinatal morbidity and mortality. There is no treatment for PE other than terminating the pregnancy. Therefore, understanding the pathophysiological basis is important to uncover therapeutic targets and also to develop diagnostic and prognosis markers. Recent studies have shown that PE is a protein misfolding disorder. There are indications that the urinary misfolded proteins can be used as a biomarker for the diagnosis and prognosis of PE. Furthermore, several drugs that are under development for modulating the protein folding pathway may be repurposed for the treatment of PE.

The aim of this study was to examine the role of protein misfolding in PE. The objectives of this study were to validate the association between protein misfolding and PE in Indian population and then explore the role of protein oxidation and gene mutations in protein misfolding.

A case-control study was conducted by involving 65 normotensive pregnant women and 63 PE women. Midstream urine samples were collected for protein misfolding and oxidation studies. Blood samples were collected for mutation analysis. Protein misfolding was determined by Congo red dot assay. Protein carbonyl was chosen as a marker for protein oxidation and it was measured by ELISA technique. Misfolding linked mutations of the Serine Proteinase Inhibitor, Clade A, member 1 (*SERPINA1)* gene was selected for genetic studies as the product of this gene *viz*., Alpha-1 Antitrypsin (A1AT) is misfolded in PE. Mutations in *SERPINA1* gene were determined by PCR-RFLP method.

Abstract

Protein misfolding was significantly higher in PE women than in normotensive pregnant women ($p < 0.001$). Furthermore, clinical variables of PE such as gestational age of onset, severity, and co-morbidities did not alter the elevation. Protein carbonyl level was significantly higher in the PE women than in the normotensive pregnant women ($p = 0.002$). The correlation between protein misfolding and protein carbonyl level was significant but weak ($r = 0.3$). *SERPINA1* gene mutations were absent in all the PE women.

The overall results of this study support the conclusion that protein misfolding is associated with PE and it arises mostly due to protein oxidation and unlikely due to mutations in *SERPINA1* gene.

INTRODUCTION

Preeclampsia is a common complication that affects 2-8 % of pregnancies worldwide (Ghulmiyyah and Sibai, 2012). It is characterized by *de novo* hypertension and other features *viz*., proteinuria, pulmonary edema, new-onset renal insufficiency, cerebral or visual disturbances (ACOG, 2013; Brown *et al*., 2018). PE is associated with risk factors such as primigravida, family history, placental abnormalities, obesity, pre-existing vascular disease, and thrombophilia (Dutta, 2013).

PE is the major cause of maternal and fetal morbidity and mortality (Steegers *et al*., 2010; Mayrink *et al*., 2018). Women who develop PE in first pregnancy have increased risk of developing PE in subsequent pregnancies (Dukler *et al.*, 2001; Makkonen *et al.*, 2000; Langenveld *et al*., 2011; Bramham *et al*., 2011). It is one of the leading causes of maternal and fetal complications during pregnancy explicitly in low to middle income countries (McCarthy *et al.*, 2018; Firoz, 2011; Duley, 2009). Also, women with PE are likely to manifest cardiovascular diseases following pregnancy (ACOG, 2013; Wu *et al*., 2017; Thilaganathan and Kalafat, 2019; Lisowska *et al*., 2018; Valdes, 2017). If left untreated, it can cause adverse effects particularly, in low resource settings (Mol *et al.,* 2016). It is difficult to predict which women are likely to develop PE in the later stage of pregnancy since PE is associated with risk factors. Once diagnosed, there is no effective treatment other than the delivery of fetus or termination of pregnancy (Pennington *et al*., 2012; Romero and Chaiworapongsa, 2013; Jayabalan, 2013). Hence, accurate identification of pregnant women at risk is necessary for closer monitoring and timely intervention (Townsend *et al*., 2016).

Inadequate development of the placental vasculature is the main event in the pathogenesis of PE (Reynolds *et al*., 2010; Woods *et al*., 2018; Reynolds and Redmer, 2001; Granger *et al*., 2001), which is reported by various clinical, pathological and experimental findings (Roberts and Gammill, 2005). In PE, inadequate and incomplete transformation of maternal spiral arteries occurs as a result of shallow invasion of trophoblasts (Goldman-Wohl and Yagel, 2002; Silva and serakides, 2016; Van den Brule *et al*., 2005; Fisher, 2015; Kaufmann *et al*., 2003; Carter *et al*., 2015). This eventually results in reduced perfusion of the placenta (Roberts and Escudero, 2012; Natale *et al*., 2018; Verlohren *et al*., 2010). Reduced perfusion of the placenta further deteriorates the physiological conditions of the maternal-fetal interface and leads to deficiency in the blood flow to the fetus (Rana *et al*., 2019; Phipps *et al*., 2016; Vanwijk *et al.*, 2000). Angiogenic factors appear to play an important role in the abnormal vascular development of the placenta (Cheng and Zheng, 2014; Bardin *et al*., 2015). The two major factors in this connection are placental growth factor (PlGF) and soluble fms-like tyrosine kinase 1 (sFLT-1) (Karumanchi, 2016; Shibata *et al*., 2005). PlGF is a pro-angiogenic factor that belongs to the vascular endothelial growth factor family (VEGF) (Chau *et al*., 2017; Nejabati *et al*., 2017; Falco *et al*., 2002). It is secreted by placental trophoblast and plays an important role in trophoblast invasion and differentiation. In contrast, sFLT-1 is an anti-angiogenic protein since it binds to PIGF and reduces its bioavailability (Eddy *et al*., 2018). The balance between PlGF and sFLT-1 thus controls the placental vascular development (Herraiz *et al*., 2015; Palmer *et al*., 2016; Radulescu *et al*., 2016). The levels of sFLT-1 are higher in PE

thus results in an anti-angiogenic molecular environment (Abbas *et al*., 2018; Pant *et al*., 2019; Akhter *et al*., 2017; Tang *et al*., 2019; Karumanchi and Bdolah, 2004).

Reduced perfusion of the placenta results in the induction of endoplasmic reticulum stress (ER) (Veerbeek *et al*., 2015). It is characterized by an imbalance between the demand and capacity for protein folding (Karagoz *et al*., 2019; Oakes and Papa, 2015; Gross, 2006). There is no conclusive evidence on how the reduced perfusion results in the induction of ER stress. The increased load of secretory proteins, mutated proteins (Lin *et al*., 2008) and oxidatively damaged proteins (Malhotra *et al*., 2008) are some of the triggers for ER stress (Chaudhari *et al*., 2014). Protein folding itself is an oxidative process that generates reactive oxygen species (Gregerson and Bross, 2010). A higher load of secretory proteins may elevate the intraluminal levels of reactive oxygen species. Biosynthesis of polypeptide hormones, growth factors, membrane proteins occurs in the ER. Since these proteins play an important role in fetal development, ER stress leads to placental insufficiency (Hart *et al*., 2019). This condition is characterized by a reduced supply of nutrients to the fetus (Vinayagam *et al*., 2015). Eukaryotic cells respond to ER stress by activating a signal transduction pathway called as Unfolded Protein Response (UPR) (Boyce and Yuan, 2006; Kadowaki and Nishitoh, 2013; Lin *et al*., 2008; Wang and Kaufman, 2016). This response leads to the release of molecular chaperones and proteins that stimulate protein folding and degradation of misfolded proteins (Ciechanover and Kwon, 2017; Kriegenburg, 2012; Broadley and Hartl, 2009; Barral *et al*., 2004; Hartl *et al*., 2011; Kettern *et al*., 2010; Oakes and Papa, 2015; Liu *et al*., 2016). If the protein load in the ER is beyond its capacity

to process, then the ER leads to the activation of degradative mechanisms such as apoptosis and autophagy (Sano and Reed, 2013).

In addition to placenta, misfolded proteins have been shown in the urine and serum of PE women (Buhimschi *et al*., 2008; Kalkunte *et al*., 2013; Buhimschi *et al*., 2014; Cheng, 2014; Cheng *et al*., 2016; McCarthy *et al*., 2016; Jonas *et al*., 2016; Tong *et al*., 2017; Sammar *et al*., 2017). The presence of urinary misfolded proteins has spawned interest in exploiting it as a biomarker to diagnose PE. Further, pilot studies have shown that urinary misfolded proteins may appear well before the manifestation of clinical symptoms (Buhimschi *et al*., 2008; Buhimschi *et al*., 2014). If this is validated, then urinary misfolded proteins may be used as a biomarker for early identification of pregnant women who are at risk of developing PE.

Scope, Objectives and Justification

2.1. Scope of the study:

PE is a common complication of pregnancy that affects perinatal outcomes (Roberts *et al*., 2003). Till today, there is no clear idea about the pathophysiological origins of PE (Staff *et al*., 2013; George and Granger, 2011). Also, there is no specific treatment to cure PE. Therefore, understanding the pathophysiological origins of PE is necessary to uncover novel therapeutic targets and also prognostic markers. In this light, scientific literature for recent advances that may be useful for the said purposes was explored. Emerging evidence appears to indicate that PE may be linked to protein misfolding (Buhimschi *et al*., 2008; Kalkunte *et al*., 2013; Buhimschi *et al*.,2014; Cheng, 2014; Cheng *et al*., 2016; McCarthy *et al*., 2016; Jonas *et al*., 2016; Tong *et al*., 2017; Sammar *et al*., 2017). Presence of misfolded protein has been shown in the placenta, urine and plasma of PE women (Buhimschi *et al*., 2008; Kalkunte *et al*., 2013; Buhimschi *et al*., 2014; Cheng, 2014; Cheng *et al*., 2016; McCarthy *et al*., 2016; Jonas *et al*., 2016; Tong *et al*., 2017; Sammar *et al*., 2017). This is an important milestone in perinatal research since it justifies the addition of PE to the list of protein misfolding diseases. The presence of misfolded proteins in the urine is an important lead as it can be developed as a biomarker for the diagnosis and prognosis of PE (Buhimschi *et al*., 2008; Buhimschi *et al*., 2014; McCarthy *et al*., 2016). Also, several attempts are already being made to develop drugs that can restore the physiological functioning of the protein folding pathway in diseases such as Alzheimer's (Chadwick *et al*., 2012; Penke, 2017; Van Bulck *et al*., 2019; Cao *et al*., 2018). If protein misfolding plays a major role in the pathophysiological origins of PE, then these drugs can be repurposed for the treatment of PE. Therefore, we decided to first confirm the association of protein

misfolding with PE and then explore the possible causes for misfolding. These issues were addressed by formulating three objectives which are listed below.

2.2. Objectives of the study:

1. To evaluate the association between protein misfolding and preeclampsia.
2. To determine the correlation between protein oxidation and protein misfolding in preeclampsia.
3. To determine the frequency of PiS and PiZ mutations of *SERPINA1* gene in preeclampsia.

2.3. Justification for the study:

The association between urinary misfolded protein and PE has been established. However, two issues are yet unanswered. Firstly, the association has been established mainly in the western populations and there are no studies from the developing countries (Buhimschi *et al.,* 2014; McCarthy *et al.,* 2016). This is particularly significant given that the methods for the analysis of misfolded proteins are simple and therefore promises the highest benefit to maternal health management in resource-limited settings. Secondly, PE shows extensive heterogeneity in the clinical presentation in terms of the gestational age of onset, severity, and co-morbidities. Presently, there is no information on whether these clinical variables affect the association between urinary misfolded protein level and PE. The first objective of this study was aimed at filling this gap. This was achieved by evaluating the association between urinary misfolded protein levels and PE in Indian population.

Currently, there is no information about the factors responsible for protein misfolding in PE. The present study was aimed at addressing this gap in knowledge. This was achieved by evaluating factors that are strongly linked to protein misfolding. The two main reasons for protein misfolding are overexpression and loss of protein stability (Santucci *et al.,* 2008; Hartl, 2017). Some of the common factors that disturb the protein stability are oxidative damage and gene mutations. The second and third objectives of this study were aimed at examining the potential role of oxidative damage and gene mutations as the cause of protein misfolding in PE respectively.

Oxidative stress is a biochemical hallmark of PE (Aouache *et al*., 2018). This motivated the hypothesis that oxidative stress may be the cause of protein misfolding in PE. However, there was no information in the literature on the status of protein oxidation in the urine of PE women. Protein carbonylation, nitrotyrosine, dityrosine, and sulfonic acid are known protein oxidation markers. Among these protein carbonylation is an easily measurable and commonly used potential oxidative marker. Therefore, the association between protein oxidation and PE was determined first. Further, the correlation between protein oxidation and protein misfolding was evaluated to determine the causal relationship. Several important proteins have been identified in the urinary misfolded protein fraction of the PE women. Some of these are Alpha-1-Antitrypsin, ceruloplasmin, albumin, immunoglobulin free light chains, Alzheimer's β-amyloid and interferon-inducible protein 6-16 (Buhimschi *et al*., 2014). Analyzing the mutation status of all the corresponding genes was beyond the resources available for this study. Therefore, A1AT protein coding gene *SERPINA1* was selected for mutational analysis. The

mutations such as PiS and PiZ in *SERPINA1* gene were analyzed. The choice was supported by three reasons. Firstly, A1AT has been shown to be misfolded in the urine of PE women. Secondly, A1AT has been shown to ameliorate the symptoms of PE. Thirdly, PiS and PiZ are the two common mutations of *SERPINA1* gene that are responsible for the misfolding of A1AT protein.

Review of Literature

Proteins are the structural and functional molecules present in the cells and organelles of all the organisms. Proteins are synthesized on ribosome in the cell as native form, which undergoes a series of conformational changes (folding) to form an active and functional molecule. Transformation of the native form of protein to functional form is a complicated mechanism, which is a result of a series of fundamental steps and re-organization of the cellular and molecular interplay of proteins. Protein folding mechanism occurs in the endoplasmic reticulum once the translation is completed. Various mechanisms of protein folding and regulation via chaperons are acquired by organisms. Under extreme conditions, proteins may fail to fold properly and begins to misfold. Misfolded proteins are inclined to uncover the hydrophobic regions to the surrounding aqueous cellular environment wherein these regions are not exposed in case of native or folded proteins (Stefani, 2008; Shao, 2015). Oligomers formed during the aggregation process cause toxicity to cells. (Sengupta *et al*., 2016; Cheon *et al*., 2007) The chaperone role is linked with proteins in the course of oligomer formation, denaturation, and degradation via proteolysis. This provides support for proteins during unfolding when it undergoes stress, with the aid of protein quality control mechanism. When the protein quality control mechanism fails to do so, autophagy facilitates the elimination of protein aggregates (Hartl *et al*., 2011).

3.1. Protein folding and its significance:

Protein folding is a biological phenomenon wherein a polypeptide attains a specific native three dimensional structure through sequential conformational modifications. Protein folding occurs in a reproducible manner with a specific biological function. The sequence of amino acids has the necessary information for

the proper folding of the proteins. The transition of newly synthesized proteins into perfectly folded protein depends on distinct property of sequence of amino acids, side chains of amino acids, interactions, stabilization process and different contributing factors from the surrounding cellular environment (Englander *et al*., 2007). A similar type of protein may adopt distinct configuration and fold differently based on cellular location. Besides performing a series of biological activities, folding is associated with many other biological events such as regulation of cellular growth, differentiation, and transportation of molecules to specific cellular locations based on specific function (Diaz-villanueva *et al*., 2015). As the proteins are an integral part of every biological activity, their role varies from enzymes of chemical reactions to maintain the electrochemical potential across the cell membrane. Protein folding is a dynamic and rapid process, which can occur within milliseconds or seconds. Protein folding is associated with various types of bonds and interaction such as covalent bonds (peptide bond, disulfide) and non-covalent bonds (hydrogen bond, hydrophobic and ionic interactions, vander waals force). Hydrophobic force is the main driving force that appears to be responsible for the proper folding of the protein (Raschke *et al*., 2001; Chandler, 2005; Camilloni *et al*., 2016). As the bonds undergo free rotation in all directions, proteins will acquire an unlimited number of conformations.

The architecture of the proteins occurs through four levels of protein structure namely primary, secondary, tertiary, and quaternary level. The primary structure of a protein is made up of a unique linear sequence of amino acids of its polypeptide chain which is dictated by inherited genetic information. The sequence of amino acids in the polypeptide chain determines the shape and function of the

proteins. Each amino acid is joined together via a covalent peptide bond. The secondary structure of a polypeptide is organized into highly subspecific α helixes and β pleated sheets. They are held together by hydrogen bonds. Tertiary structure supports the 3-D shape of a protein. Tertiary structure is based on the interactions between the R groups of the amino acids that serve the protein. If this structure is disturbed, then the protein is said to be denatured which is chemically affected and its structure is distorted or disrupted. Quaternary structure is composed of multiple polypeptide chains, which are known as protein subunits. These subunits may consist of either homodimer or heterodimer. The quaternary structure of protein subunits interacts with one another, and assemble themselves to constitute a larger protein complex.

Significance of protein folding encompasses the preparation of proteins which have a novel or unique function, employing the specific native conformational structure of a protein in molecular drug design, utilizing the sequences for proteins to resolve the structure and shape of the protein and to prevent the protein misfolding diseases in human beings.

3.1.1. Protein folding theories:

Though countless investigators have reported the conformational structure of proteins, the pattern of the cardinal mechanism of protein folding is still controversial. Based on this, several hypotheses and protein folding theories such as anfinsen theory, levinthal theory, wetlaufer theory, diffusion-collision model, framework model, jigsaw-puzzle model, hydrophobic collapse model, nucleation condensation model and energy landscape and folding funnel were proposed

(Ahluwalia *et al*., 2012). List of different protein theories or models were given in Table 3.1.

Table 3.1: Proteins folding models with investigated proteins

Protein model	Protein investigated	Technique	Reference
Diffusion collision	B domain of protein A	Stopped flow CD and NMR hydrogen–deuterium exchange pulse labeling	Islam *et al.*, 2002
Nucleation condensation	hTRF1	Φ-value analysis, Molecular Dynamics (MD) simulations	Gianni *et al*., 2003
Diffusion model	Engrailed homeodomain	Φ-value analysis, MD simulations	Daggett and Fersht, 2003
Parallel folding pathway	Cytochrome c	Stopped Flow experiments	Gianni *et al*., 2003
Hydrophobic collapse	Bc-Csp cold shock protein	Forster Resonance Energy Transfer (FRET) kinetics	Magg and Schmid, 2004
Hydrophobic collapse	BphC enzyme	MD simulation	Zhou *et al*., 2004
Hydrophobic collapse	BBL (peripheral subunit binding domain	Ultrarapid double-jump laser-induced temperature jump, kinetics, Fluorescence	Welker *et al*., 2004
Hydrophobic collapse	TC5b	Photo CIDNP NMR	Mok *et al*., 2007
Nucleation condensation	CspB, CTL9, IM9, SpectrinR17, ubiquitin, SpectrinR16, apo-Azurin, FKBP12, IM7	ϕ-value analysis	Nolting and Agard, 2008

Hydrophobic collapse	Monellin	Time resolved -IR	Kimura *et al*., 2008
Diffusion collision	B1 domain of streptococcal protein G	MD simulations	Best and Hummer, 2011
Hydrophobic collapse	C-terminal domain of the Fas-associated death domain (Fadd-DD)	Quenched-flow hydrogen deuterium exchange (NMR)	Greene *et al*., 2012
Hydrophobic collapse	FSD-1	Action Derived Molecular Dynamics (ADMD) simulations	Lee *et al*., 2012

hTRF1: human telomeric repeat factor-1; **Bc-Csp**: Bacillus caldolyticus cold shock protein; **BphC**: Biphenyl-2,3-diol 1,2-dioxygenase C; **TC5b**: Trp-cage mini protein 5b; **CspB**: Cold shock protein B; **CTL9**: C-terminal domain of ribosomal protein L9; **IM7**: immunity protein 7; **FKBP12**: FK binding protein 12; **FSD1**: Full size D-1; **NMR**: Nuclear magnetic resonance; **CIDNP**: Chemically induced dynamic nuclear polarization

3.2. Protein misfolding:

Protein misfolding is a characteristic feature of a protein wherein the protein lacks the feature of attaining the native conformational structure. These misfolded proteins tend to deposit as large insoluble aggregates or plaques that are toxic to cells, tissues or organs both intracellularly and extracellularly (Grune *et al*., 2004; Stefani and Dobson, 2003; Aguzzi and Connor, 2010). Insoluble aggregates are also known as amyloids, which are abnormal and biologically inactive or non-functional. They often remain as unfolded or misfolded or partially folded proteins. Usually, misfolded proteins are eliminated by quality control mechanisms of cells such as proteasome complex systems and autophagy (Ding and Yin, 2008; Jackson

et al., 2016). When the misfolded proteins are overloaded, they become functionally inactive and the quality control systems of cells fail to degrade the misfolded proteins, which leads to protein misfolding diseases (Dobson, 2004; Blancas-Mejia and Ramirez-Alvarado, 2013; Jackson *et al*., 2016). Overview of the protein folding, misfolding and their degradation within the cells has been provided in Figure 3.1.

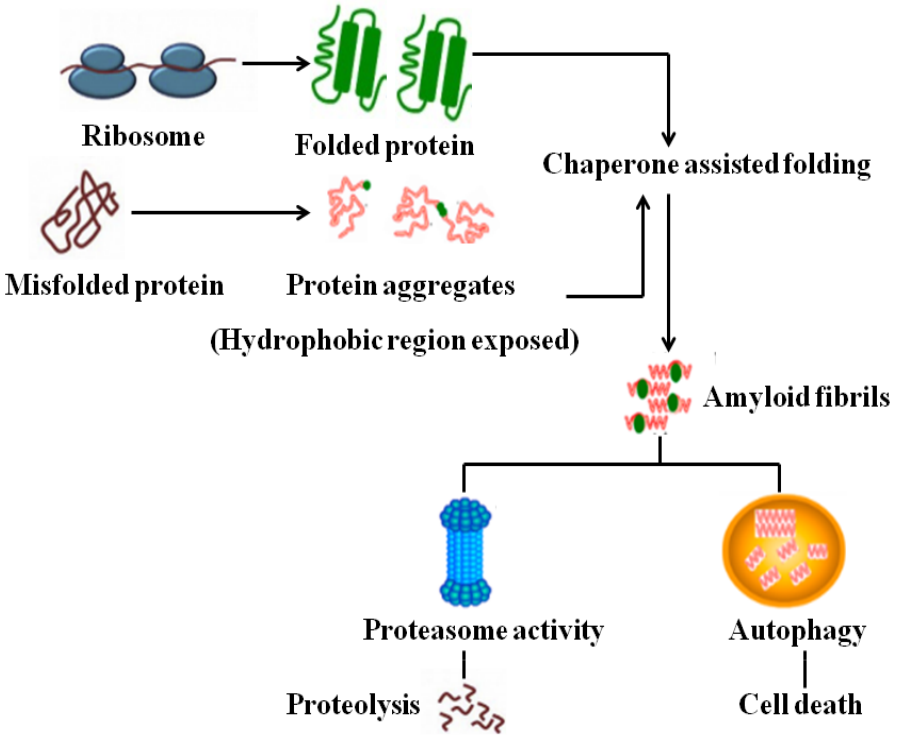


Figure 3.1: Protein folding, misfolding and degradation mechanism within the cell

3.3. Mechanism of protein aggregation:

Protein aggregation occurs through a multitude of events in a sequential and parallel manner. It occurs due to changes in internal conformation, protein-protein interactions, which leads to the formation of a dimer or oligomer. Further, it leads to subsequent aggregate growth due to sedimentation and limited solubility.

Usually, dimers and oligomers are formed by the integration of peptide and protein molecules, which further combines to form mature fibrillar amyloid aggregates (Ecroyd and Carver *et al*., 2009). Partially unfolded proteins that have distinct conformation produce aggregates. Protein aggregation is induced by intermolecular interactions of partially unfolded conformation of proteins (Krebs *et al*., 2000). For example, transthyretin (tetramer) gets disaggregated into monomer and leads to the amyloid formation (Ferreira *et al*., 2013; Yee *et al*., 2019). In the native state, factors responsible to induce protein aggregation slows down the process of aggregation. Once the native state is impaired that will initiate the protein aggregation through several factors such as pH, temperature, post-translational modification, mutation and oxidative stress (Alam *et al*., 2017). In the case of native unfolded proteins *viz, α*-synuclein and β-amyloid, protein aggregation is induced by factors such as high temperature and pH (Cohen *et al*., 2013). The following section summarizes the various mechanism of aggregation such as self-congregation of monomeric protein, accumulation of transformed monomeric protein, nucleation mechanism and surface promoted aggregation.

3.3.1. Self-congregation of monomeric protein:

Native monomers possess an innate tendency to form self-association in a reversible fashion (Philo and Arakava, 2009). Formation of reversible oligomers occurs through intermolecular interaction when they come in contact with the surface of native monomers. Consequently, reversible oligomers will lead to irreversible aggregates with the aid of covalent bonds (disulfide) when the protein concentration is elevated. Insulin which is a therapeutic protein is shown to reveal the reversible oligomer formation (Silva *et al*., 2018).

3.3.2. Accumulation of transformed monomeric protein (non-native):

The native monomer rarely loses the ability to assemble reversibly. Besides, transformed non-native monomer or the partially unfolded structure acquires the powerful inclination towards protein aggregates (Meric *et al*., 2017; Horwich *et al*., 2002). Therefore, the primary step in this event is a native state (conformational) to the non-native state (non-conformational), which differentiates it from the foregoing events. Aggregation may be induced by agents such as heat or shear forces, which further aids in the transformation of native monomer to adopt non-native state (Stathopulos *et al*., 2004).

3.3.3. Nucleation mechanism:

This mechanism is applicable when the genesis of observable aggregates or precipitate is required. During this phenomenon, the conformational monomer collapses to initiate events such as fibrillation and aggregation. Aggregates of different sizes tend to grow to a much greater extent by the addition of monomers gradually, which is termed as 'critical nucleus' (Gillam and Macphee, 2013). Usually, the mechanism of nucleation carries lag phase without possessing any apparent precipitate. Once the critical time is over, accumulation of many bulky species appears instantly for a longer period. This phenomenon is named as 'homogeneous nucleus' whereas the critical nucleus itself is aggregate of monomer products (Ghosh *et al*., 2016). However, the generation of 'heterogeneous nucleation' emerges as a result of species excluding the protein aggregates. Nucleation dependent oligomerization is a conventional method for the formation

of amyloids. In order to initiate the process, nucleus associated with the saturated protein solution outreaches the critical concentration of the amyloidogenic protein. Once the nucleus is developed, the fibril grows readily (Linse, 2017). The time interval required for the genesis of monomer and nucleus is known as the lag phase (Arasio *et al*., 2015). Seeding or allocation of nucleus to the saturated protein solution enriches the fibrillation mechanism by freezing the lag phase.

3.3.4. Surface promoted aggregation:

Surface promoted aggregation is conceived when the native monomer comes in contact with surface of the flask or container. Electrostatic interaction and hydrophobic interaction in an air liquid interface may facilitate the binding process in the container. Once the monomer binds to the surface, this may lead to conformational changes which will enhance the affinity for accumulation or aggregation either at the surface or when transformed monomer is released back to the solution (Philo and Arakava, 2009).

3.4. Factors promoting aggregation of protein:

Upon favourable environment, proteins fold into stable native conformation can produce amyloids both *in vitro* and *in vivo* by virtue of borderline cohesion and vigorous manner. The primary cause of amyloid deposition is protein unfolding or misfolding (Soto, 2013). Disruption in the protein quality control machinery or extrinsic agents facilitates the promotion of either misfolded or partially folded structures (Ciechanover and kwon, 2017). This is due to exposed hydrophobic regions and unstructured regions that are buried inside the folded conformation of protein (Yan *et al.,* 2007). In favour of safeguarding from exposure to the solvent,

the partially folded structures of proteins are subjected to hydrophobic collapse which leads to the generation of an invariable aggregated form (Jahn and Radford, 2008). Therefore, the aggregation mechanism is directed by hydrophobic forces. For instance, non-covalent interactions such as van der waals and electrostatic interaction propel the accumulation of amyloids in β-amyloid protein and monoclonal antibodies (Fitzpatrick *et al.,* 2014; Kumar *et al*., 2011; Fodera *et al*., 2013). Factors promoting protein aggregation are broadly classified into *in vitro* and *in vivo*. *In vitro* and *in vivo* factors promoting protein aggregation are discussed in the following section.

3.4.1. *In vitro* factors responsible for protein aggregation:

In vitro factors such as solvent, temperature, radiation, pressure, agitation and pH are responsible for the aggregation of the misfolded proteins.

3.4.1.1. Solvent:

Though most of the experimental investigations on protein folding imply proteins *in vitro*, causes of contiguous delimitation may convolute protein misfolding *in vivo*. An unfolded state of protein may become destabilized when protein alone is delimited. Whereas delimiting the protein as well as solvent results in a solvent intermediate cause that destabilizes the original native state. Confining both solvent and proteins indicates unfolding of proteins to a condensed unfolded structure which is distinct from the unfolded structure observed to a greater extent. Delimiting the solvent has a remarkable effect on thermodynamics and kinetics of proteins (Lucent *et al*., 2007).

3.4.1.2. Temperature:

Once the nascent polypeptide is released from ribosome, it comes in contact with the surrounding cellular environmental temperature. According to the established evidence, the rate of protein folding is fast and affected by elevated temperature and low in a cold surrounding environment. The inability of the protein to adopt is brought about by dynamic coercion on protein stability. Deposition of misfolded protein in the cell at high temperature results in heat shock response (Feller, 2018).

3.4.1.3. Radiation:

It is a well-known fact that radiations affect protein structures by fragmentation and aggregation. Along with proteins, amino acids also get degraded nonetheless, mechanisms of modification due to radiation are enigmatic (De Pomeraia *et al*., 2003).

3.4.1.4. Pressure:

High pressure may facilitate the interaction between protein-solvent and protein-protein via volume changes. Besides, it leads to change in the equilibrium of three dimensional proteins between native and denatured forms. Due to the distinct property, this will alter the thermodynamics and kinetics of protein aggregation (Kim *et al.*, 2006).

3.4.1.5. Agitation:

Agitation is a type of stress which stimulates the protein and disrupts the structure of proteins. Agitation induces the nucleation of protein species at the interface of water and air. The collision created by agitation between nuclei and other surrounding molecules causes the generation of large particles to facilitate aggregation (Shieh and Patel, 2015).

3.4.1.6. pH:

pH is a primary agent that affects the conformation of protein structure. Usually, when the net charge of a protein elevates, pH diverges from an isoelectric point. Besides, pH distant from the isoelectric point can cause the protein structure to unfold and promote protein aggregation (Chi *et al*., 2003).

3.4.2. *In vivo* factors responsible for aggregation of proteins:

There are several *in vivo* factors responsible for the aggregation of proteins. Impairment of proteasome complex, overexpression of proteins, mutations induced by pathogens, oxidative stress, impairment of autophagy and aging are major among the *in vivo* factors.

3.4.2.1. Proteasome impairment:

The ubiquitin proteasome pathway (UPP) is responsible for the degradation of unwanted proteins and retaining the required via quality control systems in cells. Besides performing normal protein degradation, it plays a vital role in regulating the quality control mechanism by degrading misfolded or abnormal folded proteins (mutated). UPP constitutes a multiprotein network termed as proteasome. Various

factors including aging leads to the impairment of proteasome complex and cause unusual ubiquitin-protein aggregates implicated in the advancement of neurodegenerative diseases (Myeku *et al*., 2011). UPP is mainly involved in the rapid and selective degradation of aggregates via polyubiquitination.

3.4.2.2. Overexpression of proteins:

Overexpression is a part of post-translational error due to peptide extensions, which possess a net negative charge to a greater extent. Elevated levels of proteins and enzymes can damage the cells and various metabolic activities due to activation or overburden of certain pathways and distorting regulation mechanism. Besides, it can perturb the stability and integrity of protein network and leads to depletion of resources available in the cell to generate and carry proteins (Zhang *et al*., 2004).

3.4.2.3. Pathogenic mutations:

Serpin are the inhibitors of proteolytic enzymes, whose function is involved in coagulation of blood, tissue remodeling, and other vital physiological roles. The mutations which lead to protein misfolding deactivates serpin and further causes overactivity of the proteolytic enzyme (Law *et al*., 2006; Huntington, 2011).

3.4.2.4. Oxidative stress:

Oxidative stress is caused due to the generation of reactive oxygen species which plays a fundamental role in the advancement of number of diseases. Reactive oxygen species are produced due to various physiological and metabolic processes occurring in the living system. Oxidative stress induces certain stress response pathways in the cellular organelles and damages the macromolecules such as

proteins, nucleic acids or lipids. Macromolecular damage is mainly brought about by reactive oxygen species and reactive nitrogen species (Sies *et al*, 2017).

3.4.2.5. Impaired autophagy:

Usually, protein aggregates are unloaded from cells by UPP or autophagy mechanism. UPP and autophagy mechanism are the well regulated pathways to selectively segregate and degrade oligomers and aggregates of misfolded proteins (Lamark and Johansen, 2012). Autophagy mechanism is largely known as non-selective degradative pathway. Its main role is to reinforce the cellular quality control mechanism by removing the specific protein aggregates and impaired cellular organelles.

3.4.2.6. Aging:

Aging is one of the important factors for protein aggregation and misfolding. The molecular mechanism of age related disease commencement and the interaction of protein aggregates relying on age factor is largely unknown. One probability is that the aggregates associated with age supporting factors enhance disease related aggregates of protein by inducing the cell to undergo stress. Another probability is that the disease related proteins and age related aggregates of proteins may accumulate and interact with each other (Tuite and Melki, 2007; Kikis *et al*., 2010).

3.5. Classification of protein aggregates:

Protein aggregates have been classified in different ways, as there is no precise definition exists. Protein aggregates are broadly classified into *in vivo, in vitro,* fibrillar and amorphous. For example, amyloid fibrils are named as fibrillar

or ordered aggregates that are found in both in vivo and in vitro, although inclusion bodies are termed as amorphous or disordered aggregates that are found in vivo. Likewise, in vitro aggregates are termed as disordered aggregates as it is formed at high protein concentrations during refolding. Other classifications of protein aggregates comprise physical (non-covalent), chemical (covalent), soluble and insoluble particles, reversible and irreversible (Horwich, 2002; Jahn and Radford, 2008).

3.6. Analytical methods to monitor protein aggregation:

To detect and understand the mechanism of formation of amyloid deposits combination of multiple analytical methods are necessary. The key features of commonly used methods to detect and analyze the aggregates of protein are discussed in the following section.

3.6.1. Dye-binding assays:

Various dye-binding assays are carried out to detect the protein aggregates (amyloid or amorphous). The details of the dye-binding assays are summarized below in Table 3.2.

Table 3.2: Dyes used for binding of protein aggregates (Hawe *et al.*, 2008)

Dye used	Applied aspect
Congo red	Detection of amyloids
Thioflavin T(ThT)	Detection of amyloids
Nile Red	Detection of folding and unfolding intermediates, amyloids and surface hydrophobicity
DCVJ and CCVJ	Detection of protein solvent viscosity
ANS and Bis-ANS	Detection of folding and unfolding intermediates, amyloids and surface hydrophobicity.

DCVJ: 9-(Dicyanovinyl)-julolidine; **CCVJ:** 9-(2-carboxy-2-cyanovinyl)-julolidine; **ANS:** Anilino-1-naphthalenesulfonate; **Bis-ANS**: 4,4'-Dianilino-1,1'-binaphthyl-5,5-disulfonate

3.6.2. Circular Dichroism Spectroscopy:

Circular Dichroism (CD) spectroscopy is mainly employed in protein chemistry and structural biology. It is used to predict and analyze the secondary and tertiary structure of proteins with the wavelength ranging from (190–250 nm) and (250–320 nm). Transformation of amyloid aggregates into protofibrils is brought about by the generation of β-sheet structure, following distinct minima in the range of 215–218 nm in the far-UV CD region. CD can also be used to obtain the qualitative data on amyloid assemblies and kinetics of structural intermediates linked with aggregation (Ranjbar and Gill, 2009).

3.6.3. Fluorescence Measurements:

Fluorescence measurements such as fluorescence intensity (FI) and emission maxima (λ max) are the sensitive parameters to the conformational changes and the polarity of protein chromophores. Commonly used protein chromophores such as Trp and Tyr are employed to evaluate and analyze the protein folding. Fluorescence of aromatic moieties has been employed to trace structural dynamics and self-integration of many amyloid proteins such as α-synuclein, β-amyloid, and prions (Antosiewicz and Shugar, 2016).

3.6.4. Fluorescence Microscopy:

Fluorescence microscopy (FM) is an indispensable tool used for the detection of protein aggregates. Congo red, Thioflavin T and Nile red (NR) dyes are used as fluorophores to track protein aggregates. Apple green birefringence was displayed by congo red and to locate the presence of amyloid deposits thioflavin fluorescence increases.

Fluorescence microscopy has been employed efficiently at elevated concentrations of protein, by reducing the changes in the local protein surrounding for the identification and to distinguish the aggregates. Nile Red (phenoxazone) cohere with hydrophobic surfaces of proteins and displays powerful fluorescence (Demeule *et al*., 2007).

The current perspective comprises confocal and two- or multi-photon FM and total internal reflection. Confocal FM is effectively used for 3D resolution investigation. Two- or multi-photon FM has been used for the investigation of UV-excitable fluorescence if confocal FM is not utilized. Total Internal Reflection

Fluorescence Microscopy (TIRFM) has been utilized for the examination of plasma membrane and progression of fibrils (Schneckenburger, 2005).

3.6.5. Transmission Electron Microscopy:

Transmission electron microscopy (TEM) has been effectively utilized for immediate examination of self-integrated proteins and protofibrils, oligomers and mature fibrils. Qualitative data on protein aggregates can be obtained. By employing TEM, protein aggregates such as α-synuclein, β-amyloid, lysozyme have been investigated (Yagi *et al*., 2005 and Han *et al*., 2017).

3.6.6. High Resolution Transmission Electron Microscopy:

High resolution transmission electron microscopy (HRTEM) is an instrumental tool utilized for tracking the generation of *in vitro* amyloid fibrils. It gives the illustration of various protein aggregate species such as circular species, prefibrillar species, and mature fibril species. Furthermore, it gives the information on the appearance of protein aggregates generated via various misfolding mechanisms such as amorphous aggregates.

HRTEM is a convenient tool to rapidly carry out the experimental investigations on the formation of fibrils. HRTEM also verifies the morphology of fibrillar protein aggregates. However, this technique will not verify the appearance of the unique β-sheet structure. To support this feature, auxillary techniques are essential in addition to HRTEM for obtaining the information on morphology, arrangement, and organization of fibrils and other amyloids. It gives a much better resolution to other techniques such as confocal FM and scanning electron microscopy (SEM) (Anderson and webb, 2011).

3.6.7. Field Emission Scanning Electron Microscopy:

Amyloid aggregates have a unique β-sheet structure that may be produced in vitro as well as in vivo environments. SEM tool is used to examine the 3D appearance of the image of amyloid, however, it is not in widespread use as compared to other techniques even though it has comparatively higher resolution up to the nanometer level. In SEM, specimen emits secondary electrons and these are utilized to design the final image. In field-emission scanning electron microscopy (FESEM), a field-emission cathode supplies narrow limiting conducting beams with high and low electron energy, which provides the increased resolution and reduced sample damage of the images (Takai *et al*., 2014).

3.6.8. Atomic Force Microscopy:

Biological sciences have utilized the efficiency of AFM to supply 3D structural features and images of various protein samples or fibrils with better resolution. AFM will not involve the preparation of a sample which will harm the sample. Its principle implies examining the extremity over the samples, and the laser beam perceives the deflection. It can be utilized in the investigation of living cells which possess supramolecular structures and extension of amyloid aggregates. AFM furnishes the information on qualitative as well as quantitative analysis at the nanometer level (width, length and distinct features of surface protein aggregates). Besides, it is a useful technique to track and analyze the dehydrated and hydrated samples under atmospheric conditions (Adamcik and Mezzenga, 2012).

3.7. Consequences of protein misfolding:

If the proteins acquire correct folding, it must achieve its functionality within the cell. Many chaperon mechanisms are essential to fold correctly. Besides, cellular degradation mechanisms are required to degrade the non-essential incorrectly folded proteins. Due to mutations, proteins acquire misfolded state and deposits in the ER. As a result of these, various pathological manifestations arise which are primarily established as protein folding issues. As a result of these consequences, protein misfolding leads to four important events. Each event is summarized in the following section.

3.7.1. Improper localization:

Several proteins that are confined to specific cellular organelles must possess proper conformational native structure to transport properly, mutations that weaken the conformational structure causes improper sub-cellular localization. This leads to dysfunction and further causes loss of function of protein and gain of function (due to toxicity) once it deposits in an improper location. For instance, mutations in Alpha-1-Antitrypsin (protease inhibitor), causes emphysema and liver damage due to loss of function and gain of function condition respectively (Perlmutter, 2011).

Mutant proteins fail to attain native conformation and settle in the ER. If the misfolded proteins are not eliminated, it deposits in ER (hepatocytes) and leads to liver damage. Due to mutation, A1AT is not released and fails to carry out its normal cellular function such as inhibition of protease activity (neutrophil elastase) in the lung. This further leads to severe damage to connective tissue in the lungs.

Though the damage to the lungs can be controlled by enzyme replacement therapy, the accumulation of protein aggregates in the liver has become a challenge to cure the disease (Hidvegi *et al*., 2005).

3.7.2. Improper degradation:

Endoplasmic Reticulum Associated Degradation (ERAD) and autophagy mechanism play a vital role in preventing the deposition of misfolded proteins (non-functional). Sometimes they become inefficient in being overactive, eliminating the mutant proteins and maintains some activity. Hence, improper degradation of proteins may lead to the manifestation of a severe form of the disease. For example, in the case of cystic fibrosis, a mutation in cystic fibrosis transmembrane conductance regulator (CFTR) gene is a result of amino acid residue phenylalanine at position 508. This mutation facilitates the proteins to be misfolded and directed for the elimination process (Meacham *et al*., 2001; Wang *et al*., 2006).

3.7.3. Dominant negative mutations:

Protein misfolding leads to disease via dominant negative mutations wherein the protein antagonizes the role of wild type (WT) protein and facilitates the loss in the protein activity in heterozygote condition. In epidermolysis bullosa simplex (a disorder of connective tissue), mutant keratin proteins such as KRT5 and KRT14 cause blistering in the skin due to injury.

Keratin produces elongated filaments that give the structure to the epidermis of the skin. If the keratin possesses disease linked mutations, then it causes the protein to be misfolded and forms aggregates due to mechanical stress. An individual with heterozygote can have filaments with WT as well as the mutant type

(MT) of protein when the filament is made up of multiple keratin molecules. Hence, the dominant feature of the disease can be described by the fact that the MT protein found in the filaments fails to function due to the compromising function of the whole filament. Recent reports on chemical chaperons have identified the role that can hinder the aggregation of MT keratin. The mechanism of action of these chaperones is not elucidated completely (Chamcheu *et al*., 2011).

3.7.4. Gain of toxic function:

When a protein attains non-native conformation, it leads to toxicity due to the dominant phenotype exhibited by protein structural modifications. One example is the lipid transport molecule, apolipoprotein E (APOE). One copy of the *APOE4* allele is exhibited by more than 60 % of individuals suffering from AD. *APOE4* polymorphism maintains a modified conformational fold of the protein whereas other alleles elongate the structure of the domain which is compromised by an additional salt bridge of *APOE4*. This further mediates the lipid affinity of *APOE4*, distorts the function of mitochondria and fails neurite outgrowth. The *APOE4* polymorphism is also linked with elevated levels of β-amyloid, which is found aggregated in the brain of individuals suffering from AD (Chen *et al*., 2011).

3.8. Protein misfolding diseases:

Growing evidence indicates that protein misfolding is associated with a variety of diseases. Both indigenous and extraneous factors are reported to contribute to protein misfolding diseases. Major factors responsible for protein misfolding encompasses the inability of ubiquitin proteasome complex and molecular chaperones to eliminate the aggregated proteins (Howlett, 2003). It is

also related to inefficient proteolytic activity, disruption in the normal cellular transfer of proteins and mutations. Furthermore, it is also associated with excess protein synthesis, aging, and environmental stress. More than 20 protein misfolding diseases have been identified so far. Earlier investigations on protein misfolding were confined majorly on neurodegenerative diseases but the recent reports highlight the role of protein misfolding in non-neurodegenerative diseases. A list of misfolded proteins and the associated diseases in humans has been given in Table 3.3 (Boshoff *et al*., 2014; Chaudhari and Paul, 2006).

Table 3.3: List of misfolded proteins responsible for human diseases

S.No.	Misfolded Proteins	Human Diseases
1	β - amyloid, tau	Alzheimer's disease (AD)
2	α – Synuclein, tau	Parkinsons's disease (PD)
3	Huntingtin	Huntington's disease (HD)
4	Prion	Spongiform encephalopathies
5	Tau	Multiple tauopathies
6	Superoxide dismutase 1	Amyotrophic lateral sclerosis (ALS)
7	Transthyretin	Familial amyloidotic polyneuropathy
8	PrP	Creutzfeldt-Jakob disease (CJD)
9	Desmin and α β-crystallin	Desminopathy
10	Rhodopsin	Retinitis pignemtosa (inherited blindness)
11	p53, SRC (non-receptor tyrosine kinase)	Cancer
12	Aquaporin-2/vasopressin	Nephrogenic diabetes insipidus
13	A1AT	COPD, Emphysema

14	Cystic fibrosis transmembrane conductance regulator, (CFTR)	Cystic fibrosis
15	PrP	Familial insomnia
16	α1-Antitrypsin	α1-Antitrypsin deficiency
17	MYH7, MYBPC3 and TTN	Hypertrophic and dilated cardiomyopathy
18	KRT5 and KRT4	Epidermolysisbullosa simplex
19	GNRH	Hypogonadotropic hypogonadism
20	β-glucosidase	Gaucher's disease
21	Antibody light chain	Multiple myeloma
22	α-galactosidase	Fabry
23	HbS	Sickel cell anemia
24	Atrophin-1	Dentatorubropallido-Luysian atrophy
25	Ataxin	Spinocerebellar ataxia (SCA)
26	NPC1	Niemann Pick Type C disease
27	Amylin	Type II diabetes
28	Lipofusin	Neuronal ceroid lipofuscinosis
29	Tau	Pick's disease
30	Frataxin	Friedreich's disease
31	Transthyretin	Senile systemic amyloidosis
32	α-Synuclein, tau	Hallervorden-Spatz disease
33	Tau	Frontal-temporal dementias
34	GFAP, tau-2	Alexander's disease
35	Transthyretin	Familial amyloid polyneuropathy I
36	α-Synuclein	Dementia with Lewy bodies
37	Apolipoprotein AI	Familial amyloid polyneuropathy III

38	β_2-Microglobulin	Haemodialysis-related amyloidosis
39	Ig light chains	Primary systemic amyloidosis
40	Calcitonin	Medullary carcinoma of the thyroid
41	Serum amyloid A	Secondary systemic amyloidosis
42	SCAD	Ethylmalonic aciduria
43	Fibrillin	Marfan syndrome
44	Procollagen pro aaa	Osteogenesis Imperfecta
45	PAH	Phenylketonuria
46	Androgen receptor	Spinal and bulbar muscular atrophy
47	α-Crystallins	Cataracts
48	Serum amyloid A	Rheumatoid arthritis
49	Gelsolin	Finnish amyloidosis
50	Prolactin	Prolactinomas

A1AT: Alpha-1 Antitrypsin; **PrP**: Protease-resistant-protein; **MYH7**: Myosin heavy chain 7; **MYBPC3**: Myosin binding protein C 3; **TTN**: Titin; **KRT5**: Keratin type-II cytoskeletal 5; **KRT4**: Keratin type-II cytoskeletal 4; **GNRH**: Gonadotropin releasing hormone; **HbS**: Hemoglobin S; **NPC1**: Niemann-Pick, type C1; **GFAP**: Glial fibrillary acidic protein; **SCAD**: Short chain acyl CoA dehydrogenase; **PAH**: Phenylalanine hydroxylase; **COPD**: Chronic obstructive pulmonary disease

3.9. Therapeutic strategies for inhibition of protein aggregation:

Living organisms tend to adopt several mechanisms to prevent unnecessary protein aggregates and generation of amyloids at elevated concentrations of proteins. The inhibition of protein self-congregation is vital to serve two purposes. The first possibility is, developing and designing strategies to control the protein

aggregation and the second is the detection and designing the inhibitors that control the generation of amyloid aggregates.

Various molecules have been investigated both in-vivo and in-vitro to determine the anti-amyloidogenic efficacy. Chaperones, vitamins, flavonoids, nanoparticles, polyphenols, etc. have been tested to control the amyloid fibrillation of proteins (Stefani and Rigacci, 2013; Sharma and Ghosh, 2019).

The efficacy of nanoparticles is also tested on amyloid aggregates, for example, curcumin gold nanoparticles possess anti-amyloidogenic properties. Recently, investigators have reported the role of native proteins which act as chaperones and control the protein aggregation, in combination with protein folding. Anti-amyloidogenic molecules that inhibit the aggregation by the regulation of native proteins, controlling the expression of amyloidogenic proteins and controlling the protein aggregates with the help of small molecules or by vaccination (Eisele *et al*., 2015). Recent investigations on ethyl ammonium nitrate (EAN) and non-detergent surfactants *viz.* sulfobetaines (NDSBs) have been reported to effectively control chemical and heat induced aggregation (Dolan *et al*., 2015; Bezancon *et al.*, 2003).

Amino acids, drugs, imidazole derivatives, and metal ions have been shown to possesses an efficient inhibitory role in the aggregation of various proteins. Apart from this, osmolytes have been implicated to suppress the aggregation in various cases. Artificial chaperones designed to elevate the efficiency of refolding of a variety of proteins and control the aggregation (Machida *et al*., 2000).

3.9.1. Targeting production, misfolding, and aggregation of proteins:

In the case of β-amyloid (Aβ), it was initially believed that fibrils were pathogenic agents in AD. However, recent reports imply that aggregation deposits and intermediates are key factors. In Parkinson's disease indistinguishable finding has been revealed with discrete species of α-synuclein. Due to the presence of various intermediates that emerges in the course of oligomerization mechanism, this pathway becomes more complicated. Current investigations have shown that the aggregated proteins such as Aβ and α-synuclein possess distinct conformational species, with a unique distribution mechanism and distinct degrees of neurotoxicity (Lam *et al*., 2016).

3.9.2. Targeting chaperones:

Chaperones are another class of alternative which involves the therapeutics for protein misfolding diseases. As the chaperones play a vital role in the folding of proteins and maintaining proteostasis balance, they provide efficient therapeutic intervention in dealing with toxic proteins. Knowledge on the interaction of chaperones with the disease and respective proteins is limited. As the chaperones offer various mechanisms of action based on the type of chaperones and disease, it becomes a challenge for the development of drugs. Some indication has been provided at the interface of mutations of unique chaperones and some neurodegenerative disease. Mutations in the chaperones such as Hsp40 and Hsp70 have been associated with parkinson's disease (Ebrahimi-Fakhari *et al.,* 2011). The presence of mutations in co-chaperone valosin containing protein has been

associated with ALS. Moreover, chaperones have been implicated in neuroprotection in over exuberant cells (Carman *et al*., 2013; Witt *et al*., 2013).

3.9.3. Targeting degradation:

The inefficiency of UPP, as well as autophagy mechanism of degradation of proteins, are indicated in neurodegenerative diseases. One example is mutations in the gene (*PINK-1* and *DJ-1*), which is the source of parkinson disease encode proteins associated with UPP as well as autophagy. As the number of proteins are required with chaperones, designing the promising drugs that focused on UPP and autophagy mechanism is strenuous. Moreover, in most of the diseases, the type of unfolded proteins and cellular toxicity with respect to proteins are not elucidated. While understanding the protein degradation, the type of pathway or mechanism encountered during degradation of proteins (UPP or autophagy) vary based on the state of the protein (fibrillar or soluble) and certain post-translational modification it carries (Cook *et al*., 2012; Ciechanover and Kwon, 2015).

3.9.4. Targeting extracellular clearance of folded proteins:

Targeting the extracellular clearance of misfolded proteins are explored via antibodies to mediate the removal of Aβ. The antibodies are believed to function by one of the two or both the pathways. One pathway is that it crosses the blood brain barrier (BBB) to cohere with Aβ found in the extracellular space. Another pathway is via "peripheral sink" achievement (Wisniewski and Goni, 2015). The majority of misfolded proteins exhibit extracellular leakage and hypothetically it can be refined by exceptionally discriminating antibodies (Genereux and Wiseman, 2015). One such example is that antibodies have been designed for α-synuclein as

well as Aβ which reveals more than 1000 fold increased affinity for monomeric and oligomeric proteins (Savage *et al*., 2014; Genst *et al*., 2014; Vaikath *et al.,* 2015).

3.9.5. Rebalancing the proteostatic network:

The efficiency of proteostatic system to combat proteotoxic agents declines with age and also arbitrated by mutations and clinical manifestation of the disease. Another efficient approach is to develop drugs that incorporate rebalancing of large scale proteostatic network. For instance, a transcriptional activator, heat shock factor 1 (HSF1) mediates the heat shock response. The heat shock response and proteotoxic response declines with age and also in neurodegenerative diseases. Besides current investigation implies that HSF1 degradation is increased in α-synucleinopathy of humans and mouse. Over exuberant has reported being neuroprotective in cell models of neurodegenerative diseases. It decreases the production of polyglutamine aggregate and increases the lifetime in a mouse model of HD (Switonski *et al.*, 2012; Hayashida *et al.*, 2010). It is also shown to decrease the androgen receptor pathogen deposits and neurotoxicity in a mouse model of spinobulbar muscular atrophy. Recent studies on molecules of relatively small size have been reported to be indicated as neuroprotectors in animal models of neurodegenerative diseases and cells (Neef *et al*., 2011; Verma *et al*., 2014).

3.10. Issues in interpreting preclinical findings to clinical trials for misfolded proteins associated diseases:

Misfolded proteins associated with neuropathies have been tested for the development of drugs. Favorable candidates for ALS, Alzheimer's disease, Huntington's disease, and Parkinson's disease have been examined in preclinical studies. However, in clinical trials, only a few candidates have proven to show a

remarkable gain. In general, the failure in translating to clinical trials has troubled the designing of drugs for neurological diseases. The significant origin is the inability to develop robust candidates that provide therapeutic output. The unpredictability lies in which process to focus, which proteins to focus, inability to show beneficial approaches and outcome in preclinical investigations in animal models that do not have to anticipate rationality (Shineman *et al*., 2011).

Furthermore, in clinical investigation, there is a secure requirement to detect convertible biomarkers in animal models. Eventually, the safety of drugs and pharmacokinetics presents remarkable challenges in designing drugs for misfolded protein diseases. Currently, investigations are carried out upon structural examination of the interaction of epitopes with antibodies on Aβ and correlation of these interactions with clinical results (Karran and Hardy, 2014).

Another approach is the detection of novel candidates. For instance, the domain of cell type specific candidates has not been explored. An important feature is that in protein misfolding diseases the proteins implicated are either shown in several cell types or all types of cells, however, this leads to pathological manifestations only in certain types of neurons. Hence, drug design must focus on examining the misfolded protein species encountered as well as the influence on various inhabitants of neurons (Ashraf *et al*., 2014).

3.11. Protein misfolding in PE:

Protein misfolding has been implicated in preeclampsia in western populations. The misfolded proteins have been identified in urine, serum and placenta of pregnant women. A recent study on pregnant women (n=284) showed a unique proteomic signature in the urine. In the first phase, 59 severe preeclamptic

women required mandatory delivery based on clinical investigation, in the second phase, 225 high risk and low risk preeclamptic women were screened and of these, 19 women were examined for the presence of misfolded proteins in the urine throughout the pregnancy. Besides, protein to creatinine ratio and soluble fms-like tyrosine kinase-1 to placental growth factor ratio was also considered. Elevated and atypical immunoreactivity to *SERPINA1* was shown in the placenta, serum, and urine. Fragment of *SERPINA1* and albumin was identified in the urine of preeclamptic women (Buhimschi *et al*., 2008).

To show that the preeclamptic women exhibit urinary congophilia, an investigation was carried out on 80 pregnant. Out of 80 pregnant women, 40 were severe preeclamptic and another 40 were normotensive pregnant. The urine of severe preeclamptic women indicated urinary congophilia. Investigators recorded that congophilic substance of urine contains misfoldome of *SERPINA1*, Alzheimer's β-amyloid, ceruloplasmin, albumin, immunoglobulin free light chains and interferon-inducible protein (Buhimschi *et al*., 2014).

The presence of urinary congophilia was also reported in 28 women with chronic kidney disease, 23 preeclamptic and 31 normotensive pregnant women. This study demonstrates that urinary congophilia is higher in preeclamptic women and women with chronic kidney disease than in normotensive pregnant women (McCarthy *et al*., 2016).

A pilot study was conducted on 81 pregnant women by employing Congo red assay. The study results showed that out of 81 pregnant, 12 were reported to be preeclamptic. Further the study was extended on 642 pregnant women, the results suggest that 105 women were preeclamptic. This study implies that the urinary

congophilia shows the highest accuracy at the time of developing the symptoms of preeclampsia (Sammar *et al*., 2016). There were no reported studies on protein misfolding with respect to preeclampsia in Indian population. Therefore, the present thesis was aimed to measure the misfolded protein levels in urine samples of indian PE women.

3.12. Protein oxidation in PE:

Oxidative stress is known to cause damage to biomolecules *viz* proteins, nucleic acids, and lipids. Protein oxidation has been reported in the development of preeclampsia. Various oxidative stress markers were used to detect the presence of oxidative stress in preeclampsia. Protein carbonyl is most widely used marker to study the protein oxidation in preeclamptic women. There are reported studies on protein oxidation in the placenta, cord blood and plasma samples of preeclamptic women. But there is no reported study on protein oxidation in the urine samples of preeclamptic women. Moreover, there were no studies to demonstrate that protein oxidation leads to protein misfolding in preeclamptic women. To fulfill this gap, the protein carbonyl levels were measured in the urine of preeclamptic women, which is a measure of protein oxidation.

Protein carbonyl derivatives act as a sensitive biomarker to detect protein oxidation damage caused by Reactive Oxygen Species (ROS). Preeclamptic, normotensive pregnant and normotensive non-pregnant women were examined for the presence of protein carbonyl levels. Protein carbonyl levels of plasma were measured in 47 preeclamptic, 45 normotensive pregnant and 22 normotensive non-pregnant women. A significant statistical difference in the protein carbonyl levels of plasma was recorded between preeclamptic and normotensive pregnant women.

Normotensive pregnant women showed elevated protein carbonyl levels than in normotensive non-pregnant women. This indicates that preeclamptic women had higher protein carbonyl levels than in normotensive pregnant women (Zusterzeel *et al.*, 2000).

Protein carbonyls in serum and lipid peroxides in serum, placental, and decidual tissues were investigated on 30 mild preeclamptic, 30 severe preeclamptic and 50 normotensive pregnant women in the third trimester of pregnancy. Apart from this antioxidant were also measured. Protein carbonyls in serum, lipid peroxides in the placenta, deciduas basalis and were found to be increased in severe preeclamptic women when compared to mild preeclamptic and normotensive pregnant women. Whereas, total carotene and vitamin E levels were found to be decreased significantly in severe preeclamptic women than in mild preeclamptic and normotensive pregnant women. Moreover, a significant correlation was reported with serum protein carbonyl, lipid peroxides, decidual, placental tissues, and diastolic blood pressure. A significant correlation was also observed between protein and lipid oxidation levels and antioxidant vitamin in severe preeclamptic women. Logistic regression analysis conducted on the above parameters indicates that levels of all these parameters were linked with preeclampsia (Serdar *et al.*, 2003). Investigation on protein carbonyl levels in plasma was carried out in 14 normal pregnant women and 17 preeclamptic women. Preeclamptic women showed higher levels of protein carbonyl when compared to normotensive pregnant women (Tsukimori *et al*., 2008).

71 preeclamptic women and 72 normotensive women were studied to examine the levels of protein carbonyls, total antioxidants, and malondialdehyde.

Also, cord blood investigation was conducted for the above parameters. Protein carbonyl levels of preeclamptic mothers were significantly correlated with protein carbonyl levels of cord blood. It has been reported that protein carbonyl levels of preeclamptic mothers were associated with early prenatal outcomes (Bharadwaj *et al*., 2018). A study was conducted on 35 preeclamptic and 35 normotensive pregnant women to test the levels of protein oxidation, protein carbonyl. It was recorded that preeclamptic women had elevated levels of protein carbonyl (Bernardi *et al*., 2008).

Placenta and plasma of 33 preeclamptic women and normotensive pregnant women were examined to determine the levels of protein carbonyl. It was demonstrated that protein carbonyl levels were significantly increased in preeclamptic women (Bernardi *et al*., 2012). Protein carbonyl, total antioxidant, and vitamin C levels were measured in cord blood of 15 preeclamptic women and 20 normotensive pregnant women. Protein carbonyl levels were found to be elevated in the cord blood of preeclamptic women. Besides, vitamin C and total antioxidant levels were significantly decreased (Howladar *et al*., 2009).

Protein oxidation (protein carbonyl), DNA oxidative damage (8-hydroxy-2-deoxyguanosine), vitamins (E, C, and A), iron, nitrite, catalase, and total antioxidant levels were measured in cord blood of 19 preeclamptic women, 14 eclamptic women and 18 normotensive women. Higher levels of protein oxidation, DNA damage, nitrite, iron and lower levels of vitamins (E, C, and A), catalase and total antioxidants were reported. The significant increase in the level of protein carbonyl indicates that preeclampsia is associated with protein oxidation status (Negi *et al*., 2014). 50 severe preeclamptic women which comprises 25 HELLP

syndrome and 22 normotensive pregnant women were studied to determine the levels of protein carbonyl antioxidant (ferric reducing ability of plasma) in the placenta. Protein carbonyl levels were found to be increased in the placenta of preeclamptic women, HELLP syndrome than normotensive pregnant women. Also, antioxidant levels were significantly decreased (Zusterzeel *et al.*, 2001).

3.13. PiS and PiZ mutations in *SERPINA1* gene:

Presently**,** the cause of protein misfolding in preeclampsia is not clearly known. Genetic mutations are linked to protein misfolding diseases. A1AT has been reported to be misfolded in the urine of preeclamptic women (Buhimschi *et al*., 2014). A1AT levels have been measured in the serum of 23 severe preeclamptic women and 18 normotensive pregnant women. A1AT level was found to be decreased in severe preeclamptic women than in normotensive pregnant women. This shows that lower levels of A1AT are associated with severe preeclampsia (Twina *et al*., 2012). A1AT levels have been investigated in the placenta of 89 preeclamptic women and 120 normotensive pregnant women. Lower levels of A1AT was detected in preeclamptic patients. Besides, exogenous A1AT injection is found to lower urine protein levels and alleviate the blood pressure in mouse models (Feng *et al*., 2016).

Neutrophil elastase an endogenous inhibitor of A1AT has been investigated in the plasma of 50 preeclamptic women and 50 normotensive pregnant women. Neutrophil elastase was found to be significantly increased in severe preeclamptic women than in mild and normotensive pregnant women. Besides, levels of A1AT was found to be reduced in severe and mild preeclamptic women than in normotensive pregnant women (Kunder *et al*., 2017). A1AT has become a known

risk factor for COPD. An investigation was carried out on the PiS and PiZ alleles of *SERPINA1* gene with respect to lung function. The main role of A1AT is to suppress the function of neutrophil elastase which breaks down the pulmonary elastic fibers. Also, deficiency alleles such as PiS and PiZ are established risk factors for COPD (Thun *et al*., 2012). A1AT deficiency is a genetic disorder that shows mutant misfolded A1AT protein which accumulates and polymerizes in the hepatocytes of the liver. This condition further causes liver disease in some individuals. The reports indicate that oxidative stress is one of the causative factors in the A1AT deficiency model with respect to liver disease (Marcus *et al.,* 2012). Based on the reported studies on the role of mutations in protein misfolding, the frequency of PiS and PiZ mutations in *SERPINA1* and its association with PE was investigated.

Materials and Methods

4.1. Study design:

This study was carried out by following the prospective case-control design. The case group comprised of women whose pregnancy was complicated with preeclampsia. The control groups comprised women with an uncomplicated pregnancy.

4.2. Ethical issues:

The study was conducted after obtaining approval from the Institutional Ethics Committee of Sri Devaraj Urs Medical College, Kolar, India (DMC/KLR/IEC/07/2017-18). PE women and normotensive pregnant women were recruited from 2017 to 2019. Informed consent was obtained from the patients in written form before enrolling for the present study.

4.3. Selection of study participants:

The study participants were recruited from the Department of Obstetrics and Gynaecology which is affiliated to R. L. Jalappa Hospital and Research Centre, Kolar, Karnataka, India. PE women were enrolled upon satisfaction of the inclusion and exclusion criteria.

4.3.1. Inclusion criteria:

i. Pregnant women diagnosed with PE
ii. Superimposed eclampsia
iii. Singleton and multiple gestations
iv. Primigravida and multigravida

v. Fetal complications such as Intrauterine Growth Retardation and stillbirth

4.3.2. Exclusion criteria:

i. Pregnant women with a history of hypertension

ii. Co-morbidities such as diabetes mellitus, heart disease, respiratory disease, and epilepsy.

4.3.3. Diagnosis of PE:

Pregnant women diagnosed to have PE were based on the following criteria (ACOG guidelines, 2013):

a) New-onset hypertension (two readings of systolic blood pressure ≥ 140 mm Hg or diastolic blood pressure ≥ 90 mm Hg measured, while the patient is on bed rest between 4 h apart.

b) ≥ 20 weeks of gestation.

c) New onset proteinuria (>300 mg protein for 24 h of urine or +1 on dipstick).

d) In the absence of proteinuria, other symptoms such as Hemolysis Elevated Liver Low Platelet counts syndrome (HELLP) edema, thrombocytopenia, impaired liver function, new-onset cerebral or visual disturbances and renal insufficiency (in the absence of other renal diseases) severe headache, nausea and convulsions.

4.4. Sample size calculation:

The sample size was calculated based on the incidence of PE in the Indian population which is about 10 % of pregnancies (National Health Portal, 2016). The sample size was calculated using an open-source web-based tool viz., OpenEpi

Version 3.01. The number of patients required for a study with a power of 80 % was found to be 60 per group.

4.5. Clinical sample collection and processing:

Urine and blood samples were collected from the normotensive pregnant women and PE women. Midstream urine sample was collected in a sterile Uricol container (Himedia, Mumbai. India) and centrifuged at 2500 rpm for 5 min to remove the debris. The supernatant was stored at -80º C until analysis. 3 ml of the peripheral blood sample was collected in EDTA vacutainer and stored at 4º C until the preparation of genomic DNA.

4.6. Estimation of protein concentration:

The total protein content of the urine sample was estimated by the Bradford method using standard Bovine Serum Albumin as the standard (Bradford, 1976).

4.7. Normalization of protein concentration of urine samples:

The urine samples were normalized to a fixed concentration. Dilute samples were normalized by concentrating it by using the dialysis method (Mcfarlane, 1964). Concentrated samples were normalized by dilution with MilliQ water.

4.8. Estimation of misfolded proteins:

Misfolded proteins were estimated by using Congo Red Dot (CRD) assay (Buhimschi *et al*., 2016). Congo Red Retention (CRR) was used as a measure of protein misfolding. The protein concentration of all the urine samples was normalized to 15 µg/ml. 5 µl of Congo red solution was prepared in water (5 µg/ml). The prepared Congo red solution was mixed with 100 µl of the normalized urine

sample. Blank was made by mixing 5 µl of congo red dye with water. Once the sample was added to congo red dye, it was vortexed for 1 h at room temperature. Then 5 µl of the vortexed mix was spotted on a supported nitrocellulose membrane strip (#SF107A, 0.22 µm Himedia, Mumbai, India). Then the spot was left for air drying for 15 min, and washed with Milli-Q water for 3 min to remove the unbound stain. The assay was performed in duplicates.

Image of the nitrocellulose membrane strip was recorded using Image Lab software available in the Gel Doc Molecular imager (Bio-Rad, Hercules, USA). After recording the image, the nitrocellulose membrane strip was then washed with 50 % methanol for 3 min, 70 % methanol for 1 min and 90 % methanol for 10 min respectively to make sure that the red spot disappears completely from the blank. Once the spot was washed with methanol, it was again recorded as before. The color intensity of the retained spot was measured and CRR was calculated using the formulae as shown below. CRR was expressed as a percentage.

$$\text{CRR (\%)} = \left(\frac{Spot\ intensity\ after\ wash}{Spot\ intensity\ before\ wash}\right) 100$$

4.9. Estimation of total urinary protein carbonyl:

The protein carbonyl levels of the urine samples were measured by ELISA using a commercially available kit (STA-310, Cell BioLabs, San Diego, CA). The protein concentration of the urine samples was normalized to 10 µg/ml before the procedure.

4.10. Preparation of genomic DNA:

Genomic DNA was isolated by the salting-out method (Miller *et al*., 1988). About 2 ml of the blood sample collected in EDTA vacutainer was vortexed and

then transferred into a sterile 15 ml falcon tube. Erythrocyte lysis buffer (ELB) was added to the falcon tube containing the blood sample in the ratio of 1:4, followed by thorough mixing. The sample was then incubated for about 30-45 min on ice to induce hemolysis. The haemolysed sample was then centrifuged at 3000 rpm for 10 min. The supernatant was discarded and 10 ml of ELB was added to the pellet. The suspension was subjected to centrifugation at 3000 rpm for 10 min. The supernatant was discarded and the pellet was resuspended with 5 ml of ELB. The suspension was then supplemented with 270 µl of 20 % SDS and 30 µl of proteinase K. The suspension was incubated at 37° C, overnight in a water bath. Following this, 500 µl of 5 M sodium chloride and an equal volume of 100 % isopropyl alcohol were added to the falcon tube to precipitate the DNA. The DNA was then transferred to a 1.5 ml microcentrifuge tube containing freshly prepared 500 µl of 80 % ethanol. The sample was incubated for 15 min at room temperature and was centrifuged at 12000 rpm for 5 min. The supernatant was discarded and the pellet was washed with 80 % alcohol 3 times. The pellet was air-dried and then resuspended in 500 µl of Tris EDTA (TE) buffer. The sample was then incubated at 65° C in a water bath for 30 min. Following this, the sample was kept in a rotator overnight to dissolve the DNA completely. The sample was then stored at -80° C until further analysis.

4.11. DNA quantification and purity analysis:

The concentration and purity of the DNA preparation were determined by spectrophotometry. Measurements were carried out on the UV-Vis spectrophotometer (Perkin Elmer model Lambda 35, Waltham, MA, USA) was used to check the concentration and purity of the DNA. The amount of DNA was estimated using formula: dsDNA concentration = 50 µg/ml x OD_{260} x dilution

factor. The ratio of absorbance at 260 and 280 nm in the range of 1.7 to 2.0 was regarded as pure.

4.12. PCR amplification:

PCR reactions were performed on a gradient thermal cycler (Bio-Rad, California, USA). PCR mix comprised of 1 pM of each primer, 1 mM dNTPs, 1.5 mM $MgCl_2$, 100 – 300 ng of genomic DNA, and 1 unit of *Taq* DNA polymerase (Bangalore Genei, India) in a final volume of 25 µl. PCR parameters employed for the genotyping of the PiS and PiZ alleles are summarised in Table 4.1. The PCR amplicon was analyzed by electrophoresis on 2 % agarose gel.

Table 4.1: PCR parameters employed for the genotyping of PiS and PiZ alleles of *SERPINA1* gene

Parameters	PiS allele	PiZ allele
Primers (5'-3') • Forward (3'-5') • Reverse	TGA GGG GAA ACT ACA GCA CCT C AGG TGT GGG CAG CTT CTT GGT CA	TAA GGC TGT GCT GAC CAT CGT C GGA GAC TTG GTA TTT TGT TCA ATC
Thermal cycles (35 X) • Initial denaturation • Cycle denaturation • Annealing • Extension • Final extension	94º C (5 min) 94º C (30 s) 60.4º C (30 s) 72º C (30 s) 72º C (5 min)	94º C (3 min) 94º C (30 s) 61.3º C (30 s) 72º C (30 s) 72º C (5 min)
PCR amplicon size (bp)	121	144

bp: Base pairs; **s:** Seconds; **min:** Minutes

4.13. Genotyping of PiS and PiZ alleles of *SERPINA1* gene:

The genotype of the *SERPINA1* gene was determined by PCR-RFLP method. 10 µl of PCR amplified product was incubated at 65º C with 10 U of Taqα1 restriction enzyme (New England Biolabs, USA) for 16 h and the digestion pattern was analyzed on 3 % agarose gel. PCR-RFLP pattern for the genotyping of PiS and PiZ alleles are depicted in Table 4.2.

Table 4.2: PCR-RFLP pattern for genotyping of PiS and PiZ alleles of *SERPINA1* gene

Genotype	PiS allele (bp)	PiZ allele (bp)
Wt/Wt	100+21	123+21
Wt/mut	121+100+21	144+123+21
mut/mut	121	144

Wt: Wild type allele; **mut:** Mutant allele; **bp:** Base pairs

4.14. *SERPINA1* gene sequencing:

PCR reaction was performed as mentioned above. PCR thermal cycle conditions were as follows: initial denaturation at 94º C for 5 min, 35 cycles of denaturation at 94º C for 30 s, annealing at respective temperature for 30 s, extension 72º C for 30 s and final elongation at 72º C for 5 min. The sequence of the primers used and their corresponding annealing temperatures are shown in Table 4.3. The PCR amplicons were analyzed on 1 % agarose gel. The amplified PCR products were purified by using a commercially available kit (Gene JET PCR purification kit, Thermofisher Scientific, USA). The purified PCR products were used for Sanger sequencing. The chain termination method was carried out using

the BigDye Terminator v3.1 cycle sequencing kit as per manufacturer's instructions (Applied Biosystems, Foster City, California, USA). The samples were analyzed using ABI-3500 Genetic Analyser (Thermofisher Scientific, CA, USA).

The nucleotide sequence generated by the genetic analyzer was compared with the reference sequence retrieved from the National Center for Biotechnology Information (Sequence ID: NC_000014.9). The functional impact of the variations was analyzed by using web-based tools namely Polyphen-2 and SIFT (Adzhubei *et al*., 2010; Vaser *et al*., 2016).

Table 4.3: List of primers used for PCR amplification of *SERPINA1* gene

Exon number	Primer sequence (5'-3')	Annealing temperature (ºC)	Amplicon size (bp)
2	TTG GCA CAG GCT GGT TTA ACT GAA GAA TCC ACG CTG AA	61.3	898
3	GCA GCT TGG ATG GTC AGT TT ACG AGA CCT TTA CCT CCT CA	60.3	682
4	CCA ACA CTA CAG GGC AAG AT CAG TGA ATC ACG GGC ATC TT	57.3	580
5	CTC TTC CCT GTT CTG AGT TGT G TTA CCT GGA GCC CAC ATA CA	59	607

bp: Base pairs

4.15. Statistical analysis:

Statistical analysis was carried out by using International Business Machine Statistical Package for Social Sciences (SPSS. Version-21, IBM, New York) statistics for windows. The data were checked for normality using the Shapiro-Wilk test. Paired 't' test was performed when the data from two groups follows the

normal distribution. If the data did not follow the normal distribution, then medians of the groups were calculated and Mann-Whitney 'U' test was performed. For the data followed normal distribution, the means of more than two groups were compared using ANOVA with post-hoc test. Pearson's correlation coefficient was used to assess the correlation between the variables. P-value ≤ 0.05 was considered as statistically significant.

Results

5.1. Levels of urinary misfolded protein are elevated in PE women:

Urinary misfolded protein level was measured in PE women (n = 62) and normotensive pregnant women (n = 65). Profile of Congo Red Retention (CRR) in the urine of normotensive pregnant women and PE women before and after the wash is shown in Figure 5.1. The mean CRR (%) of PE women was 77.9 ± 11.5 which is 2.1 times higher as compared to mean CRR value of normotensive pregnant women which was 37.9 ± 4.1 The difference in the mean CRR of the two groups was found to be statistically significant ($p < 0.001$). Profile of mean CRR (%) values in the normotensive pregnant women and PE women is shown in Figure 5.2. The data was analyzed after stratifying the PE patients into clinical subgroups such as severity, gestational age of onset. In the PE women, the mean CRR (%) value in early-onset (70.5 ± 9.0) and late-onset (82.7 ± 10.3) was found to be 1.9 and 2.2 times higher as compared to the mean CRR of normotensive pregnant women. In the two groups, the mean CRR (%) values were significantly different from that of the normotensive pregnant women ($p < 0.001$). Profile of mean CRR (%) values in normotensive pregnant women to gestational age of onset is shown in Figure 5.3. In the mild subtype of PE, the mean CRR (%) value was (61.2 ± 3.2) and in a severe subtype of PE, the mean CRR (%) value was (82.4 ± 8.4) and found to be 1.6 and 2.2 times higher than that of normotensive pregnant women. In the mild and severe groups, the mean CRR values were significantly different from that of the normotensive pregnant women ($p < 0.001$). Profile of mean CRR (%) values in normotensive pregnant women with respect to severity is shown in Figure 5.4. Data was compared after distinguishing with respect to the involvement of co-morbidities as shown in Figure 5.5. In PE women superimposed with eclampsia,

the mean CRR (%) value (89.4 ± 2.0) was 2.4 times higher than that of normotensive pregnant women ($p < 0.001$). In PE subgroup with fetal complications i.e., IUGR and stillbirth, the mean CRR (%) value (74.6 ± 5.8) was 2.0 times higher as compared to normotensive pregnant women ($p < 0.001$) as depicted in Figure 5.6.

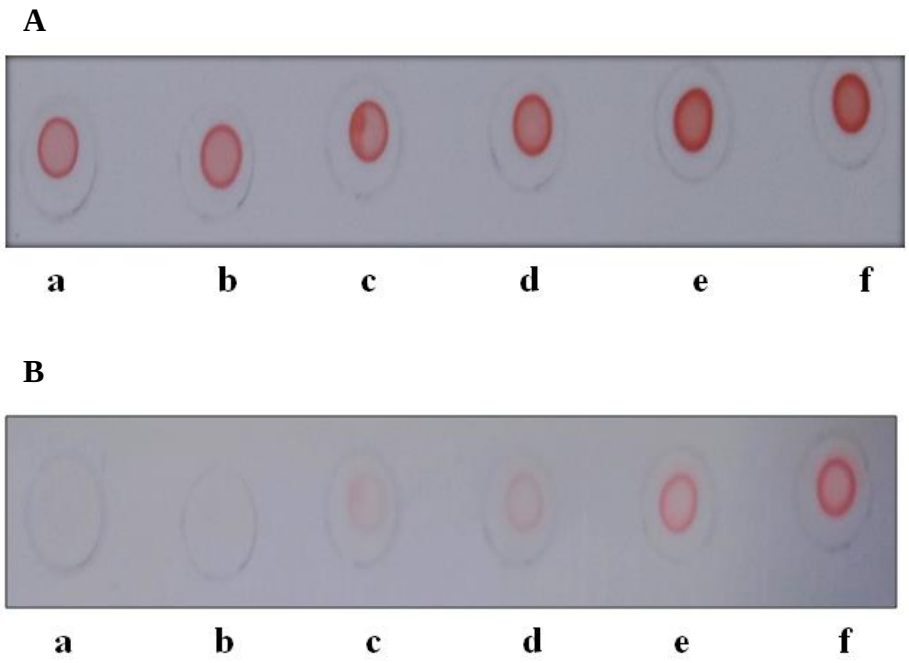
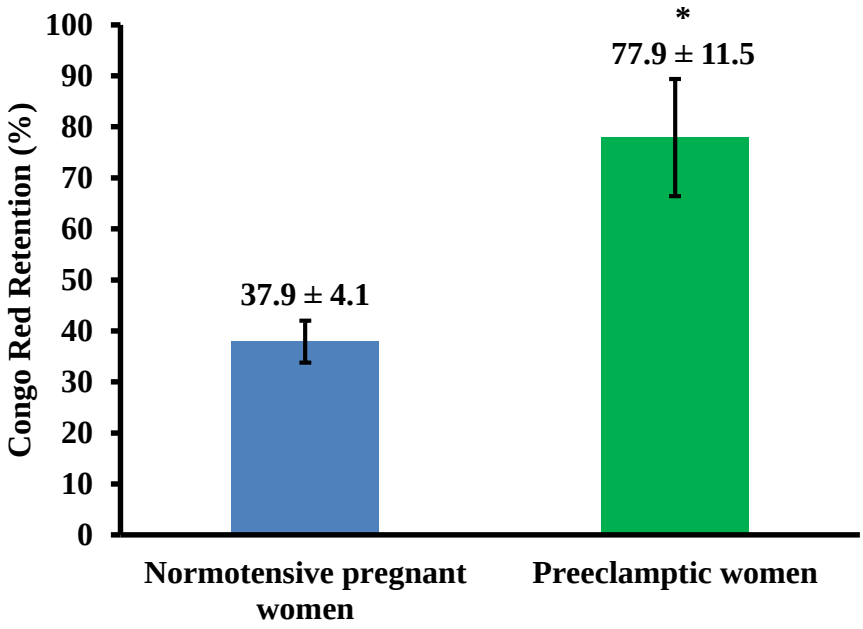
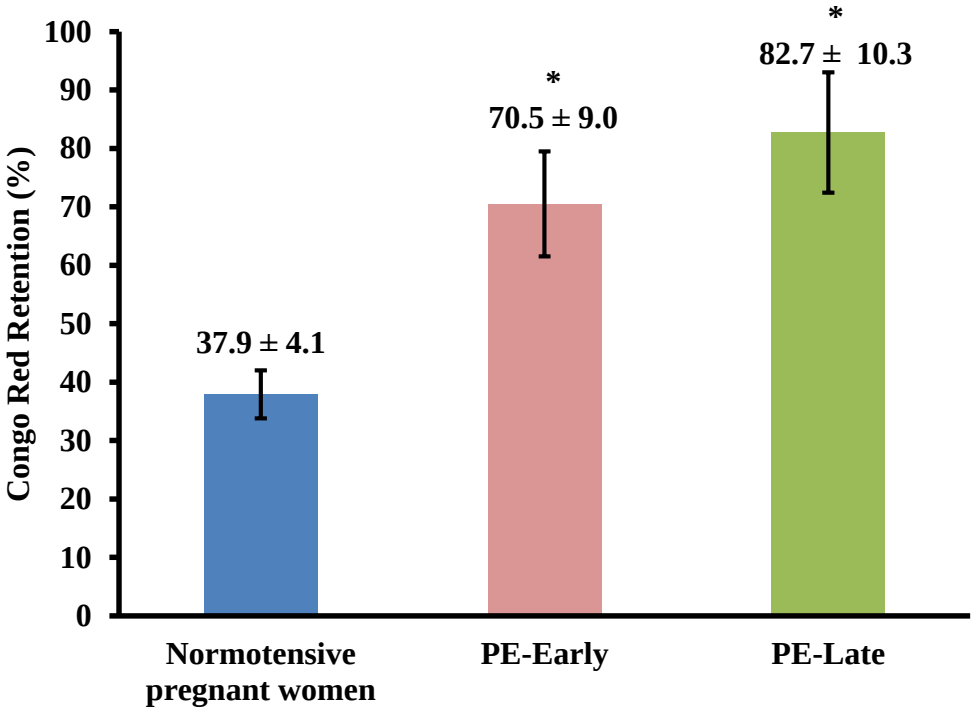


Figure 5.1: Profile of CRR in the urine of normotensive pregnant women and PE women (A) before and (B) after wash. Blank (a and b), normotensive pregnant women (c and d), and PE women (e and f)



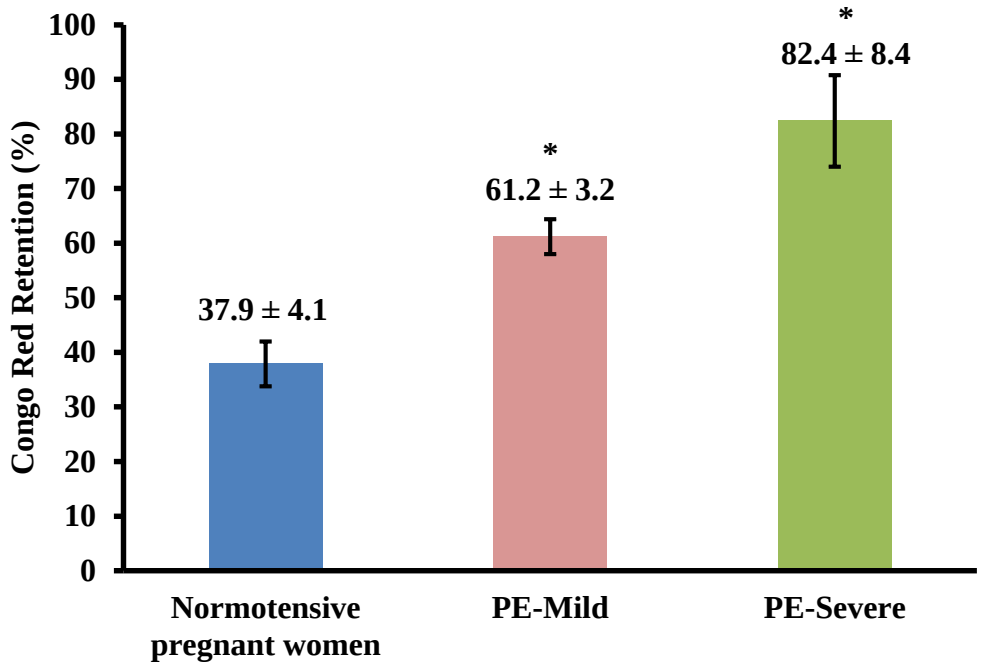
***** p < 0.001; w.r.t. normotensive pregnant women

Figure 5.2: Profile of mean CRR (%) values in normotensive pregnant women and PE women



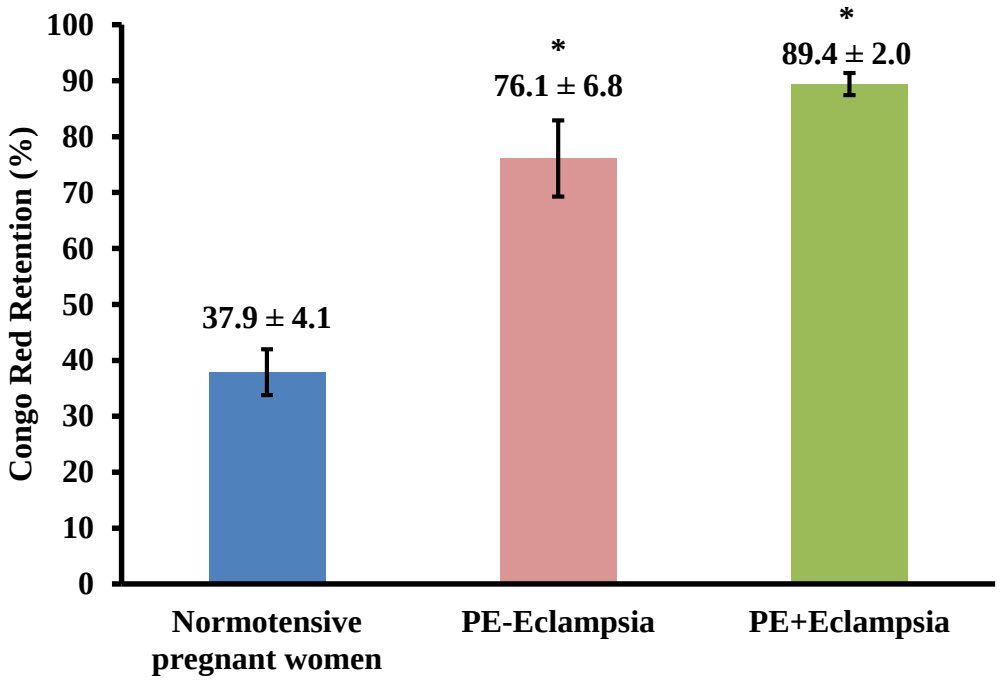
***** p < 0.001; w.r.t. normotensive pregnant women; **PE:** Preeclamptic women

Figure 5.3: Profile of mean CRR (%) values in normotensive pregnant women with respect to the gestational age of onset



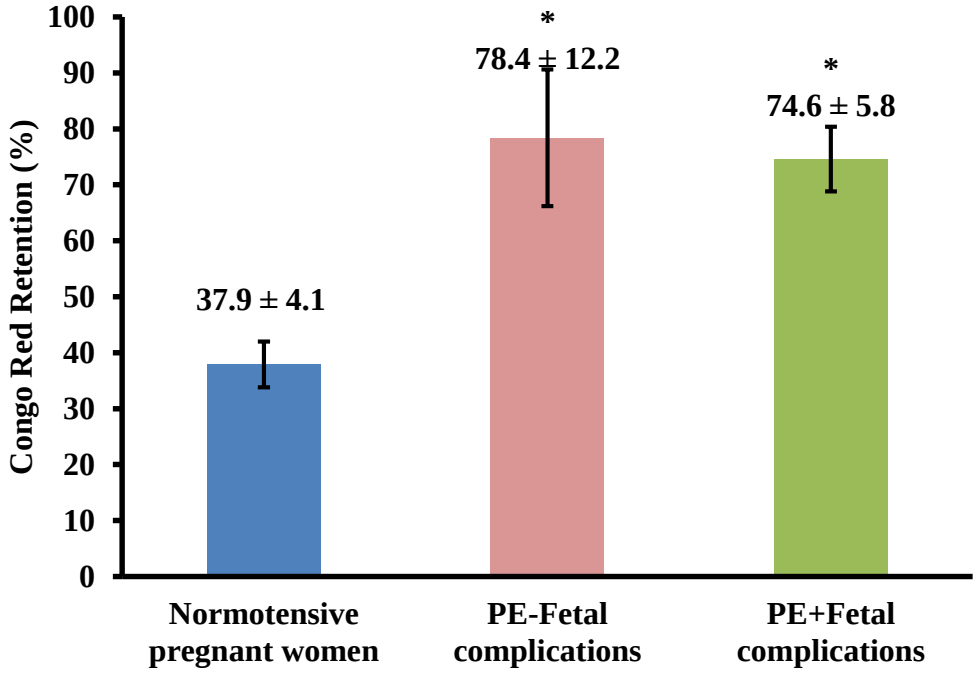
***** p < 0.001; w.r.t. normotensive pregnant women**; PE:** Preeclamptic women

Figure 5.4: Profile of mean CRR (%) values in normotensive pregnant women with respect to severity



***** p < 0.001; w.r.t. normotensive pregnant women**; PE:** Preeclamptic women

Figure 5.5: Profile of mean CRR (%) values in normotensive pregnant women with respect to PE superimposed with eclampsia



***** $p < 0.001$; w.r.t. normotensive pregnant women; **PE:** Preeclamptic women

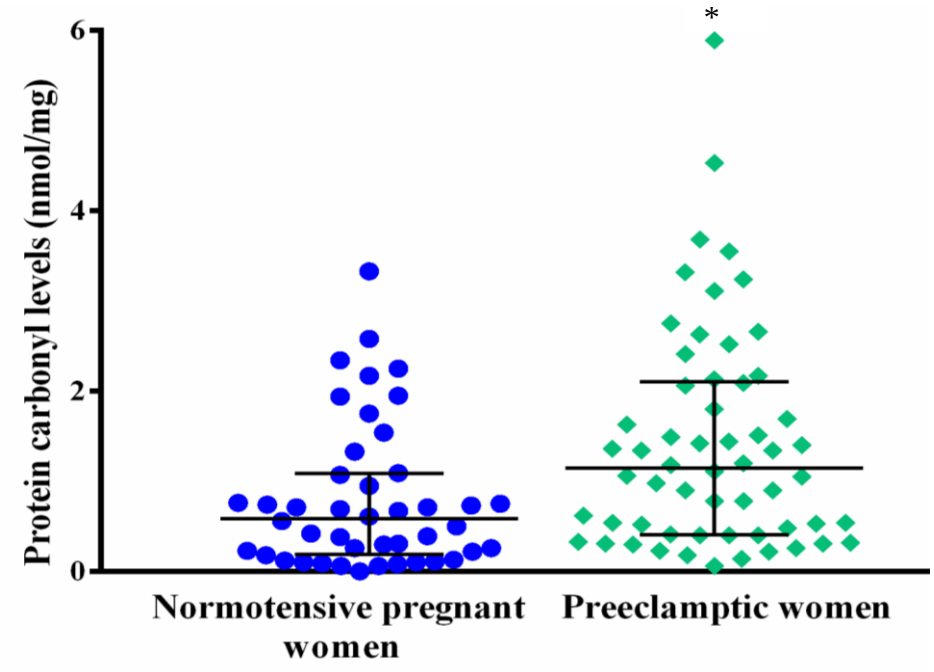
Figure 5.6: Profile of mean CRR (%) values in normotensive pregnant women with respect to PE with fetal complications

5.2. Levels of urinary protein oxidation are elevated in PE women:

The median protein carbonyl levels in the urine of PE women and normotensive pregnant women was 1.1 nmol/mg (IQR = 0.4 - 2.1 nmol/mg) and 0.58 nmol/mg (IQR = 0.19 - 1.08 nmol/mg) respectively. The protein carbonyl levels in the urine of PE women were 1.96 times higher as compared to normotensive pregnant women, which is statistically significant ($p = 0.002$; Mann-Whitney U test; 2-tailed) (Figure 5.7). PE patients were categorised based on the severity and gestational age of onset. The median urinary protein carbonyl levels obtained from normotensive pregnant women and the early-onset group ($p = 0.11$) was not significant. However, the late onset group showed a significantly higher protein carbonyl levels compared to normotensive pregnant women ($p = 0.001$) (Figure 5.8).

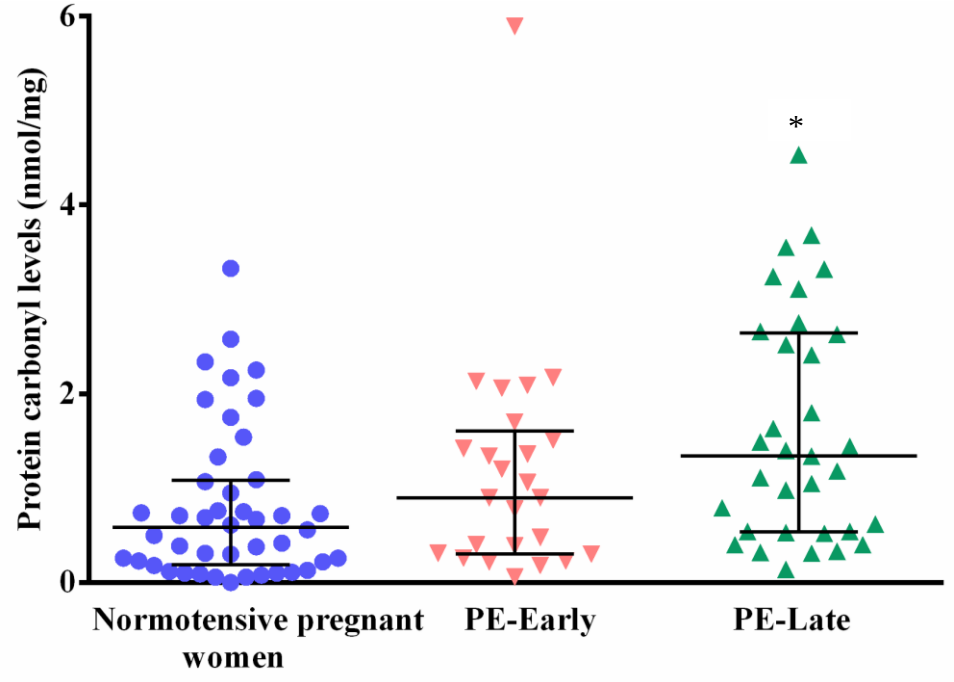
The median urinary protein carbonyl levels between normotensive pregnant women and the mild group was not statistically significant (p = 0.92). However, the difference in the median urinary protein carbonyl levels between normotensive pregnant women and the severe group was found to be statistically significant (p = 0.001) (Figure 5.9).

Further, the PE patients were categorised based on co-morbidities. The median urinary protein carbonyl levels obtained from normotensive pregnant women and PE superimposed with eclampsia was found to be statistically significant (p = 0.01) (Figure 5.10). In PE subgroup with fetal complications i.e., IUGR and stillbirth, the median urinary protein carbonyl levels were not statistically significant (p = 0.22). A comparison of urinary protein carbonyl levels between normotensive pregnant women and PE women with fetal complication is depicted in Figure 5.11.



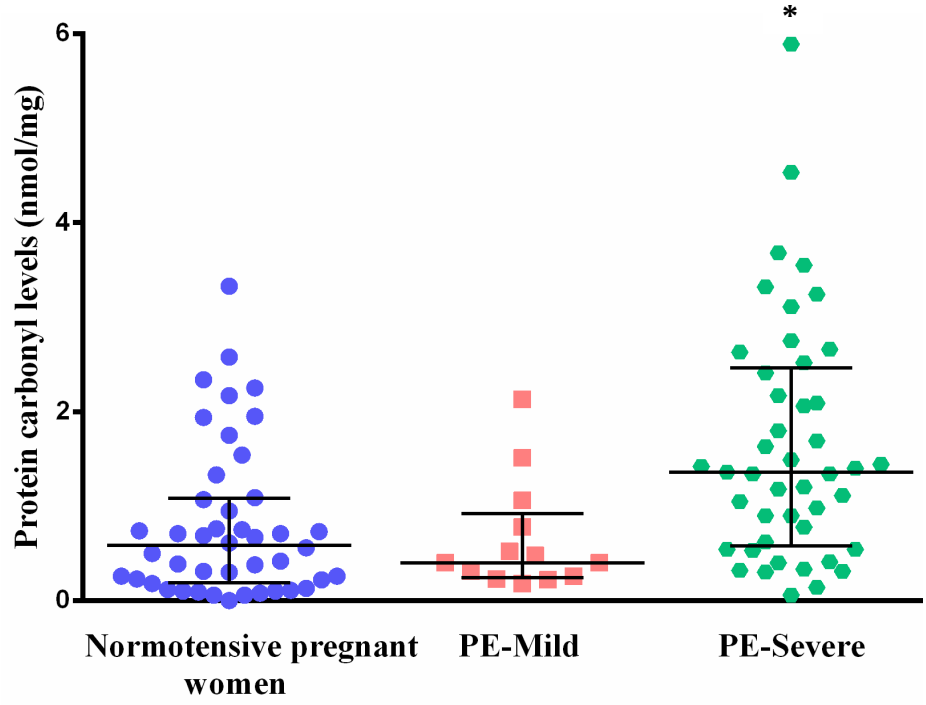
* p = 0.002; w.r.t. normotensive pregnant women

Figure 5.7: Comparison of urinary protein carbonyl levels in normotensive pregnant women and PE women



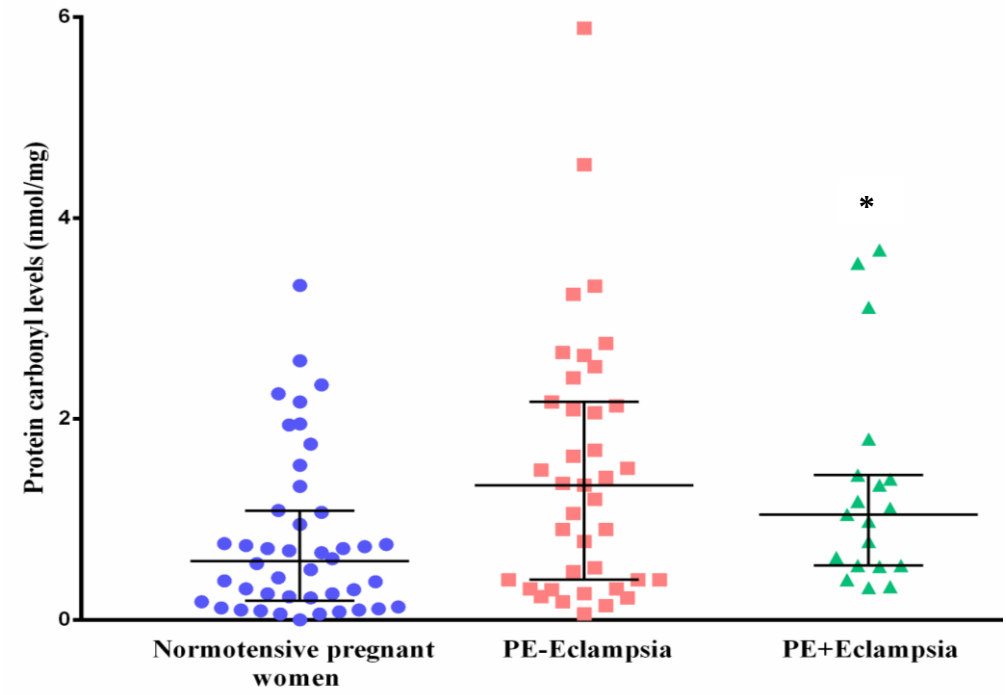
* p = 0.001; w.r.t. normotensive pregnant women; **PE**: Preeclamptic women

Figure 5.8: Comparison of urinary protein carbonyl levels between normotensive pregnant women and gestational age of onset



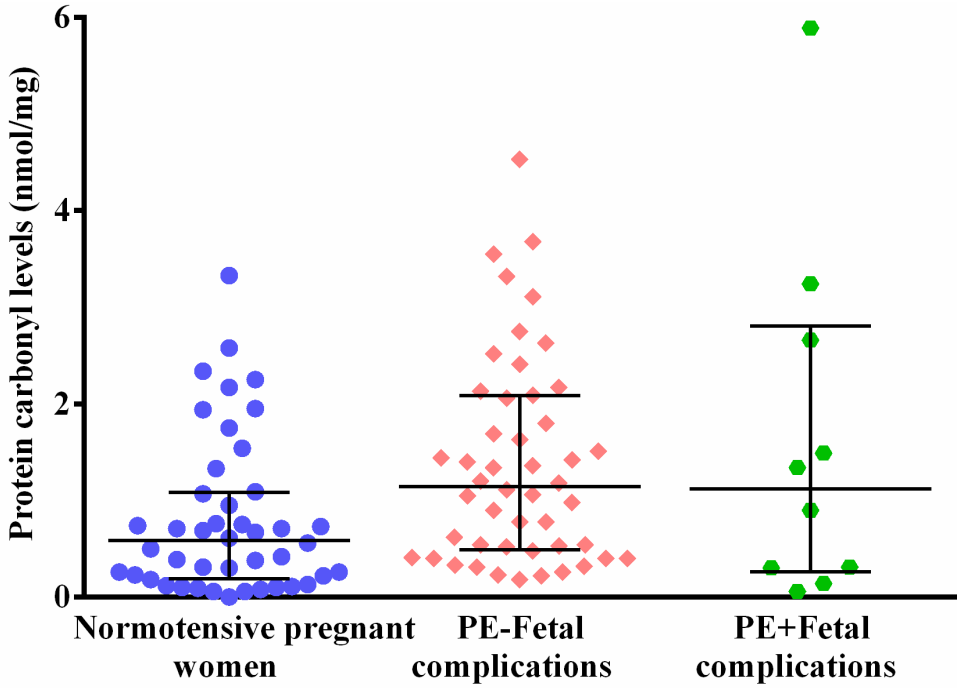
* p = 0.001; w.r.t. normotensive pregnant women; **PE:** Preeclamptic women

Figure 5.9: Comparison of urinary protein carbonyl levels between normotensive pregnant women and severity



* p = 0.01; w.r.t. normotensive pregnant women; PE: Preeclamptic women

Figure 5.10: Comparison of urinary protein carbonyl levels between normotensive pregnant women and PE superimposed with eclampsia



PE: Preeclamptic women

Figure 5.11: Comparison of urinary protein carbonyl levels between normotensive pregnant women and PE with fetal complications

5.3. Correlation between urinary protein misfolding and oxidation:

To find the association between protein carbonyl levels and CRR of the urine samples obtained from PE women Pearson's correlation analysis was performed. Correlation between urinary protein carbonyl levels and CRR in PE women is shown in Figure 5.12. Significant difference was observed in the correlation between the two parameters ($p = 0.018$) but it was found to be weak ($r = 0.3$).

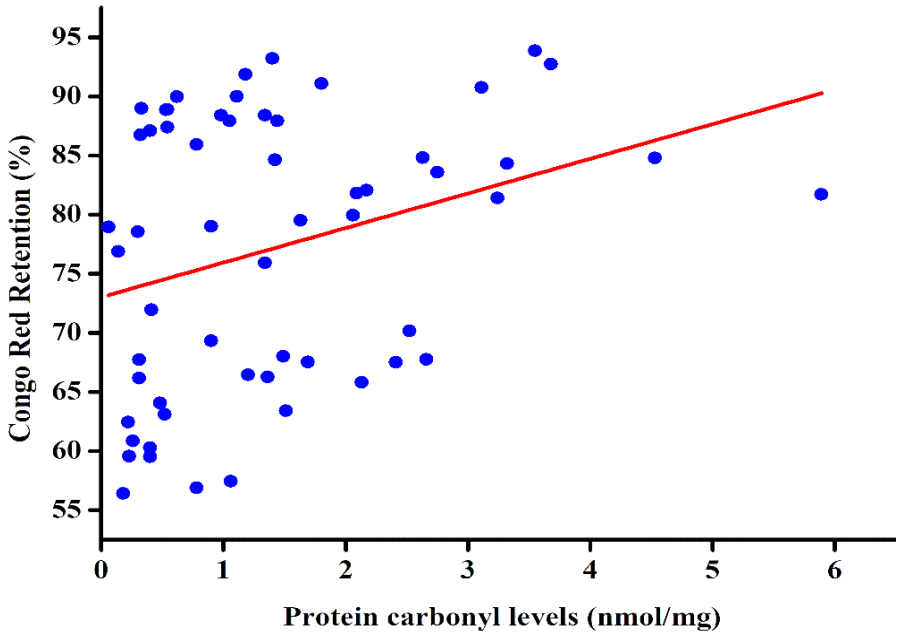


Figure 5.12: Correlation between urinary protein carbonyl levels and CRR in PE women

5.4. Frequency of PiS and PiZ alleles of *SERPINA1* gene in PE:

All the PE patients were genotyped for PiS and PiZ mutations of *SERPINA1* gene. Protein misfolding occurs only in PE women and not in normotensive pregnant women. Therefore, PiS and PiZ mutation were checked only in the PE women. PiS and PiZ mutations were not observed in any of the patients either in homozygous or heterozygous conditions: i.e all the samples were normal (homozygous for wild type allele). Representative images of PCR-RFLP band

pattern of PiS and PiZ on agarose gel electrophoresis are shown in Figure 5.13 and 5.14 respectively. Since all the samples showed negative results by PCR-RFLP method, a subset of the samples was subjected to complete sequencing for the confirmation. Furthermore, the study was extended to find any other mutations were involved. Ten samples were randomly chosen for the complete sequencing of *SERPINA1* gene by the Sanger sequencing method. Five unique mutations in PE women were found. The representative image of PCR amplicons of *SERPINA1* gene exons 2-5 on agarose gel electrophoresis is recorded in Figure 5.15. Electrophoregrams of *SERPINA1* gene mutations recorded in PE women is illustrated in Figure 5.16. Bioinformatics analysis indicates that these mutations had a benign effect on the stability of A1AT. The identified mutations were tabulated (Table 5.1).

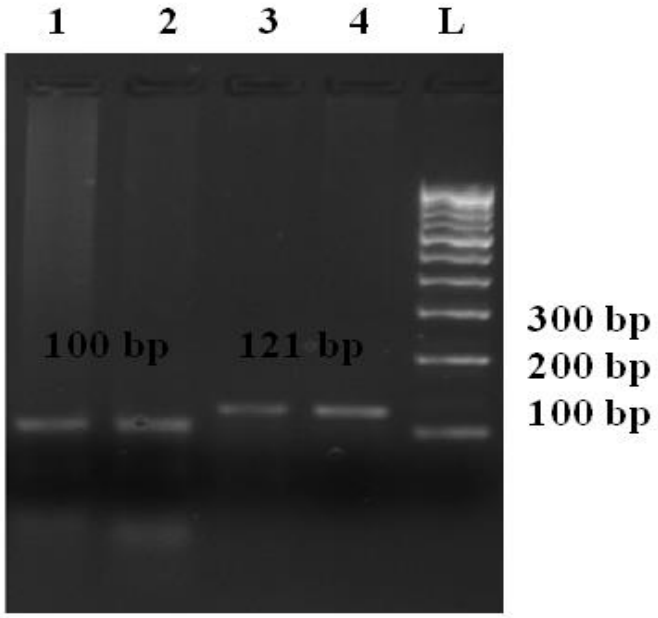


Figure 5.13: Representative image of PCR-RFLP band pattern of PiS on agarose gel electrophoresis. PCR-RFLP product (100+21 bp) (**Lanes-1 and 2);** PCR amplicon (121 bp) (**Lanes-3 and 4**); 100 bp DNA ladder **(L)**

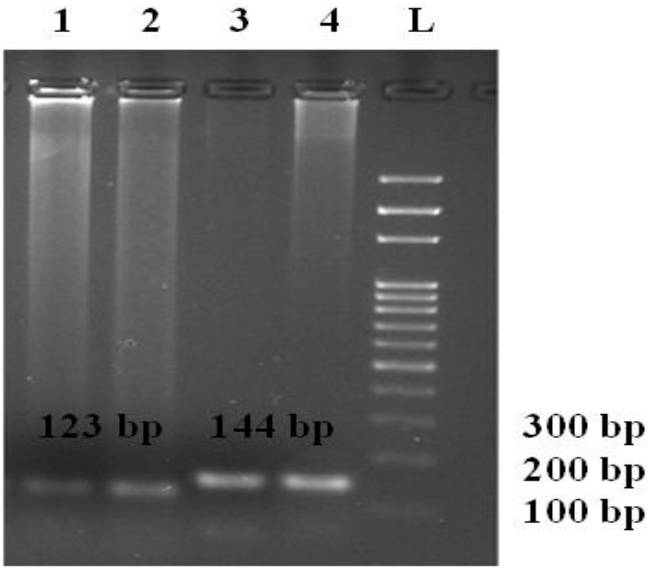


Figure 5.14: Representative image of PCR-RFLP band pattern of PiZ mutation on agarose gel electrophoresis. PCR-RFLP product (123+21 bp) (**Lanes-1 and 2**); PCR amplicon (144 bp) **(Lanes-3 and 4)**; 100 bp DNA ladder **(L)**

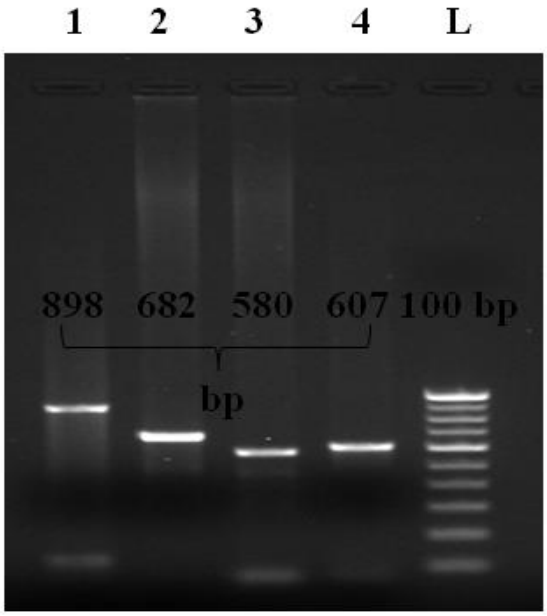


Figure 5.15: Representative image of PCR amplicons of *SERPINA1* gene exons 2-5 on agarose gel electrophoresis. Exon 2 (898 bp) **(Lane-**1); Exon 3 (682 bp) (**Lane-2**); Exon 4 (580 bp) (**Lane-3)**; Exon 5 (607 bp) (**Lane-4)**; 100 bp DNA ladder **(L)**

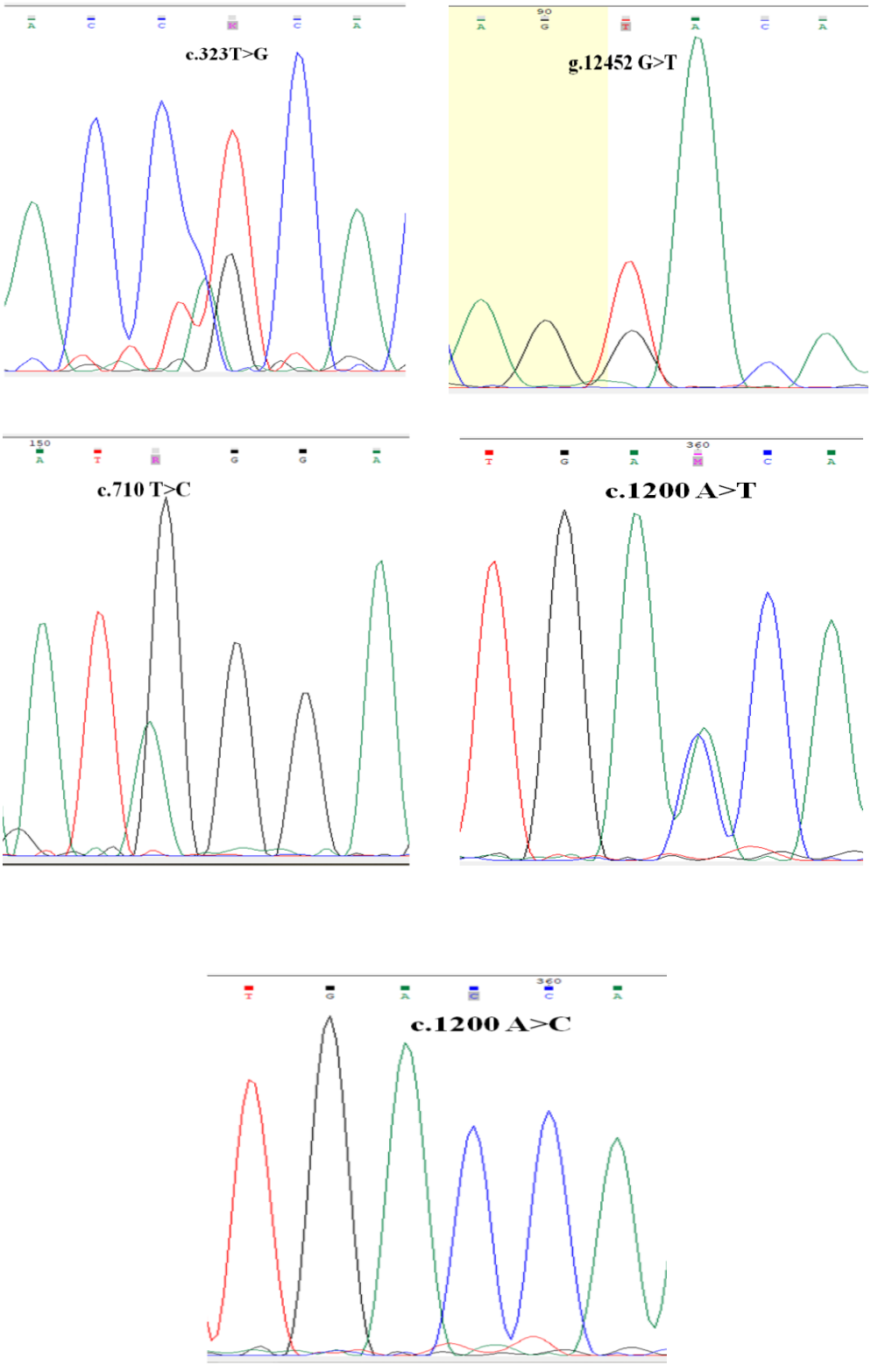


Figure 5.16: Electrophoregrams of *SERPINA1* gene mutations observed in PE women.

Table 5.1: Profile of mutations in *SERPINA1* gene among PE women

Exon Number	Mutation locus	Amino acid position	Frequency (n = 10)	Mutation type	Impact*
2	c.323 T>G	p. Leu108Arg	1 (10 %)	Missense	Benign
	g.12452 G>T	NA	4 (40 %)	Intronic variant	Benign
3	c.710 T>C	p.Val237Ala	2 (20 %)	Missense	Benign
5	c.1200 A>T	p. Glu400Asp	1 (10 %)	Missense	Benign
	c.1200 A>C	p.Glu400Asp	2 (20 %)	Missense	Benign

NA: Not applicable; * Impact on protein stability/mRNA splicing

Discussion

The aim of this study was to determine the role of protein misfolding within PE and its molecular and biochemical origin. The main findings of this study are:

i. Urinary misfolded protein levels are elevated in PE.
ii. Urinary misfolded protein levels are elevated in all the clinical subgroups of PE.
iii. Urinary protein carbonyl levels are elevated in PE and show a significant correlation with urinary protein misfolding.
iv. PiS and PiZ mutations of *SERPINA1* gene are absent in PE.

Together, these results support the inference that urinary misfolded protein level is associated with PE and this arises mainly due to oxidative damage.

Inference 1: The urinary misfolded protein level is associated with PE:

This study shows that urinary congophilia is associated with PE in Indian pregnant women. This is the first study to show this relationship in the Indian scenario. Previous investigations reported urinary congophilia mainly in the western populations (Buhimschi *et* al, 2014; McCarthy *et al*., 2016). The power of the study was > 90 %. This indicates the strength of the study.

In the present study, the mean CRR value was 37.9 % in the normotensive pregnant women. This is approximately two-fold higher than that reported in the previous studies (15 and 16 %). The variation in the mean CRR value might be due to the difference in the chemical composition of the nitrocellulose membrane used for the CRD assay. A supported form of nitrocellulose membrane was used in this study whereas the unsupported form of nitrocellulose membrane was used in the previous study (McCarthy *et al*., 2016). The supported form is supplanted with

additional chemicals that increase the strength of the membrane and make it convenient for handling. In contrast, the unsupported membrane is made up of pure nitrocellulose and is fragile to handle. Plasticizers like cellulose acetate are added to supported nitrocellulose membranes. The supplemented impurities in the supported membrane might have interfered with the removal of unbound Congo Red dye in the washing step. The results suggest that there is a statistically significant difference between normotensive pregnant women and PE women despite of using an impure supported membrane. This observation emphasizes the robustness of the CRD assay concerning the choice of the membrane, which permits the assay to translate into a bed-side clinical test.

Inference 2: Clinical variables do not affect the association between urinary misfolded protein levels and PE:

The clinical presentation of PE is not uniform but highly heterogeneous. The two major criteria for heterogeneity are gestational age of onset and severity on its association with urinary misfolded protein level was not known. The impact of clinical variables of PE on its association with urinary misfolded protein level was not known. An assay can be used as a clinical test only if the impact of the clinical variables is known. Therefore, the influence of gestational age of onset and severity on the levels of urinary misfolded protein in PE patients was analysed. Elevated levels of urinary misfolded protein were detected in all clinical subgroups of PE.

The urinary misfolded protein levels were also compared between the subgroups. There was a significant difference between early and late-onset PE. It was higher in the late-onset group than in the early-onset group Figure 5.3. This

observation sheds light on the potential origins of the misfolded proteins. Emerging evidence indicates that early-onset and late-onset PE are distinct types with separate pathophysiological basis (Raymond and Peterson, 2011; Vasapollo *et al*., 2008; Aksornphusitaphong and Phupong, 2013; Sulistyowati, 2017; Dadelszen *et al*., 2003; Herzog *et al*., 2017; Redman, 2017; Lisonkova and Joseph, 2013; Stergiotou *et al*., 2013; Gerasimova *et al*., 2019). Early-onset PE appears to be mainly linked to placental factors (Valensise *et al*., 2008). In contrast, late-onset PE appears to be linked to maternal factors such as pro-inflammatory cytokines and anti-angiogenic proteins that induce the activation of maternal endothelial cells through varied pathways (Chatterjee *et al*., 2014; Raghupathy, 2013; and La Marka *et al*., 2007).

Recent studies have shown that there is a difference in the protein misfolding pattern in the placenta of late-onset and early-onset PE. This was demonstrated based on the expression of proteins of the Unfolded Response Pathway (URP) (Yung *et al*., 2014). This pathway is activated as a counter mechanism to alleviate the effects of misfolded protein accumulation such as endoplasmic reticulum stress. The expression pattern of the URP was found to be higher in the case of early-onset PE (Burton and Yung, 2011). However, its pattern in the case of late-onset PE was more or less similar to the pattern in normotensive placentae. That is, protein misfolding in the placenta is restricted to early-onset PE. The protein misfolding pattern observed in the urine is different from that observed in the placenta (Buhimschi *et al*., 2014). This indicates that the placenta may not be the main source of misfolded proteins in the urine. A proteomic study of the misfolded proteins fraction of the urine has been carried out to identify the misfolded proteins. It was found that the misfolded protein fraction contains major

plasma proteins *viz* serum albumin, alpha-1 antitrypsin, IgG-kappa chain, and ceruloplasmin. Furthermore, misfolded protein has been demonstrated in the plasma of PE women (Buhimschi *et al*., 2014). These observations indicate that the urinary misfolded proteins in the PE women mainly arise from the maternal plasma.

Inference 3: Urinary protein carbonyl level is associated with PE:

To the best of our knowledge, this is the first study to show that the level of protein carbonyl is elevated in the urine of PE women. Previous studies have shown protein carbonyl levels in the maternal plasma are higher in pregnant women when compared to non-pregnant women (Zusterzeel *et al*., 2000). Furthermore, it has also been shown that the protein carbonyl levels are significantly higher in PE women when compared to normotensive women. The association between plasma carbonyl levels and PE has been demonstrated in several studies (Serdar *et al*., 2003; Tsukimori *et al*., 2008; Bharadwaj *et al*., 2018; Bernardi *et al*., 2008). The relative elevation in the level of protein carbonyl was found to be in the range of 1.6 - 2.7. Elevated levels of protein carbonyl in the cord blood of PE women have also been documented (Bernardi *et al*., 2012; Howlader *et al.,* 2009; Negi *et al*., 2014). The relative increase in the protein carbonyl levels in the cord blood of PE women is about 1.6 - 2.0 times higher than that from normotensive women. Association between elevated protein carbonyl levels and PE has also been reported and verified in the placenta (Zusterzeel *et al.,* 2001). The results obtained in this study extends the spectrum of protein oxidation in PE to urine. This observation holds translational value as urine can be invasively sampled for diagnostic or prognostic purposes.

The major causes of protein misfolding are overexpression and loss of protein stability. Oxidative damage to protein is a well-established cause for its misfolding through its effect on protein stability. Elevation of both protein misfolding and oxidation in the urine of PE women motivated the evaluation of correlation between them. There is a significant but weak correlation between protein carbonyl and CRR was observed. This indicates that protein oxidation, not the exclusive but one of the causes for urinary protein misfolding in PE.

As mentioned before, proteomic analysis has identified the presence of plasma proteins like albumin, alpha-1-antitrypsin, ceruloplasmin, IgG κ-free light chain, and interferon inducible protein 6–16 of the misfolded protein fraction of urine from PE women (Buhimschi *et al*., 2014). This indicates that the urinary misfolded proteins are likely to reflect plasma proteins that are misfolded due to elevated systemic oxidative stress. Oxidative stress is a biochemical hallmark of PE. Urinary misfolded proteins have also been shown in the case of chronic kidney disease (McCarthy *et al*., 2016). Kidney damage, which is common to both PE and chronic kidney disease, may have also been a source of misfolded proteins.

Inference 4: Common pathogenic mutations of *SERPINA1* gene are not associated with PE:

Currently, there is a paucity of information on the cause of protein misfolding in PE. Genetic mutations that reduced protein stability have been linked to protein misfolding. A1AT is an important plasma protein that has been identified in the misfolded protein fraction of the urine (Buhimschi *et al*., 2014). Furthermore, several reported studies, have shown that the plasma and placental level of A1AT is deficient in PE (Feng *et* al., 2016; Twina *et al*., 2012; Kunder *et al*., 2017).

Besides, A1AT has been shown to ameliorate PE in animal model studies (Feng *et al*., 2016).

A1AT is an important inhibitor of a plasma serine protease. Its main function is to neutralize the proteolytic activity of neutrophil elastase. This enzyme catalyzes the proteolytic degradation of extracellular matrix proteins such as proteoglycan, elastin, fibrinogen, and collagen present in the vascular basement membrane. This process plays an important role in the infiltration of neutrophils into the tissues. Reduced levels of A1AT alter the homeostatic balance between A1AT and elastase (Dunlea *et al*., 2018). The excess elastase activity then leads to excessive tissue damage. Tissue damage due to excessive elastase activity arising due to A1AT deficiency forms the pathophysiological basis of chronic pulmonary obstructive disease (Tuder *et al*., 2010).

A1AT enzyme is coded by the *SERPINA1* gene. PiS and PiZ are the two common mutations in the *SERPINA1* gene that induces misfolding of A1AT enzyme (Torres-Duran *et al*., 2018). Severe A1AT deficiency arises when these two mutations are present in the homozygous condition (Thun *et al*., 2012). However, they produce borderline deficiency with little clinical manifestation when they are present in the heterozygous condition (Zorzetto *et al*., 2008). Individuals who are heterozygous for PiS and PiZ mutations are predisposed to develop severe A1AT deficiency when exposed to compounding risk factors such as oxidative stress (Marcus *et al.,* 2012). For example, heterozygous individuals do not develop the chronic pulmonary obstructive disease; however, the risk is increased when these individuals are habituated to tobacco smoking (Fregonese and stolk, 2008). This is because the oxidative factors in the tobacco smoke inactivate the residual enzyme.

It was already mentioned that oxidative stress is a biochemical hallmark of PE. Therefore, it was assumed that women who are heterozygous for PiS and PiZ mutations may not manifest the clinical symptoms of A1AT deficiency but may be predisposed to misfolding of A1AT protein during pregnancy due to oxidative stress.

New Knowledge Generated

1. This study reaffirms the association between urinary misfolded protein and PE and further shows that the association is not affected by clinical variables such as gestational age of onset, severity, and co-morbidities. This aspect affirms the translation value of urinary misfolded protein in the diagnosis of PE.

2. This is the first study to show that urinary misfolded protein is associated with PE in Indian women.

3. This study shows that the urinary protein oxidation is elevated in PE and it might be the cause for urinary protein misfolding.

Strengths and Limitations

1. The availability of urine samples from PE women of different clinical subgroups is the main strength of this study. There were a sufficient number of PE women ($p > 0.05$) with early-onset (40.3 %) and late-onset (59.7 %) thus permitting us to evaluate the impact of the gestational age of onset on the level of urinary congophilia. There was adequate power (> 90 %) both at the level of the overall group and subgroups.

2. Several misfolded proteins such as serum albumin, alpha-1 antitrypsin, IgG-kappa chain, and ceruloplasmin have been identified in the urine of PE women. The genetic studies were restricted to only one protein *viz.*, A1AT due to resource limitation. Further studies are required to confirm the role of the genetic factor in causing protein misfolding in PE.

List of Publications

S.No.	Publication	Indexation
1	**Chandrakala N**, Rangappa SS, Suryanarayna R, Balakrishna S. Urinary congophilia in preeclampsia: Experience from a rural tertiary-care hospital in India. Pregnancy Hypertension, 2018;13:83-86.	PubMed Scopus Web of Science Impact Factor 1.99
2	**Chandrakala N**, Rangappa SS, Suryanarayana R, Balakrishna S. Urinary protein carbonyl levels and its correlation with protein misfolding in preeclampsia. Hypertension in Pregnancy, 2019;38(2):124-128.	PubMed Scopus Web of Science Impact Factor 1.2
3	**Chandrakala N**, Rangappa SS, Balakrishna S. Misfolding linked mutations of *SERPINA1* gene are uncommon in PE. Archives of Medicine and Health Sciences, 2019;7(2):177-180.	UGC-CARE list Group 1

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Appendices

APPENDIX – I

COMPOSITION OF BUFFERS AND REAGENTS

S.No.	Name of the reagent	Chemicals
1	**Erythrocyte lysis buffer (ELB)**	155 mM NH_4Cl 10 mM $KHCO_3$ 1 mM EDTA pH – 7.4
2	**NaCl**	5 M NaCl in water
3	**SDS**	20 % Sodium Dodecyl Sulphate in water
4	**Proteinase K**	20 mg Proteinase K in 1 ml of water
5	**Tris-EDTA buffer (TE)**	10 mM Tris base 1 mM EDTA pH – 8.0
6	**Tris-acetate-EDTA buffer (TAE)**	40 mM Tris 20 mM Acetic acid 1 mM EDTA pH – 8.4
7	**Phosphate buffered saline (PBS)**	137 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4 1.8 mM KH_2PO_4 pH – 7.4

APPENDIX- II

ETHICAL CLEARANCE CERTIFICATE

SRI DEVARAJ URS ACADEMY OF HIGHER EDUCATION & RESEARCH

SRI DEVARAJ URS MEDICAL COLLEGE

Tamaka, Kolar

INSTITUTIONAL ETHICS COMMITTEE

Members

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2. **Dr. Jagadamba. A** **Assoc. Prof of Physiology, SDUMC (Member Secretary)**
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10. Dr. Anand Tippanna Talikoti Prof. of Anaesthesia, SDUMC
11. Dr. Srinivasa Reddy.P Prof. of Forensic Medicine, SDUMC
12. Dr. Sumathi.M.E Assoc. Prof of Biochemistry, SDUMC
13. Dr. Bhuvana. Assoc. Prof of Pharmacology, SDUMC
14. Dr. Pavan, Asst. Prof. of Surgery, SDUMC
15. Dr.Hariprasad Asst. Prof. of Orthopedics, SDUMC

No. DMC/ KLR/ IEC/ 07/ 2017-18 Date: 02-05-2017

PRIOR PERMISSION TO START OF STUDY

The Institutional Ethics Committee Sri Devaraj Urs Medical College, Tamaka, Kolar has examined and unanimously approved the Ph.D Synopsis entitled **"Molecular and biochemical studies on misfolded protein in preeclampsia"** being investigated by Ms. Chandrakala N[1], Dr. Sharath B[1] and Dr. Sheela S R[2] in the Department of Cell Biology & Molecular Genetics[1] and OGB[2] at Sri Devaraj Urs Medical College, SDUAHER, Tamaka, Kolar.

Member Secretary
Institutional Ethics Committee
SDUMC, Tamaka Kolar

Member Secretary
Ethical Committee
SDUMC, Kolar.

Chairman
Institutional Ethics Committee
SDUMC, Tamaka Kolar

CHAIRMAN
Institutional Ethics Committee
Sri Devaraj Urs Medical College,
Tamaka, Kolar

APPENDIX - III

PROFORMA

PROJECT TITLE: MOLECULAR AND BIOCHEMICAL STUDIES ON MISFOLDED PROTEIN IN PE

I. General information

1. Case No : Date:
2. IP/OP No :
3. Patient's Name:
4. Age :
5. Address :
6. Phone No :

II. Family history

7. PE in mother **Yes/No** : Details:
8. PE in sister **Yes/No** : Details:

III. Personal history

9. Married: Consanguineous : Non-consanguineous:
10. History of present complaints:
11. History of hypertension :
12. PE : Yes/No:

IV. General examination

13. Height : Weight:
14. Blood pressure : Proteinuria:
15. Edema :

V. Obstetrical history

16. Period of amenorrhea :
17. Gestational age : Weeks Days
18. Gravida: Para: Living: Aborted/Dead:
19. Present pregnancy details :
20. Previous pregnancy details : Yes: No:
21. History:

VI. Laboratory tests

22. Complete blood count :

    Hemoglobin : Packed cell volume (PCV):

    Red blood cell count : White blood cell count:

    Platelet count :

23. Renal function test :

    Blood urea : serum creatinine: uric acid:

24. Liver function test:

    Transaminases: Aspartate transaminase (AST/SGOT):

    Aspartate transaminase (ALT/SGPT):

    Total bilirubin: Direct bilirubin:

    Alkaline phosphatase level test (ALP) :

    Gamma glutamyl transpeptidase (GGT) :

    Albumin: Globulin: Albumin/Globulin:

    Serum lactate dehydrogenase (LDH): Total protein :

INFORMED CONSENT FORM

IP/OP No. **Date:**

University: Sri Devaraj Urs Academy of Higher Education and Research

Title of the study: Molecular and biochemical studies on misfolded protein in PE

Name of the Investigator: Ms. Chandrakala N.

Name of Participant:

The following details has been described to me:

1. Three ml of venous blood in EDTA vacutainer and urine sample in sterile uricol will be collected.
2. For ideal test interpretation and data analysis, family history and clinical information are very much essential.
3. Participation in the study is completely voluntary and study participants need not pay for sample collection.
4. All the data and tests results are maintained with medical confidentiality and will not be revealed until and unless required by the law.

I grant my consent to make use of blood and urine samples for aforementioned research studies and give my sample, photographs to be pre-owned for the purpose of medical research, validation of test results and educational principle as long as my privacy is protected.

I understand that I can withdraw from this study at any time and this will not affect my future care. I have read and obtained a copy of this consent form.

I have perceived the information provided in this document and have the liberty to ask questions.

Patient's signature:

Date:

Witness name and signature: 1. **2.**

Date: **Date:**

Person obtaining consent form & his/her signature:

Date:

For any clarification, participant has the liberty to contact the Principal Investigator:

Principal Investigator: **Date:**

(**Ms. Chandrakala N.**)

Pregnancy Hypertension 13 (2018) 83–86

Contents lists available at ScienceDirect

Pregnancy Hypertension

journal homepage: www.elsevier.com/locate/preghy

Urinary congophilia in preeclampsia: Experience from a rural tertiary-care hospital in India

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ARTICLE INFO

Keywords:
Preeclampsia
Misfolded proteins
Urinary Congophilia

ABSTRACT

Objectives: To evaluate the presence of urinary congophilia among Indian patients with preeclampsia.
Study design: A prospective case control study in which congophilia of urine samples from preeclamptic pregnant women (n = 62) and normotensive pregnant women (n = 65) was compared by using Congo Red Dot Blot assay.
Main outcome measures: Presence of urinary congophilia.
Results: Mean percentage of Congo Red Retention was 37.9 ± 4.1 in the normotensive pregnant group and 77.9 ± 11.5 in the preeclamptic pregnant group ($P < .001$). The mean percentage of Congo Red Retention in both early-onset (70.5 ± 9.0) and late-onset (82.7 ± 10.3) groups were significantly higher than in normotensive controls ($P < .001$). The mean percentage of Congo Red Retention in mild (61.2 ± 3.2) and severe (82.4 ± 8.4) types of preeclampsia were also as significantly higher than in normotensive controls ($P < .001$). The mean percentage of Congo Red Retention in preeclampsia superimposed by eclampsia (89.4 ± 2.0) and preeclampsia complicated by intrauterine growth restriction and intrauterine death (74.6 ± 5.8) were significantly higher than in normotensive controls ($P < .001$).
Conclusions: The results of this study confirms the presence of urinary congophilia in Indian pregnant women with preeclampsia. Furthermore, our study shows that urinary congophilia is not affected by clinical variables like gestational age of onset, severity, superimposition by eclampsia and complication by intrauterine growth restriction and intrauterine death. Urinary congophilia can be used to differentially identify preeclamptic pregnant women from normotensive pregnant women.

1. Introduction

Mature proteins are folded into a specific three dimensional confirmation. Pathophysiological conditions in some diseases can disturb the ordered folding and lead to the formation of structurally abnormal misfolded proteins. Protein misfolding may contribute to disease pathogenesis by either reducing the biological activity of the protein or due to toxicity of the misfolded proteins [1,2]. Misfolded proteins are well known for their role in the formation of amyloid plaques seen in neurodegenerative disorders like Alzheimer's disease, Parkinson's disease, Huntington's disease, Amyotrophic Lateral Sclerosis, Spongiform Encephalopathy and Familial Amyloidotic Polyneuropathy [3]. An azo dye called Congo Red exhibits a special affinity for misfolded proteins (a property referred to as congophilia) and is used as gold standard to identify amyloid fibrils [4,5]. Recent studies have shown that misfolded proteins are abundantly present in the urine of pregnant women who develop preeclampsia [6,7].

In addition to its importance in understanding the pathophysiology of preeclampsia, misfolded proteins are indicated to hold diagnostic and prognostic value [6]. Pilot studies have indicated that misfolded proteins appear in urine well before the onset of clinical symptoms [6]. This aspect along with the ease of determining the urinary misfolded proteins holds promise for early diagnosis and risk prediction of preeclampsia particularly in resource limited settings like in developing and underdeveloped nations. Urinary misfolded proteins can be detected by a simple paper-based dot-blot technique using congo red staining; the method is referred to as Congo Red Dot Blot (CRDB) [6]. Congo red is a synthetic diazo dye with specific affinity for β-sheets of

Abbreviations: CRDB, Congo Red Dot Blot; CRR, Congo Red Retention; PE, preeclampsia; IUGR, intrauterine growth restriction; IUD, intrauterine death; HELLP, Hemolysis Elevated Liver Low Platelet Count

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https://doi.org/10.1016/j.preghy.2018.05.006
Received 29 January 2018; Received in revised form 28 March 2018; Accepted 13 May 2018
Available online 14 May 2018

amyloid fibrils of misfolded proteins [8–10]. This special affinity of misfolded proteins to congo red dye is known as congophilia. In addition to the simplicity of congophilia based dot blot technique, the non-invasiveness of the technique is useful in adapting the CRBD assay for self-collected urine samples.

In order to establish the clinical utility of CRDB assay, extensive data from diverse geo-ethnic populations is necessary. At present, there are only two studies on the presence of misfolded protein in the urine of preeclamptic women. Both of these studies were carried out in western population mainly involving pregnant women of Caucasian descent [6,7]. There are no studies from developing societies where this assay promises the highest benefit to maternal health management. In this study, we have evaluated the presence of misfolded proteins in the urine of preeclamptic women in a tertiary care hospital located in a rural and economically backward area in India.

2. Materials and methods

2.1. Study design

We conducted a case control study comprising of pregnant women with and without preeclampsia. The study was approved by the Institutional Ethics Committee of Sri Devaraj Urs Medical College, Kolar, Karnataka, India. Informed consent was obtained in writing before recruiting the patients. Patients were recruited from the Department of Obstetrics and Gynecology of R. L. Jalappa Hospital and Research Centre attached to Sri Devaraj Urs Medical College between November 2016 – October 2017. A total of 127 pregnant women were recruited of whom 62 were affected with preeclampsia while the remaining 65 pregnant women were normotensive and presented no complications till delivery. Midstream urine sample was collected from all the study participants and congophilia was measured by CRDB assay.

2.2. Patient selection

Women were diagnosed with preeclampsia based on the following criteria: i) new onset hypertension (two readings of systolic blood pressure $\geq$140 mm Hg or diastolic blood pressure $\geq$90 mm Hg measured 4 h apart while the patient is on bed rest ii) $\geq$20 weeks of gestation iii) new onset proteinuria (> 300 mg protein for 24 h of urine or +1 on dipstick) iv) in the absence of proteinuria, other symptoms like Hemolysis Elevated Liver Low Platelet counts syndrome, edema, thrombocytopenia, impaired liver function, new-onset cerebral or visual disturbances and renal insufficiency (in the absence of other renal disease) nausea, severe headache and convulsions [11]. Inclusion criteria were: (i) pregnant women with preeclampsia, (ii) superimposed eclampsia, (iii) singleton and multiple gestation and, (iv) primigravida and multigravida condition. Exclusion criteria were: (i) pregnant women with chronic hypertension and (ii) co-morbidities such as diabetes mellitus, epilepsy, respiratory diseases, and heart diseases.

2.3. Sample collection and storage

Midstream urine samples were collected in sterile urine container (Himedia, Mumbai. India) and centrifuged at 2500 rpm for 5 min. The supernatant was stored at -80 °C till analysis.

2.4. Congo Red Dot Blot assay

Total protein content of the urine sample was estimated by Bradford method using Bovine Serum Albumin as the standard [12]. Protein concentration of all the urine samples were normalized to 15 μg/ml by concentration or dilution depending on the starting level. Concentration of the urine samples were carried out by using dialysis method [13]. CRDB assay was performed according to the method of Buhimschi *et al* [6]. 5 μl of congo red in water (5 μg/ml) was added to 100 μl of normalized urine sample and vortexed for 1 h at room temperature. 5 μl of vortexed mix was spotted on a strip of nitrocellulose membrane (Himedia, Mumbai, India) in duplicates. The spot was air dried for 15 min, and washed with Milli-Q water for 3 min. Image of the membrane strip was captured using Gel Doc Molecular imager (Bio-Rad, Hercules, USA). The membrane was then sequentially washed with 50% methanol for 3 min, 70% methanol for 1 min and 90% methanol for 10 min. The image of the washed spot was again captured as before. Colour intensity of the spot was measured using Image Lab software which was available in the Gel Doc instrument. Congo Red Retention (CRR) was calculated using the formulae given below and expressed in percentage.

$$CRR(\%) - \left(\frac{Spot\ \text{int}ensity\ after\ wash}{Spot\ \text{int}ensity\ before\ wash} \right) 100$$

2.5. Statistical analysis

Statistical analysis was performed with the use of IBM SPSS Statistics for Windows, Version 20.0. The quantitative data was presented using mean, standard deviation and confidence interval and qualitative data by percentages. The data was checked for normality using Shapiro-Wilk test. ANOVA was used to compare the difference in the means across the groups. Post-hoc test was used for pairwise comparison. P value < 0.05 was considered as statistically significant.

3. Results

The baseline characteristics and clinical outcome profile of the participants in the study groups is depicted in Table 1. The preeclamptic group includes clinical subgroups with respect to severity, gestational age of onset and fetal complications in the form of intrauterine growth restriction (IUGR) and intrauterine death (IUD). The CRR values were found to show normal distribution. Post-hoc analysis indicated that there was > 90% power to detect at least 15% difference in CRR value.

The mean CRR (%) of preeclamptic pregnant women was 77.9 ± 11.5 which is 2.1 times higher than that of mean CRR value of normotensive pregnant women which was 37.9 ± 4.1 (Fig. 1). The difference in the mean CRR of the two groups was found to be statistically significant ($P < .001$). We also analyzed the data after stratifying the preeclamptic patients into clinical subgroups. The mean CRR (%) value of preeclamptic women with early-onset (70.5 ± 9.0) and late-onset (82.7 ± 10.3) was 1.9 and 2.2 times higher relative to that of the mean CRR of normotensive pregnant women. The mean CRR of the two groups were significantly different from that of the normotensive control groups ($P < .001$). The mean CRR (%) of mild subtype (61.2 ± 3.2) and severe subtype (82.4 ± 8.4) of preeclampsia was 1.6 and 2.2 times higher than that of normotensive pregnant women. The mean CRR of the mild and severe groups were significantly different from that of the normotensive control groups ($P < .001$).

We also analyzed the data after stratifying w.r.t., the presence of co-morbidities. The mean CRR (%) of preeclamptic pregnant women superimposed with eclampsia (89.4 ± 2.0) was 2.4 times higher than that of normotensive pregnant women ($P < .001$). The mean CRR (%) of preeclamptic subgroup with fetal complications i.e., IUGR and IUD (74.6 ± 5.8) was 2.0 times higher than that of normotensive pregnant women ($P < .001$).

4. Discussion

This study confirms the presence of urinary congophilia in Indian women with preeclampsia. Hitherto, the data on urinary congophilia was restricted to western populations. Availability of urine samples from preeclamptic women of different clinical subgroups is main

Table 1
Baseline parameters and clinical outcome of the study participants.

Parameter	Preeclamptic pregnant women (n = 62)		Normotensive pregnant women (n = 65)
Age (years)	23.9 ± 3.6		24.2 ± 3.1
Gravida			
• Primigravida	• 33 (53.2%)		• 26 (40%)
• Multigravida	• 29 (46.8%)		• 39 (60%)
Severity			
• Mild	• 13 (20.9%)		NA
• Severe	• 49 (79.1%)		
Gestational age of onset			
• Early onset (< 34 weeks)	• 25 (40.3%)		NA
• Late onset (≥34 weeks)	• 37 (59.7%)		
Blood pressure (mm Hg)	Mild PE	Severe PE	
• Systolic Blood Pressure	144.6 ± 5.9	172.3 ± 13.5	116.9 ± 4.9
• Diastolic Blood Pressure	93.5 ± 3.8	113.5 ± 4.4	75.5 ± 5.4
Proteinuria			
• 1+	• 37 (59.6%)		NA
• 2+	• 15 (24.1%)		
• 3+	• 10 (16.1%)		
Co-morbidity			
• Eclampsia	• 23 (37.0%)		NA
• IUGR	• 7(11.3%)		
• IUD	• 3(4.8%)		
• HELLP syndrome	• 1 (1.6%)		

NA = Not Applicable.

strength of this study. There were sufficient number of preeclamptic women ($P > .05$) with early-onset (40.3%) and late-onset (59.7%) thus permitting us to evaluate the impact of gestational age of onset on the level of urinary congophilia. There was adequate power (> 90%) both at the level of the overall group and subgroups.

Our findings that, there is significant difference in the level of urinary congophilia between early-onset and late-onset of preeclampsia provides useful insight into the source of misfolded proteins. An expanding body evidences indicate that early-onset and late-onset preeclampsia are distinct entities with respect to the underlying pathophysiology [14–22]. Placental factors are indicated to be predominantly associated with early-onset while maternal factors are mostly associated with late-onset preeclampsia. Maternal factors include pro-inflammatory cytokines and anti-angiogenic factors that induces the activation of maternal endothelial cells through diverse pathways [23–25]. Recent studies have shown that the activation of Unfolded Response Pathway in the placenta is different between early-onset and late-onset preeclampsia [26]. Compared to the placenta from early-onset preeclampsia, the nature of Unfolded Response Pathway was found to be similar in both late-onset and normotensive placentae. Our findings with significant difference in urinary congophilia between early-onset and late-onset preeclampsia indicates that placenta may not be the main source of misfolded proteins in the urine. In the studies by Buhimschi and co-workers, major plasma proteins like serum albumin, alpha-1 antitrypsin, ceruloplasmin, and IgG-kappa chain were identified in the misfolded protein fraction of urine [6]. In addition, presence of misfolded protein has been demonstrated in the plasma of preeclamptic women [27]. Thus urinary misfolded proteins in preeclamptic women appear to be derived mostly from the plasma. Furthermore, a growing body of evidences indicate that endothelial dysfunction, a cardinal pathological feature in preeclampsia, is a consequence of chronic activation of Unfolded Response Pathway [28]. In

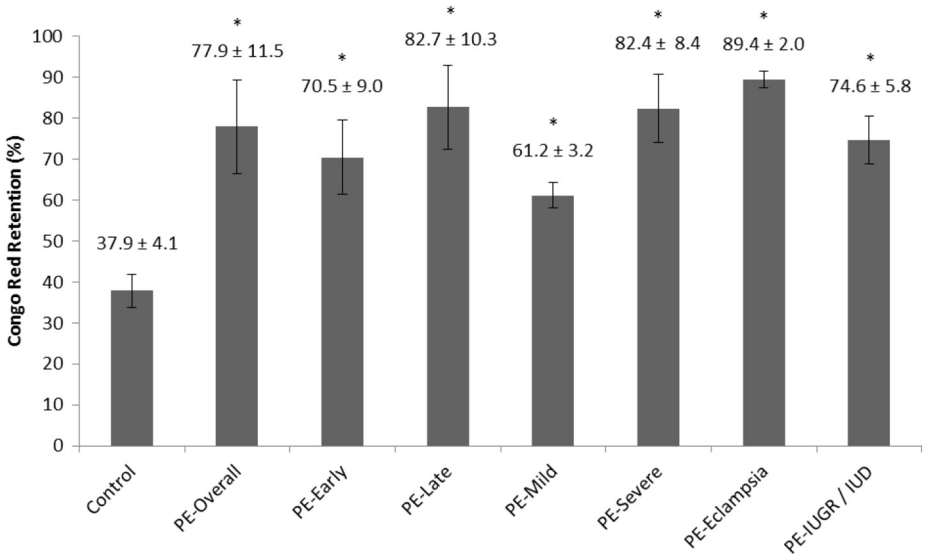

Fig. 1. Profile of mean Congo Red Retention (%) values in the study groups. ($^{*}P < .001$; w.r.t. normotensive control group) (PE: Preeclmapsia).

this light, we are compelled to assume that misfolded proteins in the urine of preeclamptic women reflects maternal and not placental etiologic factor. Furthermore, the maternal factor that leads to urinary misfolded proteins appears to be shared by both early-onset and late-onset preeclampsia and thus represents a core pathophysiological event.

Studies by McCarthy et al found that urinary congophilia was present in women with chronic kidney disease. This issue questions the clinical utility of urinary congophilia in the management of preeclampsia. At present, there is a paucity of data on the prevalence of chronic kidney disease among pregnant women in India. However, in the general Indian population, the mean age for CKD is in the range of 35.6 − 45.2 years [29]. In contrast, the mean age at first birth (given the predominance of preeclampsia in primigravida) in the Indian subcontinent is about 22.7 years [30]. It appears that the age spectrum for the risk of preeclampsia and CKD are separated by at least 10 years in the Indian population. Thus, CKD may not be a major confounding factor in the clinical utility of urinary congophilia. Furthermore, we argue that the utility of urinary congophilia is mainly as a test for screening pregnant women for the risk of preeclampsia rather than as a diagnostic test for women with symptoms indicative of preeclampsia. Screening tests are offered to large group of patients while diagnostic tests are offered to specific patients with reasonable clinical indications. The cost of the screening test is under pressure since it has to cover large group of patients of whom only a small fraction eventually derive its benefit. CRDB holds promise as a screening test as the cost of the assay consumables and the amount of labour involved are negligible.

This study confirms that urinary congophilia level is elevated in preeclamptic women compared to normotensive pregnant women in the Indian population. The level of congophilia varies across the clinical subgroups of preeclampsia but the average level remains significantly higher than the normotensive pregnant women. We are motivated to explore the utility of CRDB assay in the prediction of preeclampsia well in advance of the onset of clinical symptoms.

5. Funding:

No specific funding was available for this study.

6. Disclosure of interests:

The authors declare that they have no conflict of interests.

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Hypertension in Pregnancy

ISSN: 1064-1955 (Print) 1525-6065 (Online) Journal homepage: https://www.tandfonline.com/loi/ihip20

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To cite this article: Chandrakala Nagarajappa, Sheela Shikaripur Rangappa, Ravishankar Suryanarayana & Sharath Balakrishna (2019): Urinary protein carbonyl levels and its correlation with protein misfolding in preeclampsia, Hypertension in Pregnancy

To link to this article: https://doi.org/10.1080/10641955.2019.1590720

Published online: 26 Mar 2019.

HYPERTENSION IN PREGNANCY
https://doi.org/10.1080/10641955.2019.1590720

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Urinary protein carbonyl levels and its correlation with protein misfolding in preeclampsia

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ABSTRACT

Objective: To evaluate the association of protein carbonylation with preeclampsia and its correlation with urinary protein misfolding.

Method: Protein carbonyl and misfolded protein levels were measured in the midstream urine sample (58 preeclamptic and 44 normotensive pregnancy) by ELISA and Congo Red Dot assay respectively.

Results: Significant difference was observed in the levels of protein carbonyls (P = 0.002) and misfolded proteins (P = 0.001). Correlation between protein carbonyl and misfolded proteins levels was significant but weak (r = 0.3; P = 0.018).

Conclusion: Urinary protein carbonyl level is elevated in preeclampsia but plays a minor role in proteins misfolding.

ARTICLE HISTORY
Received 18 December 2018
Accepted 1 March 2019

KEYWORDS
Preeclampsia; protein carbonyl; misfolded proteins

Introduction

Preeclampsia is a common complication of pregnancy that is characterized by *de novo* hypertension and proteinuria. Preeclampsia is a major public health burden that affects 5–8% of pregnancies globally (1). Also, it is one of the leading cause of perinatal morbidity and mortality. Impaired placental vascularization and endothelial damage are the etiological hallmarks of preeclampsia. Endothelial damage has been documented in the vessels of the glomerulus, umbilical cord, and placenta (2). Also, markers of endothelial activation like adhesion molecules, cytokines, and pro-coagulant and anti-angiogenic factors (sFLT-1 and sENG) are elevated in the plasma of preeclamptic women (2). Endothelial dysfunction is assumed to be responsible for the proteinuria in preeclampsia (2).

Oxidative stress plays an important role in endothelial damage. Involvement of oxidative stress in preeclampsia is indicated by the elevated levels of oxidative stress markers in the placenta, maternal, and fetal plasma. Commonly observed markers of oxidative stress are protein carbonyl (due to protein oxidation), malonaldehyde (due to lipid peroxidation), and 8-hydroxy-2′-deoxyguanosine (due to DNA oxidative) (3–5). Furthermore, a reduction in the total antioxidant status has been demonstrated in the maternal plasma and placenta (6).

Urine is an important specimen in preeclampsia since it is diagnostically altered in most patients due to proteinuria. Recent studies have shown that the urine of preeclamptic women contains misfolded proteins (7–10). This observation extends the spectrum of abnormalities in the urine of preeclamptic women. Though the presence of misfolded proteins has been confirmed by subsequent studies, the cause of misfolding is still not clear. Protein oxidation may be linked to the misfolding of urinary proteins in preeclamptic women. Oxidative damage is an established factor in protein misfolding (11). The aim of this study was to evaluate the urinary levels of protein carbonyl in preeclamptic pregnant women and its correlation with urinary protein misfolding.

Materials and methods

Study design

We conducted a case–control study by including 44 normotensive and 58 preeclamptic pregnant women, respectively. Subjects were enrolled between November 2016 – October 2017 from the Department of Obstetrics and Gynecology of R. L. Jalappa Hospital and Research Centre attached to Sri Devaraj Urs Medical College. The study was approved by the

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Institutional Ethics Committee of Sri Devaraj Urs Medical College, Kolar, Karnataka, India. Written informed consent was obtained prior to the recruitment of the subjects. Midstream urine sample was collected from the study participants and used to evaluate the levels of protein carbonyl and misfolded proteins.

Patient selection

Preeclampsia was diagnosed in the pregnant women on the basis of the following criteria: (i) *de novo* hypertension (systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg measured 4 h apart twice while the patient is on bed rest, (ii) ≥20 weeks of gestation, (iii) new onset proteinuria (≥300 mg protein in 24-h urine sample or +1 on dipstick), (iv) in the absence of proteinuria, other symptoms like Hemolysis Elevated Liver Low Platelet counts syndrome, edema, thrombocytopenia, impaired liver function, new-onset cerebral or visual disturbances and renal insufficiency (in the absence of other renal diseases) nausea, severe headache, and convulsions (12). Inclusion criteria for the cases were: (i) pregnant women diagnosed with preeclampsia, (ii) superimposed eclampsia, (iii) singleton and multiple gestation, and (iv) primigravida and multigravida condition. Exclusion criteria for the cases were: (i) pregnant women with chronic hypertension and (ii) co-morbidities such as diabetes mellitus, epilepsy, respiratory diseases, and heart diseases. Pregnant women were defined as "normotensive" if they did not develop any complications till the time of delivery.

Sample collection and storage

Midstream urine sample was collected in a sterile urine container (Himedia, Mumbai, India) and centrifuged at 2,500 rpm for 5 min at room temperature. The supernatant was preserved at – 80°C till further analysis.

Estimation of protein concentration

Total protein content of the midstream urine sample was determined by the Bradford method using standard Bovine Serum Albumin (13). Urine samples were normalized to the required protein concentration by dilution or concentration by dialysis method (14).

Estimation of urinary protein carbonyl

Protein concentration of the urine samples was normalized to 10 µg/ml. Protein carbonyl levels of the normalized sample were determined by the ELISA method using a commercial kit (Cell BioLabs, San Diego, CA).

Estimation of urinary misfolded proteins

Protein concentration of the urine samples was normalized to 15 µg/ml. Misfolded protein levels were determined by Congo Red Dot assay (8). One hundred µl of the normalized urine sample was mixed with 5 µl of Congo Red (5 µg/ml in water) and vortexed for 1 h at room temperature. Five µl of the mix was spotted in duplicates on a strip of supported nitrocellulose membrane (Himedia, Mumbai, India), air dried for 15 min, and then washed with Milli-Q water for 3 min. Image of the nitrocellulose membrane strip was recorded using Gel Doc Molecular imager (Bio-Rad, Hercules, USA). Nitrocellulose membrane strip was then sequentially washed with 50% methanol for 3 min, 70% methanol for 1 min and 90% methanol for 10 min. The image of the washed spot was recorded as before. The color intensity of the spot was measured by using Image Lab software (Bio-Rad, Hercules, USA). Percentage of Congo Red Retention (CRR) was determined using the formula given below.

$$CRR\ (\%) = \left(\frac{Spot\ intensity\ after\ wash}{Spot\ intensity\ before\ wash}\right) 100$$

Statistical analysis

Statistical analysis was carried out using SPSS Statistics V22.0 (International Business Machine Corporation, Armonk, New York). Quantitative variables were represented as mean, standard deviation and confidence interval. Qualitative variables were represented as percentages. Shapiro–Wilk test was performed with Q–Q plots and normality plots. Mean was determined if the data showed normal distribution; otherwise, the median was calculated. Means of the two groups was compared using student t-test while medians of the two groups were compared using the Mann–Whitney U test. Pearson's correlation coefficient was used to assess the correlation between the variables. P value <0.05 was considered to be statistically significant.

Results

The clinical parameters of the study participants are summarized in Table 1. A total of 102 pregnant women were included in the study. Of these, 58 women were preeclamptic and 44 women were normotensive pregnant. The

Table 1. Clinical parameters of the study groups.

Parameter	Preeclamptic pregnant women (n = 58)	Normotensive pregnant women (n = 44)
Age (years)	23.9 ± 3.5	24.6 ± 3.3
Gravida	• 32 (55.2%)	• 19 (43.2%)
• Primigravida	• 26 (44.8%)	• 25 (56.8%)
• Multigravida		
Severity	• 13 (22.4%)	NA
• Mild	• 45 (77.6%)	
• Severe		
Gestational age of onset	• 25 (43.1%)	NA
• Early onset (< 34 weeks)	• 33 (56.9%)	
• Late onset (≥ 34 weeks)		
Blood pressure (mm Hg)	• 166 ± 17.1	• 117.0 ± 4.6
• Systolic Blood Pressure	• 108.9 ± 9.4	• 75.9 ± 4.9
• Diastolic Blood Pressure		
Dipstick proteinuria	• 36 (62.1%)	NA
• 1+	• 14 (24.1%)	
• 2+	• 08 (13.8%)	
• 3+		
Co-morbidities	• 19 (32.8%)	NA
• Eclampsia	• 7(12.1%)	
• IUGR	• 3(5.2%)	
• Stillbirth	• 1 (1.7%)	
• HELLP syndrome		

NA = Not Applicable

protein concentration of the urine samples was normalized to ensure that the differences observed in the study parameters reflected baseline modifications and were not due to proteinuria.

Firstly, we determined the urinary levels of protein carbonyl both in preeclamptic and normotensive pregnant women (Figure 1). The protein carbonyl levels did not show normal distribution. Therefore, we calculated the median and interquartile range (IQR) for both the patient groups. The median protein carbonyl levels in the urine were 1.1 nmol/mg (IQR = 0.4–2.1 nmol/mg) in the preeclamptic women and 0.58 nmol/mg (IQR = 0.19–1.08 nmol/mg) in the normotensive women. The urinary protein carbonyl levels in the preeclamptic pregnant women were 1.96 times higher than in the normotensive pregnant women. The difference in the urinary protein carbonyl levels between the two groups was statistically significant (P=0.002; Mann–Whitney U test; 2-tailed).

Secondly, we determined the urinary levels of misfolded protein by measuring the percentage of Congo Red Retention (Figure 2). Misfolded protein levels in both preeclamptic and normotensive pregnant women followed the normal distribution. Therefore, we calculated the mean and standard deviation for each patient group. The mean Congo Red Retention of the urine was 77.2 ± 11.5% in the preeclamptic women and 38.0 ± 4.3% in the normotensive women. The mean Congo Red Retention of urine from preeclamptic pregnant women was 2.03 times higher than that of normotensive pregnant women. The difference in the means of Congo Red Retention between the two groups was statistically significant (P=0.001; student t-test).

Thirdly, we carried out Pearson's correlation test to determine the correlation between protein carbonyl levels and Congo Red Retention of the urine from preeclamptic

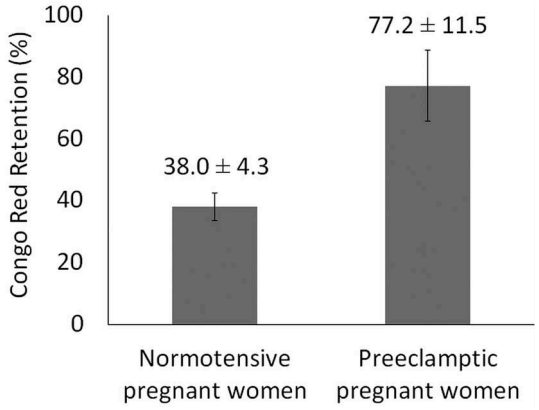

Figure 2. Comparison of urinary misfolded protein levels in preeclamptic and normotensive pregnant women.

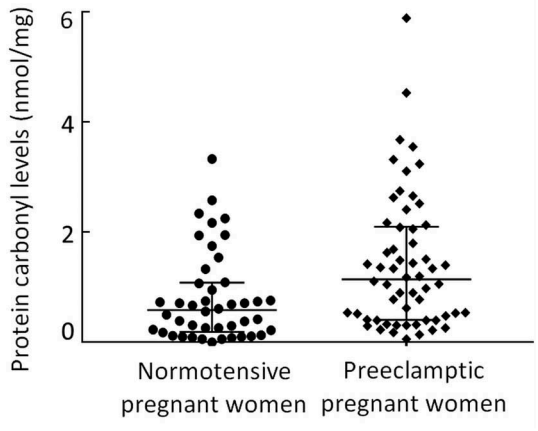

Figure 1. Comparison of urinary protein carbonyl levels in preeclamptic and normotensive pregnant women.

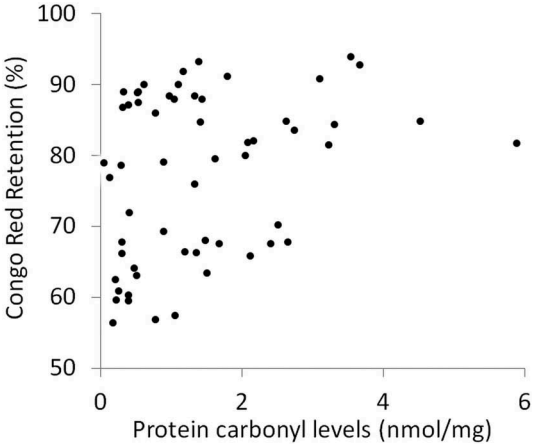

Figure 3. Correlation between urinary protein carbonyl levels and Congo red retention in preeclamptic pregnant women.

pregnant women (Figure 3). The correlation between the two parameters was statistically significant (P = 0.018) but weak (r = 0.3). We also analyzed early-onset and late-onset subgroups for correlation separately. Positive correlation was observed with early-onset but not with late-onset. However, the results were not significant (early-onset: r = 0.22; P = 0.27; late-onset: r = −0.02; P = 0.92). This appears to be due to fractioning of the sample size and the consequent loss of normal distribution in the data (early-onset: skewness = 0.08 $_{misfolding}$, 2.63 $_{carbonyl}$; late-onset: skewness = −1.15 $_{misfolding}$, 0.71 $_{carbonyl}$).

Discussion

In this study, we have evaluated the association of urinary protein carbonyl levels with preeclampsia. To the best of our knowledge, this is the first study to determine the urinary levels of protein carbonyl with preeclampsia. Furthermore, we have examined the association of protein carbonyl with urinary protein misfolding. The main findings of this study are: (i) urinary protein carbonyl levels are elevated in preeclampsia and (ii) elevation in protein carbonyl levels showed a significant but weak correlation with protein misfolding. These results show that urinary proteins in preeclampsia are subjected to oxidative damage and this may be one of the causes of misfolding.

Elevated levels of protein carbonyl have been demonstrated in the plasma of the preeclamptic women. It is seen that the protein carbonyl levels in the maternal plasma are higher in pregnant women than in non-pregnant women (3). Furthermore, the protein carbonyl levels are significantly higher in preeclamptic women than in normotensive women. The association of plasma carbonyl levels with preeclampsia has been confirmed by several studies (6,15–17). The elevation in protein carbonyl levels was in the range of 1.6–2.7 fold.

Elevated levels of protein carbonyl have also been documented in the cord blood of preeclamptic pregnancies (16,18–20). The relative increase in the protein carbonyl levels between preeclamptic and normotensive pregnant women was 1.6–2.0 times. Association of elevated protein carbonyl levels with preeclampsia has also been verified in the placenta (21). Our results extend the spectrum of protein oxidation in preeclampsia to urine. This observation is significant in view of the diagnostic value of urine in preeclampsia.

Recent studies have shown that preeclampsia is an amyloidosis, that is, a disorder of protein conformation. Misfolded proteins have been demonstrated in the maternal plasma, maternal urine, and placenta (7–10). In addition, unfolded protein response that counters accumulation of misfolded proteins has been shown to be elevated in preeclamptic placenta (22). Misfolded proteins are known to bind with high affinity to dyes like Congo Red, Thioflavin T, and Curcumin. The affinity for Congo Red has been exploited to develop a non-invasive test for preeclampsia using urine sample (8). The association of preeclampsia with urinary misfolded proteins is now well established. However, the cause of protein misfolding in preeclamptic women is not known. The major causes of protein misfolding are disruption of protein stability and excessive protein synthesis (23,24). Oxidative damage has been shown to result in protein misfolding (11). Our observation of a weak correlation between protein carbonyl and Congo Red Retention indicates that protein oxidation plays a minor role in the misfolding of urinary proteins in preeclampsia. Plasma proteins like albumin, alpha-1-antitrypsin, IgG κ-free light chain, ceruloplasmin, and interferon inducible protein 6–16 have been observed in the misfolded protein fraction of urine from preeclamptic women (8). Hence, the congophilia in the present study may reflect plasma proteins misfolded as a result of the elevated systemic oxidative stress during preeclampsia. Also, urinary congophilia has been demonstrated even in chronic kidney disease (9). Therefore, kidney lesions, which are common to both preeclampsia and chronic kidney disease, may have also contributed to congophilia. If placenta was the source of congophilia, then the correlation between protein carbonyl and congophilia would have been stronger with early-onset than with late-onset preeclampsia. Because, placental endoplasmic reticulum stress is comparatively higher in early-onset preeclampsia (22). Our results appear to indicate this possibility, but the correlation lacks statistical significance possibly due to insufficient samples at the level of subgroups.

To conclude, this study adds protein oxidation to the spectrum of abnormalities in the urine of preeclamptic women. Protein oxidation along with proteinuria and misfolding may serve as a comprehensive panel to screen and diagnose preeclampsia.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

No specific funding was available for this study.

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Original Article

Misfolding Linked Mutations of SERPINA1 Gene are Uncommon in Preeclampsia

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Abstract

Background: Alpha- 1 antitrypsin (A1AT) is a protease inhibitor that plays an important role in regulating oxidative stress in preeclampsia (PE). Recent studies have shown that A1AT is misfolded in PE. However, the cause of A1AT misfolding is not known. Mutations in *SERPINA1* gene is an established source of A1AT deficiency. PiS and PiZ are the two common misfolding- associated mutations in the *SERPINA1* gene. **Objective:** The purpose of this study was to evaluate the frequency of PiS and PiZ mutations in the *SERPINA1* gene in preeclamptic women. **Materials and Methods:** We carried out a cross- sectional study by including 200 preeclamptic pregnant women. PiS and PiZ mutations in the *SERPINA1* gene were genotyped by using polymerase chain reaction- restriction fragment length polymorphism method. **Results:** PiS and PiZ mutations were absent both in homozygous and heterozygous conditions in the preeclamptic women. **Conclusion:** PiS and PiZ mutations in the *SERPINA1* gene may not be associated with A1AT misfolding in PE.

Keywords: Genetics, preeclampsia, serpins

Introduction

Preeclampsia (PE) is a complication of pregnancy characterized by *de novo* hypertension and proteinuria on or after the 20th week of gestation. PE complicates 2%–8% of all pregnancies globally and constitutes a leading cause of maternal and perinatal morbidity and mortality.[1,2] The cardinal pathogenetic events in PE are defective remodeling of the uterine spiral artery, placental ischemia, endothelial dysfunction, and oxidative stress.[3] The origin of these pathogenetic modifications is not clearly understood, and hence, PE continues to be called a disease of theories.

Recent studies have shown that PE is a proteopathy, i.e., involves aberration in protein folding.[4- 9] One of the important proteins found to be misfolded in the placenta of preeclamptic women is alpha- 1 antitrypsin (A1AT).[6] A1AT is a serine protease inhibitor that plays a critical role in the regulation of proteolytic tissue damage and anti- inflammatory process.[10] A1AT appears to regulate the release of inflammatory cytokines particularly *via* the activation of mitogen- activated protein kinase pathways as well as nuclear factor- κB pathways.[11] One of the activators of this pathway is the oxidative stress generated by reoxygenation subsequent to hypoxia.[12] Hypoxia- induced oxidative stress is a hallmark of PE.[13] The importance of A1AT in PE is further supported by animal model studies, wherein administration of exogenous A1AT was found to prevent the development of PE.[14] These evidence indicate that A1AT plays an important role in the pathogenesis of PE and also holds therapeutic value. However, the origin of A1AT misfolding in PE is not known.

A1AT is highly prone to misfolding due to mutation of the corresponding *SERPINA1* gene.[15] PiS and PiZ alleles are the two common mutations in the *SERPINA1* gene that increase the propensity of A1AT to misfold.[16] PiS and PiZ mutations result in the substitution of glutamic acid with valine

Access this article online

Quick Response Code:

Website: www.amhsjournal.org

DOI: 10.4103/amhs.amhs_79_19

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Received : 10 June 2019 **Revised :** 20 October 2019
Accepted : 06 November 2019 **Published :** 16 December 2019

How to cite this article: Nagarajappa C, Rangappa SS, Balakrishna S. Misfolding linked mutations of SERPINA1 gene are uncommon in preeclampsia. Arch Med Health Sci 2019;7:177-80.

at position 288 and glutamic acid with lysine at position 366. The wild- type allele is denoted as PiM. We hypothesized that mutations in the *SERPINA1* gene may be the cause for A1AT misfolding in PE. To test our hypothesis, we undertook this study, wherein the primary objective was to determine the frequency of PiS and PiZ mutations in the *SERPINA1* gene in a cross section of preeclamptic women.

Materials and Methods

Study design

We carried out this cross- sectional study by including pregnant women diagnosed with PE. The blood sample was collected from each patient and used to determine the presence of the *SERPINA1* gene mutation. The study was approved by the Institutional Ethics Committee. The patients were enrolled during the period of November 2016–February 2018. All the patients provided informed consent at the time of enrollment.

Sample size

The sample size was calculated using OpenEpi version 3.01 statistical web tool (http://www.openepi.com/Menu/OE_Menu.htm). The sample size for 99% confidence limit was found to be 196 preeclamptic patients based on the prevalence of PE in the Indian population which is ~8%.[17]

Patient selection

The study population included pregnant women diagnosed with PE. Diagnosis criteria for PE were as follows: (i) new- onset hypertension (two readings of systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg measured 4 h apart while the patient is on bed rest; (ii) ≥20 weeks of gestation; (iii) new- onset proteinuria (>300 mg protein for 24 h of urine or + 1 on dipstick); and (iv) in the absence of proteinuria, other symptoms such as hemolysis elevated liver low platelet (HELLP) syndrome, edema, thrombocytopenia, impaired liver function, new- onset cerebral or visual disturbances, and renal insufficiency, and in the absence of other renal diseases, nausea, severe headache, and convulsions.[2] Inclusion criteria were as follows: (i) pregnant women with PE, (ii) superimposed eclampsia, (iii) singleton and multiple gestations, and (iv) primigravida and multigravida conditions. Exclusion criteria were: (i) pregnant women with chronic hypertension and (ii) comorbidities such as diabetes mellitus, epilepsy, respiratory diseases, and heart diseases.

Genotyping of PiS and PiZ alleles of *SERPINA1* gene

Blood samples were collected in an ethylenediaminetetraacetic acid vacutainer used for the isolation of genomic DNA by salting- out method.[18] Quantity and purity of the DNA samples were checked by a spectrophotometer. PiS and PiZ alleles of the *SERPINA1* gene were determined by polymerase chain reaction (PCR)- restriction fragment length polymorphism method. PCR conditions are summarized in Table 1. PCR was performed in 25- μl final volume containing 1 pM of each primer, 1- mM deoxynucleoside triphosphates, 1.5- mM $MgCl_2$, 100- ng genomic DNA, and 1 unit of Taq DNA polymerase. An aliquot of the amplicon in each case was incubated at 65°C with ten units of Taqα1 restriction enzyme for 16 h, and the digestion pattern was analyzed on 3% agarose gel.

Results

A total of 200 preeclamptic pregnant women meeting the inclusion criteria were included in the study. Baseline parameters and clinical outcome of the study participants enrolled in the study are listed in Table 2. The cohort included patients of all the major clinical subtypes. Almost 56.5% of the preeclamptic women were primigravida and 43.5% were multigravida. This is in agreement with the general trend of PE being more common in primigravida than in multigravida. Nearly 47% of the preeclamptic women presented with mild type, whereas the remaining 53% showed severe type. Overall 15% of the preeclamptic patients were superimposed with eclampsia. HELLP syndrome was a rare complication that

Table 1: Polymerase chain reaction-restriction fragment length polymorphism parameters used for the genotyping of PiS and PiZ alleles of *SERPINA1* gene

Parameter	PiS allele	PiZ allele
PCR primers		
Forward	5’ TGA GGG GAA ACT ACA GCA CCT C 3’	5’ TAA GGC TGT GCT GAC CAT CGT C 3’
Reverse	5’AGG TGT GGG CAG CTT CTT GGT CA 3’	5’ GGA GAC TTG GTA TTT TGT TCA ATC 3’
PCR conditions (35 cycles)		
Initial denaturation (min)	94°C (5)	94°C (3)
Cycle denaturation (s)	94°C (30)	94°C (30)
Annealing (s)	60.4°C (30)	61.3°C (30)
Extension (s)	72°C (30)	72°C (30)
Final extension (min)	72°C (5)	72°C (5)
PCR amplicon size (bp)	121	144
RFLP pattern (bp)		
Wt/Wt	100+21	123+21
Wt/mut	121+100+21	144+123+21
mut/mut	121	144

bp: Base pairs, Wt: Wild- type allele, mut: Mutant allele, PCR: Polymerase chain reaction, RFLP: Restriction fragment length polymorphism

Table 2: Baseline parameters and clinical outcome of the study participants (*n*=200)

Parameters	Observation	
Age (years)	24.8±3.5	
Gravida, *n* (%)		
Primigravida	113 (56.5)	
Multigravida	87 (43.5)	
Severity, *n* (%)		
Mild	94 (47)	
Severe	106 (53)	
Blood pressure (mmHg)	Mild PE	Severe PE
Systolic blood pressure	137.3±8.5	175.3±15.7
Diastolic blood pressure	95.0±5	116.5±8
Dipstick proteinuria, %		
1+	48	
2+	20	
3+	32	
Gestational age of onset (weeks), *n* (%)		
Early onset (<34)	75 (37.5)	
Late onset (≥34)	125 (62.5)	
Comorbidities, *n* (%)		
Eclampsia	30 (15)	
IUGR	21 (10.5)	
Stillbirth	8 (4)	
HELLP syndrome	2 (1)	

IUGR: Intrauterine growth restriction, HELLP: Hemolysis elevated liver low platelet, PE: Preeclampsia

was observed only in 1% of the patients. Fetal complications in the form of intrauterine growth restriction and stillbirth were seen in 10.5% and 4% of the patients, respectively. Nearly 37.5% of the patients showed the early- onset type of PE, whereas the remaining 62.5% showed the late- onset type. All the preeclamptic patients were genotyped for PiS and PiZ mutations in the *SERPINA1* gene. PiS and PiZ mutations were not observed in any of the patients either in homozygous or heterozygous conditions.

Discussion

Protein folding is a process by which the linear polypeptide chain assumes a thermodynamically stable and a functional form.[19] Folding ensures that the hydrophobic groups are buried away in the core, whereas the charged groups are freely exposed to the surrounding aqueous medium. The collapse of the stable folding pattern leads to the loss of protein function and exposes the core hydrophobic groups to the aqueous medium. Hydrophobic interactions between the exposed core groups result in the formation of aggregates called amyloid deposits that build up in tissues.[20] Misfolded proteins are commonly observed in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease; it is also associated with conditions such as Type II diabetes and medullary carcinoma of thyroid.[21]

Emerging evidence indicates that PE is a proteopathy.[4- 9] Misfolded proteins have been reported in the placenta, urine, and plasma of preeclamptic women.[4- 9] This is further supported by the demonstration of elevated activation of the "unfolded protein response (UPR)" pathway in the placenta of preeclamptic women.[22] UPR promotes protein folding by activating chaperone expression and induces apoptosis if the restoration is insufficient. We have recently shown urinary misfolded proteins equally represented in both early- and late- onset PE.[9] Major proteins identified in the urinary misfolded proteome of preeclamptic women include A1AT, ceruloplasmin, immunoglobulin- free light chains, albumin, interferon- inducible protein 6- 16, and Alzheimer's β- amyloid.[6]

At present, there is little information on the origins of protein misfolding in PE. Genetic involvement in the misfolding of A1AT protein is a classical textbook example.[23] Therefore, we chose to evaluate the involvement of common mutations in the *SERPINA1* gene with PE. *SERPINA1* gene codes for A1AT protein. A1AT is an important plasma serine protease inhibitor whose main function is to neutralize the proteolytic effect of neutrophil elastase. This enzyme facilitates infiltration of neutrophils into tissues through proteolytic degradation of extracellular matrix proteins such as elastin, proteoglycan, collagen, and fibrinogen present in the vascular basement membrane. Reduction in A1AT level disturbs the homeostatic balance between A1AT and elastase, and the excess elastase activity leads to tissue damage. Elastase- mediated tissue damage arising due to A1AT deficiency is well documented in the case of chronic pulmonary obstructive disease.[24]

A1AT deficiency arises due to point mutations that lead to misfolding of the mutant enzyme.[25] However, the clinical manifestation of A1AT deficiency involves both genetic predisposition and environmental modifiers. The active oxygen intermediates produced due to cigarette smoking accelerate the severity of clinical manifestations by functionally inactivating the residual AIAT enzyme.[25] PiS and PiZ are the common mutations in the *SERPINA1* gene that render A1AT prone to misfolding. These mutations cause severe A1AT deficiency when present in recessive condition. However, under the heterozygous condition, they produce borderline deficiency with little clinical manifestation.[24] Individuals who carry PiS and PiZ mutations under heterozygous conditions are at an increased risk of developing severe A1AT deficiency when exposed to compounding risk factors such as oxidative stress. For instance, heterozygous individuals *per se* may not develop chronic pulmonary obstructive disease but are at an increased risk when habituated to tobacco smoking.[26] We assumed that a similar interaction may be involved in the pathogenesis of PE because the involvement of oxidative stress is already well established.[27] Therefore, women who carry PiS and PiZ mutations under heterozygous conditions may not manifest any symptoms of A1AT deficiency but may be prone to A1AT misfolding during pregnancy due to oxidative stress. Another line of evidence that motivated us to choose A1AT is the consistent observation from several studies that its level is deficient in the plasma and placenta of preeclamptic

women.[14,28,29] Furthermore, animal studies on preeclamptic mouse model have shown that intravenous injection with exogenous A1AT ameliorates PE.[14]

Conclusion

In this study, we evaluated the frequency of PiS and PiZ mutations in the *SERPINA1* gene among preeclamptic women. We found that the frequency of these mutations was zero in these women. This encourages us to conclude that common mutations in the *SERPINA1* gene may not be associated with the risk of developing PE.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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MOLECULAR AND BIOCHEMICAL STUDIES ON MISFOLDED PROTEIN IN PREECLAMPSIA

Thesis submitted to
Sri Devaraj Urs Academy of Higher Education and Research

in partial fulfillment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY

in

CELL BIOLOGY AND MOLECULAR GENETICS
under the Faculty of Allied Health and Basic Sciences

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March 2020

Summary and Conclusions

- The purpose of this study was to evaluate the association between protein misfolding and PE.
- Protein misfolding was evaluated by using the Cong Red Dot assay. The levels of misfolded protein were elevated in the urine of PE women. This shows that Congo Red Dot assay can differentiate PE and normotensive pregnant women.
- Next, protein oxidation and genetic factors were evaluated, as the potential source of protein misfolding.
- Protein oxidation was evaluated by measuring protein carbonyl levels by ELISA method. The levels of protein carbonyls were higher in the urine of PE women than in normotensive pregnant women ($p = 0.002$). Furthermore, protein carbonyl levels showed a statistically significant correlation with protein misfolding. This indicates that protein oxidation may be a cause for protein misfolding.
- The frequency of *SERPINA1* gene mutations namely, PiS and PiZ were screened as the potential genetic factor for misfolding in PE women. PiS and PiZ are common misfolded associated mutations in *SERPINA1* gene. Both PiS and PiZ mutation was not found among PE women either in homozygous or heterozygous conditions.
- Overall, this study shows that urinary protein misfolding is associated with PE and it arises mostly due to oxidative damage and unlikely due to mutations in *SERPINA1* gene.