Research article

Relevance of Placental protein 13 in combination with Xanthine oxidase activity and Asymmetric dimethylarginine in early prediction of Preeclampsia with an evidence of LGALS13 gene promoter variant

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ABSTRACT

Introduction and Aim: Preeclampsia is a pregnancy specific hypertensive disorder and its complications are associated with maternal morbidity and mortality. The intention of the study is to determine early placental and endothelial dysfunction markers in first and second trimesters of pregnancy for the prediction of preeclampsia.

Materials and Methods: Study design was prospective which enrolled 268 pregnant women during their first antenatal visit. Blood samples were analysed for Placental protein13 (PP13), Asymmetric dimethyl arginine (ADMA) by ELISA and Xanthine oxidase (XO) activity by colorimetric assay. On follow-up, 22 subjects developed preeclampsia, 12 had spontaneous abortions and the rest remained normotensive till delivery. PP13 gene, LGALS13 was sequenced in preeclampsia cases to know if any polymorphism was present.

Results: Results were analysed by Wilcoxon Signed Ranks test know the level of statistical significance, correlation analysis by Spearman’s correlation and diagnostic performance of the measured parameters was assessed by receivers operating characteristic (ROC) curve. Pregnant women who developed Preeclampsia had low PP13 levels in first trimester and elevated ADMA in second trimester (p<0.001). Gene sequencing of LGALS13 in preeclampsia showed -98C/A SNP at promoter region. ROC curve showed AUC for first trimester PP13 (0.932), second trimester PP13 (0.955) second trimester XO activity (0.967) and second trimester ADMA (1.000).

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

Keywords: Placental protein 13; LGALS13; xanthine oxidase activity; asymmetric dimethylarginine.

INTRODUCTION

Preeclampsia is a pregnancy-specific multisystem rapidly progressive disorder diagnosed with new onset of Hypertension (≥ 140/90 mmHg) and Proteinuria (> 0.3g/24 hr) or thrombocytopenia with multi organ dysfunction after 20 weeks of gestation (1). It affects both mother and foetus and hence is the leading cause of maternal and foetal morbidity and mortality globally. Other symptoms are edema, headache, disturbed sleep, nausea, epigastric / right upper quadrant pain, diminished urinary output and blurred vision. Fetal Complications are neonatal respiratory distress syndrome, intrauterine growth retardation, preterm delivery, placental abruption and intrauterine death. Early screening for detection of preeclampsia during antenatal care is essential and challenging. The aetiology of preeclampsia is poorly elucidated and is directly linked to placental function. Impaired placental implantation and elevated oxidative stress at the maternal-fetal interface leads to vascular inflammation and endothelial cell dysfunction. Maternal immunity and genetic factors also contribute to the disease process (2). The incidence of preeclampsia worldwide is 2-10%, in India 8-10% and 7.9% in Karnataka (3).

Placental protein 13 (PP13) synthesized on the free ribosomes in the syncytiotrophoblast belongs to the β-galactoside binding soluble-type galectin super-family. It is a 32kDa glycoprotein with 139 amino acid residues. It is a homo-dimer with each subunit of 16kDa stabilized by disulfide bonds (4).PP13 is secreted into maternal circulation as early as 5-6 weeks of gestation, its concentration increases as gestation progresses and declines after 4-5 weeks of delivery. PP13 has a Carbohydrate Recognition Domain (CRD) which has high affinity to N-acetylgalactosamine, N-acetyl galactosamine and mannose of the extracellular matrix glyco-conjugates of the endometrium which is a prerequisite for placental implantation (5). PP13 activate, attract and bring about apoptosis of the maternal T cells to provide immune tolerance to the semi-allogeneic fetus and also to facilitate uninterrupted spiral artery remodeling. PP13 is necessary for the differentiation of villous trophoblast and also syncytialization for the synthesis of immune proteins and placental hormones for the development of the embryo. Few animal model experiments also demonstrated the role of preeclampsia.
LGALS13 gene encoding PP13 is located on chromosome 19 at loci q13.1. The expression of the gene is down-regulated due to the presence of single nucleotide polymorphism (SNP) in promoter region, single nucleotide deletion (delT221) in exon 3 and mutations at the exon-intron boundaries. These DNA variants results in diminished levels of PP13 or truncated/mis-folded/ functionally altered PP13 leading to preeclampsia by virtue of disruption of normal placentation and implantation. Apparently, PP13 levels and LGALS13 gene polymorphism in preeclampsia risk is needed to be established in South Indian population (8).

Insufficient trophoblast invasion or defective placental results in intermittent blood flow resulting in ischemia- reperfusion injury in the intervillous space. This causes increased trophoblast cellular destruction leading to augmented breakdown of ATP which accumulates hypoxanthine/xanthine. Xanthine oxidase (XO) catalyzes the oxidation of hypoxanthine / xanthine to uric acid, with liberation of superoxide radical and hydrogen peroxide thus elevating placental oxidative stress. The significance of XO activity which is known to be an ischemia reperfusion injury marker is less known in preeclampsia (9).

Protein methylation is a post translational modification catalyzed by an enzyme protein arginine methyltransferase (PRMT) which transfer the methyl group from S-adenosyl methionine. This results in the formation of methylated proteins and S-adenosylhomocysteine. The cellular processes regulated by methylated proteins are RNA processing, transcriptional regulation, signal transduction and DNA repair. During protein turnover of methylated proteins Asymmetric dimethylarginine (ADMA) is formed which has antagonizing effect on Nitric oxide by inhibiting endothelial Nitric oxide synthase (10).

Diagnosis of early onset of preeclampsia is of greater clinical importance for timely management or to reduce the severity of the symptoms. So the present research study is prioritized on screening and identification of new markers during early gestation with good predictive value. As per literature review (2008-18), there exist a need on the data of plasma PP13 levels during early pregnancy and on LGALS13 gene polymorphism in Indian women during normal pregnancy and in preeclampsia. Elevated XO activity is associated with preeclampsia but estimation of XO activity in a longitudinal manner is not reported. In addition to this determination of ADMA in a longitudinal manner was requisite to assess the endothelial dysfunction in early pregnancy.

MATERIALS AND METHODS

Materials

A prospective study was conducted in the Department of Biochemistry in collaboration with Department of Obstetrics and Gynaecology in R L Jalappa Hospital and Research Centre, Kolar, Karnataka, India. The study design and patient recruitment criteria were approved by the University Central Ethics Committee of Sri DevarajaUrs Academy of Higher Education and Research Centre. The study enrolled 268 pregnant women in the age group of 20-35 years attending their first antenatal visit to the Department of Obstetrics and Gynaecology in R.L. Jalappa Hospital and Research Center. Written informed consent was obtained from all the study participants. The cohort was followed till delivery to know the pregnancy outcome. During follow-up, subjects who developed preeclampsia were categorised based on ACOG guidelines (11). For the genetic analysis cord blood was collected post-partum from preeclampsia and from normotensive women.

Inclusion criteria

Pregnant women with singleton pregnancy, primigravida, with no major fetal anomalies were included in the study.

Exclusion criteria

Pregnant women with history of chronic hypertension and on treatment or with renal disease, thyroid disorder, gestational diabetics, epilepsy, hypertensive encephalopathy, cardiovascular disease and with twin pregnancies were excluded from the study.

Blood sampling

Four ml of venous blood was collected in a sterile EDTA tube from participants in first (8-11 weeks) and second trimesters (20-24 weeks) during their routine antenatal check-up. Samples were centrifuged at 3000 rpm for 10 minutes to obtain plasma which is stored at -80°C until subsequent analysis. After delivery, 2mL of cord blood was collected in a sterile EDTA tube from the foetal side of umbilical cord from preeclampsia and normotensive women for DNA extraction.

Biochemical Analysis

Measurement of plasma PP13 and ADMA were assayed by ELISA kit as per the protocol of the manufacturer (Cusabio Biotech Co. Ltd, USA). The activity of XO was measured by colorimetric method using commercial kit procedure of manufacturer (BioVision Incorporation, USA).

Determination of XO activity

Procedure: To the standard wells 10, 20, 30, 40, 50μL of the 0.2 mM H₂O₂ standard was added. The total volume was made up to 50μL in each well with

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distilled water to generate 0.2, 4.6, 8.10 nmol/H2O2 standards. 50 μL of test samples were added to sample wells. For the positive control, 5 μL of positive control solution was added and the volume was made up to 50 μL/well with distilled water. 50 μL of reaction mix (Assay buffer+ substrate mix+ enzyme mix+OxiRed™) was added to each well containing standard, positive control and test samples. The plate was measured immediately at 570 nm at T1 and A1 was recorded. The plate was incubated away from light at 25°C for 10-20 minutes and measured again at T2 to read A2. The signal generated by XO is ΔA = A2 - A1.

B = amount of H2O2-generated by xanthine oxidase from standard curve (nmol)
T1 = time of the first reading (A1) (in min)
T2= time of the second reading (A2) (in min)
V = pre-treated sample volume added into the reaction well (in ml)

Cord blood collection, DNA extraction and sequencing of LGALS13 gene

To know LGALS13 gene polymorphisms cord blood was collected after delivery in aseptic conditions from fetal side of umbilical cord in sterile EDTA tubes. The tubes were stored at 4°C and extraction was done within three days after the sample was collected. The DNA extracted from cord blood was checked purity by using spectrophotometer against TE buffer as blank. The absorbance of DNA sample mixed with TE buffer was measured at 260nm and 280nm. The purity was represented by the ratio between 260/280 nm, the DNA samples having the ratio between 1.7-1.9 were considered for PCR reactions. The DNA samples with expected purity were used for PCR reactions. Reference sequence of LGALS13 gene (Accession No: NC_000019.10) was retrieved from National Centre for Biotechnology Information (NCBI) and primer pairs were designed using software IDT(Integrated DNA technologies, Inc, Coralville, IO, USA) and were procured from Sigma Merck company and were checked by In-silico PCR. The DNA was amplified by PCR at optimum annealing temperature using primers (Table 1) and was confirmed by Agarose gel electrophoresis. The bands were visualized under UV illumination in Bio Rad Gel Doc System. PCR products were purified using GeneiPure™Quick PCR purification kit. The purified DNA was sequenced for all the exons using BigDye® Terminator v3.1 Cycle Sequencing Kit in ABI-3500 Genetic Analyzer (Applied Biosystems, USA). Chromas software was used to get the FASTA sequence. Mutational analysis was performed using Clustal Omega multi-sequencing software by comparing with the reference sequence.

Table 1: Details of LGALS13 primers

| Coding region | Primer | GC% | Primer sequence | Amplicon Size | Annealing temperature (°C) |
|---------------|--------|-----|----------------|---------------|--------------------------|
| Exon 1        | Forward| 55% | CCTGGTAAACCAATCCACAG AATCCCCACAAGCATCTCAG | 458           | 61.3                     |
|               | Reverse| 50% |                       |               |                          |
| Exon 2        | Forward| 50% | GTCTGCCCCTTTCTATCTCCAA CCAACCCACTGTTCTCT | 401           | 61.3                     |
|               | Reverse| 55% |                       |               |                          |
| Exon 3        | Forward| 50% | TTTTACTGAGGTAGTGGAG GATACTCAGCAGCTGACTCC | 322           | 61.3                     |
|               | Reverse| 50% |                       |               |                          |
| Exon 4        | Forward| 50% | CGTCTAGAGGATGATGTGGAAAC GGTCAACGAGAGPGTCTC | 383           | 64.3                     |
|               | Reverse| 50% |                       |               |                          |

Statistical Analysis

The data was not normally distributed so the results were presented in median and interquartile range [IQR]. The level of significance between the measured parameters in first and second trimesters was derived from Wilcoxon signed rank test. Receivers Operating Characteristic (ROC) curves were plotted to know the diagnostic utility of PP13, XO activity and ADMA and in first and second trimesters of pregnancy. SPSS licensed version IBM 22.0 was used for statistical analysis and p-value<0.05 was considered significant.

RESULTS

The demographic details were recorded, and blood samples were collected from the enrolled pregnant women in their first trimester (8-11 weeks). Twelve women had spontaneous abortions after their first trimester so a total of 256 women entered second trimester. Blood samples were again collected in the second trimester (20-24 weeks). Plasma PP13, XO activity and ADMA were measured in both first and second trimester plasma samples. The number of subjects that developed preeclampsia during follow up was 22 (cases) and 234 women (controls) remained normotensive till delivery. After delivery
cord blood was collected from 22 women who developed preeclampsia and from the same number from control group. The first and second trimester data of the women who developed preeclampsia and the control group were compared to find out for any research evidence of the study parameters that can serve as predictive biomarkers for preeclampsia risk. Baseline demographic, hematological and biochemical characteristics of the study participants (n=268) are presented in table 2.

Table 2: Base line characteristics of the study population

| VARIABLES                        | n = 268 |
|----------------------------------|---------|
| Maternal age (years)             | 24      |
| Body weight (kg)                 | 53      |
| Height (m)                       | 1.56    |
| BMI (kg/m²)                      | 21.3    |
| SBP (mmHg)                       | 110     |
| DBP (mmHg)                       | 70      |
| MAP (mmHg)                       | 83.3    |
| Haemoglobin (gm%)                | 12.2    |
| Platelets (10⁹/µL)               | 266     |
| Total Count (mm³)                | 8.2     |
| MCV (Ft/red cell)                | 87.1    |
| MCH (pg/cell)                    | 28.42   |
| RBS (mg/dL)                      | 100.5   |
| Creatinine (mg/dL)               | 0.7     |
| Blood Urea (mg/dL)               | 24      |
| Calcium (mg/dL)                  | 9.9     |
| Uric acid (mg/dL)                | 3.3     |

Table 3 and fig. 1 illustrates the values of PP13, XO activity and ADMA in I and II trimesters of women who remained normotensive and who developed preeclampsia. In normotensive women there was a gradual rise of PP13 concentration as gestation progressed. However, in preeclampsia women PP13 concentration was 110.98 pg/ml in first trimester which further showed a steep rise in second trimester to 429.78 pg/ml. XO activity was not increased to an appreciable level in second trimester in the normotensive group but in women that developed preeclampsia its activity doubled. ADMA level in second trimester of normotensive group was increased by 2-fold and by 4-fold in women who subsequently developed preeclampsia.

Table 3: Placental protein 13, Xanthine oxidase activity and ADMA in I and II trimesters of normotensive group and Preeclampsia

| Variables                        | Trimester – I (n = 246) Normotensive | Trimester-II (n = 234) Normotensive | p     |
|----------------------------------|-------------------------------------|-------------------------------------|-------|
| PP 13 (pg/mL)                    | Median 205.91 IQR 102-296           | Median 323.38 IQR 189-463           | <0.001*|
| XO activity (mU/mL)              | 12.5 IQR 10.8-14.7                  | 13.5 IQR 12.3-16.4                  | <0.001*|
| ADMA (ng/mL)                     | 20.99 IQR 16.4-24.7                 | 45.6 IQR 37.9-51.0                  | <0.001*|
| PP 13 (pg/mL)                    | Median 110.98 IQR 99-221            | Median 429.78 IQR 344-540           | <0.001*|
| XO activity (mU/mL)              | 12.2 IQR 10.8-13.7                  | 33.9 IQR 22.15-42.5                 | <0.001*|
| ADMA (ng/mL)                     | 21.6 IQR 19.6-23.3                  | 84.69 IQR 74.5-96.3                 | <0.001*|

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

Fig. 1: Depicting the median A] PP13. B] XO activity C] ADMA in cases developed Preeclampsia and normotensive group in first and second trimesters of pregnancy
In our study 22 women developed preeclampsia. Cord blood samples were collected from preeclampsia and from the same number of healthy women after delivery for DNA extraction. Sequence analysis of control group showed cytosine in -98 position of the promoter region. Out of 22 preeclampsia samples, 12 samples showed an SNP at -98 position in the promoter region of LGALS13 gene i.e., Cytosine is replaced by Adenine as shown in Figure 2A and 2B.

The predictive performance of PP13, XO activity and ADMA was plotted graphically as ROC curves in first and second trimesters of pregnancy as shown in table 4. The diagnostic performance of first trimester PP13 was represented by ROC curve with showed 95% sensitivity and 78% specificity at a cut-off of 117.77 pg/mL with AUC of 0.93 at 95% confidence interval (0.855-1.000) and p<0.001 as shown in figure 3. In the second trimester at a cut-off of 371.25 pg/mL PP13 showed 95% sensitivity, 87% specificity with an AUC of 0.95 at 95% confidence interval (0.887-1.000) and p<0.001 as shown in figure 3. In the second trimester at a cut-off of 17.25 mU/mL XO activity showed 95% sensitivity, 78% specificity 78% with an AUC 0.96 at 95% confidence interval (0.921-1.000) and p<0.001 as shown in figure 4. The diagnostic performance of second trimester ADMA showed 100% sensitivity and 100% specificity at a cut-off of 50.36 ng/mL with an AUC as 1.00 at 95% confidence interval (1.000-1.000) and p<0.001 as shown in figure 4. AUC showed best accuracy for ADMA in second trimester for the prediction of preeclampsia.

### Table 4: Diagnostic performance of the study parameters in first and second trimesters of pregnancy

| Parameter     | Cut-off | Sensitivity (%) | Specificity (%) | AUC     | 95% CI Range | p      |
|---------------|---------|-----------------|-----------------|---------|--------------|--------|
| **TRIMESTER I** |
| PP13 (pg/mL)  | 117.77  | 95              | 78              | 0.93    | 0.85-1.00    | <0.001 |
| ADMA (ng/mL)  | 19.7    | 72              | 78              | 0.73    | 0.92-1.00    | 0.008  |
| **TRIMESTER II** |
| PP13 (pg/mL)  | 371.25  | 95              | 87              | 0.95    | 0.88-1.00    | <0.001 |
| XO activity (mU/mL) | 17.25 | 95              | 78              | 0.96    | 0.92-1.00    | <0.001 |
| ADMA (ng/mL)  | 50.36   | 100             | 100             | 1.0     | 1.00-1.00    | <0.001 |

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DISCUSSION
Oxidative stress implicated in pathogenesis of preeclampsia is due to ischemia reperfusion secondary to inadequate trophoblast invasion. Hypoxicated trophoblastic cells with endoplasmic reticulum and mitochondrial stress affected by free radical injury irreversibly damages the cellular components and cellular functions like enzyme activity, signal transduction and apoptosis. Cytotoxic factors are released from the ischemic placenta due to an imbalance of the apoptotic cascade into the maternal circulation. These factors in turn could be causative for the onset of maternal inflammatory response and endothelial cell activation in preeclampsia.

Several markers have been identified to predict the onset of preeclampsia in early pregnancy. However, these markers have less clinical utility. So, there is a scope to screen new biomarkers which facilitates the early diagnosis of preeclampsia. PP13 and XO are biomarkers that secrete into maternal circulation during pregnancy. PP13 secretes before 6th week of gestation from exosomes or micro-vesicles and remains even after 2-5 weeks of delivery in maternal circulation. The functional role of PP13 is embryo implantation and for proper placental development. To the best of our knowledge, PP13 and its importance as a marker for early diagnosis of preeclampsia in Indian population is seldom. However, few studies conducted in Bulgaria, Austria, Florida, and Israel population reported measuring PP13 level at first trimester showed predictive value in preeclampsia (12-15). Women who developed preeclampsia showed a 4-fold rise of PP13 concentration from its basal value which reflects the impact of oxidative stress and elevated placental apoptosis in preeclampsia. Whereas only two-fold increase of PP13 level was observed in second trimester in the women who remained normotensive. Therefore, screening for low PP13 levels at first trimester and/or elevated serum PP13 level at second trimester indicate the possibility of development of preeclampsia.

In contrary Ceylan et al., reported no differences in the first trimester PP13 or PAPP-A level in the preeclampsia women compared to control group (16). Seravalli et al., also reported that measuring PP13 in first trimester doesn’t improve the prediction of
preeclampsia nor small for gestational age (17).

XO an enzyme heightens the oxidative stress by release of hydrogen peroxide and thus regarded as an enzyme oxidant. Its level is increased in second trimester than first trimester in normotensive group and further elevated in cases developed with preeclampsia. The possible explanation for the increased XO activity could be due to release of xanthine and hypoxanthine substrates. Therefore, increased substrate availability in placental hypoxia due to increased break down of ATP and conversion of cytokine activated xanthine dehydrogenase to its oxidase form increases XO activity that releases into maternal circulation from placenta (18).

ADMA is a metabolic by product of protein methylation process produced in vivo into cytoplasm and known as inhibitor of nitric oxide synthase that catalyze the conversion of Arginine to Nitric oxide and Citrulline. ADMA generally gets cleared by the hydrolytic reaction catalyzed by Dimethyl arginine dimethyl amino hydrolase (DDAH). Oxidative stress has an impact on the DDAH expressed in placenta i.e., the thiol group of cysteine-249 of the active site is sensitive to oxidative inactivation causing ADMA to accumulate (19). Therefore, in the present study, ADMA levels in normotensive group in first trimester (21 ng/mL), second trimester (46 ng/mL) and in subjects that developed preeclampsia (85 ng/mL) was observed. The diagnostic performance of ADMA was very good with an AUC of 1.0. Increased ADMA levels in the system compromises nitric oxide synthesis and further accentuates oxidative stress hence contributing to vascular endothelial dysfunction. Few longitudinal studies have reported elevated ADMA in second trimester before the onset of preeclampsia (20,21). Contradictory finding by Osmanagaoglu et al., reported no difference in the levels of ADMA in normotensive and preeclampsia (22). The current study research findings link the role of ADMA and its clearance to endothelial dysfunction & onset of preeclampsia under prevailing oxidative stress. Thus, this current study creates scope for determination of factors/ therapeutic strategy to reduce ADMA levels for better women and child health. Our study results also supports the importance of PP13 concentration during pregnancy for better obstetric management.

Preeclampsia is a complex genetic disorder comprising of numerous genetic variants occurs because of mutation or genetic polymorphisms. Many candidate genes were studied in providing genetic evidence related to preeclampsia complications. Wider scope still exists to study the products expressed by the genes and to know the functional roles in relation to preeclampsia. Therefore, understanding the pattern of gene structure, expression in exerting molecular mechanism is essential to address etiopathogenesis linked to genetic causes. The targeted pharmacological agent for any genetic disorder is useful as an additional therapeutic approach in the diagnosis and management of the pregnancy associated complications. LGALS13 is one such gene and its expressed protein PP13 studied to a limited extent.

The low concentration of plasma PP13 was reported as a risk factor in placentation process and in later development of preeclampsia in pregnancy. As per the data, the low levels of PP13 were noticed in first trimester subsequently its level increased in second trimester. The exact reason for low PP13 level in first trimester is not clearly known. Our study findings are also in align with the studies which reported low levels of PP13 and different from other studies by collecting its gene LGALS13 sequence analysis data in an attempt to find the possible explanation. LGALS13 gene was subjected for sequence analysis by employing molecular techniques. The analysis of sequence data of LGALS13 gene of preeclampsia cases was done by comparing to reference sequence as obtained from NCBI data base. The sequence analysis of 22 preeclampsia cases revealed-98C/A SNP in the promoter region of LGALS13 gene in 12 cases. Similar observations were observed in South Africa and London cohort but the limitation in those studies was PP13 levels were not measured concomitantly (23,24). There is a paucity of information on similar SNPs in study population or Indian population.

**CONCLUSION**

This longitudinal study provides information in the change of the pattern of PP13, XO activity and ADMA under oxidative stress in first and second trimesters of normotensive and women at risk for preeclampsia. 50% reduction of PP13 in the first trimester appraises it as marker to distinguish preeclampsia in the study population. Elevated XO activity is due to ischemia reperfusion injury contribute to oxidative stress as an enzyme oxidant and increased ADMA levels in second trimester represents vascular endothelial dysfunction in preeclampsia. So, the biochemical screening such as PP13, XO activity and ADMA are vital in identifying women at risk for developing preeclampsia. A promoter variant was detected in the sequence analysis of LGALS13 gene in preeclampsia (n=12) with concomitant low PP13 levels. Study requires larger population size to generalise the results with more clinical relevance and also to find any other genotype polymorphism in the LGALS13 gene, expression of PP13 and its functional aspect associated to the regulation of blood pressure.

**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.
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