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Increased homocysteine expression associated with genetic changes in the folate pathway as a key determinant of preeclampsia: A prospective study from lower Assam, India

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ABSTRACT

Lacunae exist in the scientific understanding of preeclampsia (PE) pathogenesis, which is a global issue and a clinical challenge during pregnancy and contributes to both maternal and fetal morbidity and mortality. We aim to understand the significance of homocysteine levels and folate pathway parameters in PE pathogenesis involving pregnancy cases from lower Assam, India. PE cases {N = 145, mild-PE (n = 108), severe-PE (n = 37)} and 192 normal full-term delivery cases were evaluated for Hcy expression and Vitamin B12 levels and evaluated for association with *MTHFR* and *TYMS* polymorphisms in PE pathogenesis. Hcy level was significantly higher in PE cases with negative pregnancy outcomes (p = 0.003) and was higher in PE-preterm delivery compared to PE-term delivery cases (p = 0.301). The VitaminB12 levels were not associated with higher tHcy concentration. Distribution of *MTHFRC677T* variant genotype was significantly higher in severe-PE cases compared to mild-PE cases (p = 0.050) and was associated with an increased risk of severe PE. The combined variant genotype of *MTHFR* and *TYMSdel6* polymorphism resulted in significant increase (p = 0.014) in the risk of severe-PE compared to mild-PE. Within the PE delivery group, the combined variant genotypes showed association with higher homocysteine levels both in live birth cases as well in the PE cases with negative pregnancy outcomes. Increased homocysteine levels were associated with combined variant genotypes and were consistent in both term and preterm delivery in PE cases. The study points out the prognostic significance of *MTHFR C677T, TYMS 1494del6* genotype and tHcy levels as a risk factor for PE, negative pregnancy outcome and preterm delivery.

1. Introduction

Preeclampsia (PE) is the hypertensive disorder during pregnancy characterized by sudden rise in blood pressure, proteinuria, with or without oedema (Lorquet et al., 2010). It has serious health consequences to the affected women and their offspring, and results in enormous economic impact to the society (Liu et al., 2009). Increased mortality rates, preterm delivery, foetal growth restriction (FGR), intrauterine growth restriction (IUGR), abruptio placentae and stillbirth are significantly associated with PE (Zhang et al., 1997). On the basis of severity of hypertension and accompanying signs, symptoms and laboratory abnormalities, PE is generally subdivided into mild PE and severe PE [American College of Obstetricians and Gynaecologists (ACOG) 2011]. Women suffering from PE are at increased risk to various other co-morbidities like pulmonary oedema, thrombocytopenia, pneumonia etc. (Zhang et al., 2003). Further, women with a history of PE are more susceptible to develop heart diseases in future (Irgens et al., 2001).

Globally, the incidence of PE is estimated to be between 2%–10% of pregnancies and is the second leading cause of direct maternal and foetal death (Roberts and Lain, 2002). According to the World Health Organization (WHO), the incidence of PE is 7 times higher in developing countries (2.8% of live births) compared to be developed countries (0.4%) (Igberase and Ebeigbe, 2006); attributed mainly to late presentation of the cases followed by delayed medical intervention (Onakewhor and Gharoro, 2008). Thus, PE is a huge public health

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problem contributing to maternal and perinatal mortality and morbidity (McClure et al., 2009). In India, PE incidence is noted to be around 8–10% of the pregnancy and it is the third leading cause of maternal mortality responsible for 17% of maternal deaths (Krishna Menon and Palaniappan, 1994; MacKay et al., 2001). The National Family Health survey of India (NFHS) reports discloses that 56% of women from India under the age group of 15–49 years are iron deficiency anaemic which is also the highest in the world. NFHS report further states that Assam [72%] and Tripura [71%] two of the North eastern states top the list. Women with anaemia are shown to be at an increased risk to preeclampsia (Kitay and Harbort, 1975). Moreover, it is a serious concern because a hospital based study by Doley and Pegu, 2016 indicated that the incidence of eclampsia is high in Assam thereby indicating it to be an important obstetric emergency in the community contributing to significant perinatal and maternal morbidity and mortality.

Despite being the subject of active research for many years, unfortunately there is no data available on the underlying molecular aetiology associated with PE and its related complications and negative outcome in patients from North-eastern India who are ethnically distinct from the other parts of India. The pathogenesis of PE is complicated as numerous interactions between genetic, environmental and immunological factors are involved. Folate pathway is one of the key pathways which play a critical role in successful pregnancy (Bodnar et al., 2006). So any defect in this pathway may lead complications for the pregnant women. Folate plays a critical role in various cellular biosynthetic processes such as cell division and cell growth, DNA synthesis and methylation (Solanky et al., 2010). Scanty reports suggested that increased folate intake pose a reduced risk to PE (Bodnar et al., 2006).

Homocysteine (Hcy) is a de-methylated product formed from methionine. A high blood level of Hcy, referred to as hyperhomocytenemia, is an independent risk component of numerous pathological processes such as venous and arterial vascular systems (Antoniades et al., 2009). Pregnant women with hyper-homocytenemia may lead to numerous pregnancy complications related to pregnancy including PE (Den Heijer et al., 1996; Rajkovic et al., 1997). Hcy is recycled to methionine by the number of B vitamins. This remethylation reaction is catalyzed by methionine synthase and involving vitamin B12 as a co-factor and 5-methyl tetrahydrofolate as a methyl group donor (Castro et al., 2006).Vitamin B12 are known to reduce the plasma Hcy (Lonn et al., 2006). Deficiency of Vitamin B12 suggests one of the reasons for the deregulation of Hcy metabolism thereby elevates the serum Hcy level (Wolffenbuttel et al., 2019).

Numerous environmental and genetic factors may result in hyperhomocytenemia. *MTHFR* (5-Methylenetetrahydrofolate reductase) mutation has been identified to be associated with hyperhomocytenemia. *MTHFR* has been known to play a very essential role in folate metabolism. It can deoxidize the N5, N10- methylenetetrahydrofolate into N5-methyltetrahydrofolate, resulting in homocysteine being methylated into methionine. The most common polymorphism of MTHFR gene identified to be associated with hyperhomocytenemia is the 677C > T variant (Yang et al., 2003). The C667T mutation was shown to induce an enzyme with thermolabile properties and with decreased activity, resulting in increased plasma Hcy concentrations. There are three types of *MTHFR*: C/C, C/T, T/T. Numerous studies also suggests that 677C > T polymorphism was associated with several pregnancy complications (Frosst et al., 1995; Van der Molen et al., 2000), but the relationship with PE is controversial. Although few scanty reports (Grandone et al., 1997) suggest that *MTHFR* 677C > T allele as a genetic risk for PE but various other studies do not support this association (Kim et al., 2001).

Thymidylate Synthase (*TYMS)* is another essential enzyme of folate metabolism. It is involved in DNA synthesis and repair by taking part in the denovo synthesis of thymidine nucleotide using 5,10 MTHF as a methyl donor (Ulrich et al., 2002). *TYMS 1494 6 bp deletion* [TTAAAG] (rs16430) in 3′UTR region has been found to be connected with the change in mRNA stability of *the TYMS* gene (Mandola et al., 2004). Due to the involvement of TYMS gene in DNA synthesis, it may be presumed that *TYMS 1494del6* polymorphism may take part in pregnancy complications as DNA synthesis is important during pregnancy for foetal development. Available literature suggests that variable *TYMS* genotype may be related with adverse pregnancy outcome and unfavourable congenital anomalies.

Although considerable progress is been made to study the role of several molecular factors in the pathogenesis of preeclampsia but due to equivocal results the aetiology of this disorder is still undefined. No studies have been reported till date from Northeast India to elucidate the factors associated with the occurrence of preeclampsia. Given the background of importance in understanding the pathogenesis of preeclampsia, the present study was undertaken to evaluate the significance of deregulations in Hcy and vitamin B12 levels in PE pathogenesis and associated complications and pregnancy outcome, and its association with genetic alterations in folate pathway genes.

2. Materials and methods

2.1. Patient enrolment and stratification

A total of 337 pregnancy cases undergoing delivery from 2017 to April 2019 were enrolled for the present study from the Department of Obstetrics and Gynaecology, Fakhruddin Ali Ahmed Medical College and Hospital (FAAMCH), Barpeta, Assam. Each enrolled patient included in the study were interviewed with informed consent about patient history, demographic profile including age, blood pressure, BMI, parity, lifestyle, proteinuria, area of residence, whether suffering from any chronic kidney diseases (which is an exclusion criteria), etc. A full-thickness section of placental tissue extracts avoiding the periphery and areas of obvious infarction were collected after delivery along with 4 ml of blood samples from each enrolled cases and kept in EDTA and non-EDTA vials. Pregnancy cases was then categorised into two cohorts: PE cases (N = 145) and normal Full term delivery (NFTD) cases without any complications and no history of PE (N = 192). PE subjects (N = 145) was then further stratified as mild PE (n = 108) and severe PE (n = 37) based on ACOG 2001.

- Mild PE was defined as hypertension developing during pregnancy, accompanied by the onset of proteinuria. Proteinuria was defined as greater than or equal 300 mg/l in a 24 h urine specimen [or greater than or equal 1+ urine protein by 'dipstick' on two random specimens taken 6 or more hours apart].
- Severe PE was based on one of the following findings: BP ≥160 mmHg systolic or 110 mmHg diastolic on two occasions 6 or more hours apart; proteinuria ≥5 g in 24 h (or 3–4+ on two dipstick specimens 4 or more hours apart); oliguria (urine output < 500 ml in 24 h); abnormal liver function tests; thrombocytopenia (platelet count < 100,000/mm). Conditions that also helped to define severe PE also include: eclampsia; cerebral or visual disturbances, epigastric and right upper quadrant pain; FGR and pulmonary oedema.

The PE cases were further stratified as term delivery (n = 58) or preterm delivery cases (delivery before 37 weeks of gestation) (n = 87) based on gestation period. NFTD cases without any complications were used as a comparative control group. The study was approved by the Institutional Ethics Committee of FAAMCH, Barpeta, Assam. The plasma separated from whole blood and the serum samples from non-EDTA vials was stored at −80 °C for the estimation of total plasma homocysteine and vitamin B12 levels by ELISA. The genomic DNA was extracted from the collected placental tissue samples using the standard phenol chloroform method. A portion of the collected tissue samples were fixed in10% neutralized buffered formalin, paraffin embedded

Table 1
Demographic profile of enrolled pregnancy cases.

Cases	N	Maternal Age	Gestational period		Baby Alive	Baby Death/ IUD
			Term delivery	Preterm delivery		
Normal full term delivery (NFTD)	192	24.29 ± 3.43	192	0	192	0
PE	145	22.59 ± 3.73	58	87	132	13
Mild PE	108	22.53 ± 3.75	43	65	105	3
Severe PE	37	22.78 ± 3.72	15	22	27	10

*IUD: Intrauterine death

and was used for differential protein expression analysis of homocysteine.

2.2. Differential Homocysteine expression analysis

The plasma total homocysteine (tHcy) levels were measured by ELISA using a commercially available Homocysteine Human ELISA kit (*ITEH4011, G-Bioscience)*. All the samples were run in duplicates, and the statistical analysis was done by taking average of the two measurements. The sensitivity of the ELISA was 0.092 nmol/ml. The intra-assay and inter-assay coefficients of variation < 8% and < 10% respectively. Differential protein expression of homocysteine in PE and NFTD formalin-fixed paraffin embedded tissue sections was examined by immunohistochemistry using specific antibody (ab5154, *Abcam*), and the Super Sensitive™ One step Polymer-HRP Detection System (*Biogenex*). The results were examined, evaluated and graded by a senior pathologist.

2.3. Estimation of serum vitamin B12 levels

The serum Vitamin B12 levels were means used by ELISA using a commercially available Vitamin B12 Human ELISA kit (ITEH01544, *G-Bioscience)*. All the samples were run in duplicates, and the statistical analysis was done by taking average of the two measurements. The sensitivity of the ELISA was 3.46 pmol/l. The intra-assay and inter-assay coefficients of variation < 8% and < 10% respectively.

2.4. PCR-RFLP analysis of MTHFR C677T and TYMS (1494) 6 bp deletion polymorphism

The PCR-RFLP based approach and primers earlier reported by Frosst et al., 1995 was used for screening of *MTHFR 677C➔T* polymorphism using the *HinfI* enzyme. A single band of 198 bp characterized wild-type CC genotype for codon 677, while the presence of three bands at 198, 175 and 23 bp or 175 and 23 bp only characterized heterozygous and homozygous variant genotype status respectively. The genotyping analysis was performed by Dr. Rizwana Sultana.

For the detection of 6 bp deletion (*del*) and insertion (*ins*) polymorphism in the 3'UTR region of *TYMS*, the PCR-RFLP approach described by Ulrich et al., 2000 was followed. Briefly, the 158 bp fragment generated by PCR amplification was subjected to restriction digestion using enzyme *Dra I*. A single band of 152 bp fragment characterized as variant-type allele with 6 bp del while the presence of four fragment of 152 bp, 158 bp, 88 bp and 70 bp represented heterozygous for 6 bp insertion/deletion and the presence of two fragments 88 bp and 70 bp represented wild type allele for 6 bp insertion. For validation 20% of the samples were re-analyzed with the help of co-laboratory member in a blinded manner.

2.5. Statistical analysis

All the statistical analyses were performed by the standard methods using SPSS computer software (Version 13, SPSS Inc., Chicago, IL, USA). The results were expressed as means ± standard deviations (SD). The $\chi 2$ goodness of fit test was used for any deviation from Hardy–Weinberg equilibrium. The Mann-Whitney non-parametric test was used to analyse the differences. The significance was described as Pearson *p*-value. Student's unpaired *t*-test were used to evaluate differences between groups and the two-sided Pearson and Spearman's rho correlation coefficient were used to determine the relationship between variables. A probability of < 0.05 was regarded to be statistically significant.

3. Results

3.1. Demographical profile of the enrolled cases

For the present prospective study we enrolled patients undergoing delivery from the Obstetrics and Gynaecology Department of Fakhruddin Ali Ahmed Medical College and Hospital, Barpeta, Assam. All the samples were collected under the supervision of registered medical practitioners with clinical data and informed consent. Patients were stratified into two cohorts namely; NFTD cases ($N = 192$) and PE cases ($N = 145$); PE cases were further stratified as mild PE ($n = 108$) and severe PE cases ($n = 37$). The PE cases were further stratified as term delivery ($n = 58$) or preterm delivery cases (delivery before 37 weeks of gestation) ($n = 87$) based on weeks of gestation. The details of the enrolled cases are presented in Table 1. Majority of the enrolled subjects belong to poor socio-economic background.

3.2. Protein expression analysis of homocysteine

The total homocysteine (tHcy) levels in NFTD and PE subjects were measured by ELISA. The normal homocysteine concentrations range from 5 to 15 nmol/ml [30]. The normal reference values of homocysteine in first, second and third semester during normal pregnancy is 3.34–11 nmol/ml, 2.0–26.9 nmol/ml and 3.2–21.4 nmol/ml respectively [31]. A tHcy concentration exceeding 15 nmol/ml is termed as "hyperhomocytenemia" [32]. Moderate, intermediate, and severe hyperhomocytenemia refers to concentrations between 16 and 30, 31–100 and > 100 nmol/ml, respectively [33]. The overall range of homocysteine in all the enrolled pregnancy samples was from to 21.94 to 107.1 nmol/ml. The plasma homocysteine levels was found to be significantly higher in PE cases compared to NFTD cases ($p = 0.004$) (Fig. 1.1-A), and higher in severe PE cases compared to mild PE cases ($p = 0.088$) (Fig. 1.1-B). The homocysteine level was significantly more in PE cases with negative pregnancy outcome ($p = 0.003$) (Fig. 1.1-C), and was more in PE associated preterm delivery compared to PE term delivery cases ($p = 0.301$) (Fig. 1.1-D). Analysis with PE group clearly indicated that higher homocysteine levels were associated with negative outcome of delivery in both severe and mild PE cases (Fig. 1.1-E).Analysis within the PE group also suggested that higher homocysteine levels were associated with negative outcome of delivery in both term and preterm delivery cases (Fig. 1.1-F). The data thereby is clearly suggestive of the importance of hyperhomocytenemia in predisposition to PE and associated complications. The protein expression of homocysteine in placental tissue also showed significantly higher in the PE cases compared to normal term delivery cases ($p = 0.002$)

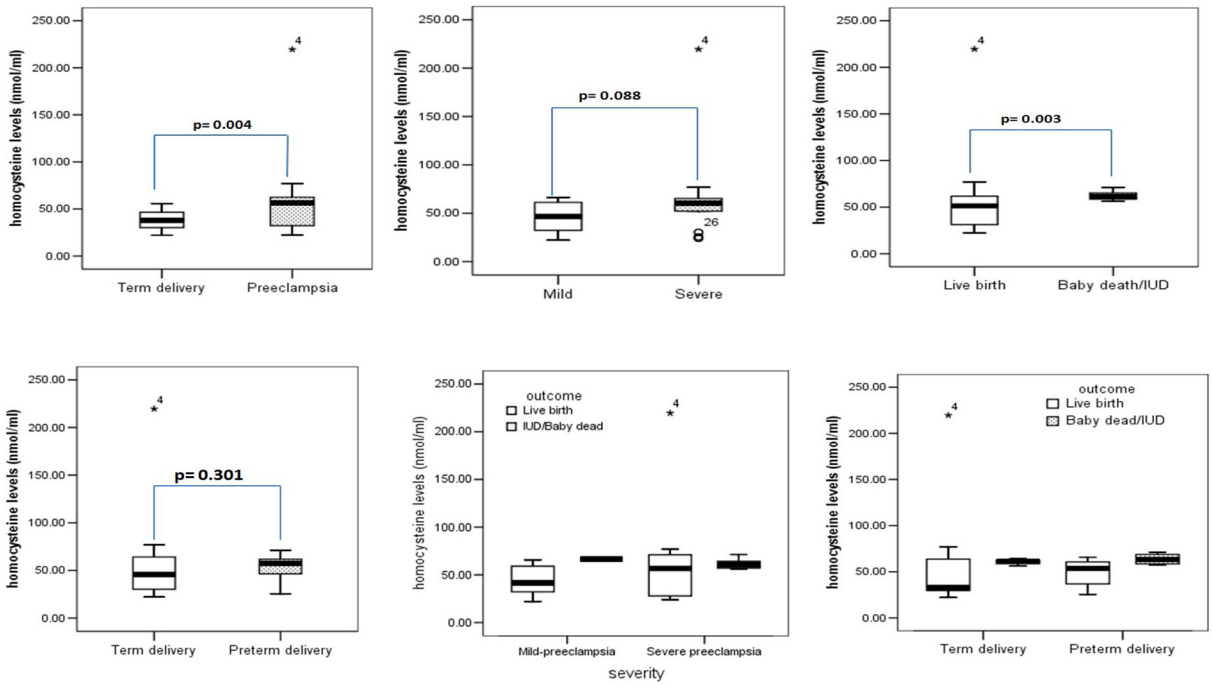

Fig. 1.1. (Left to Centre to Right), {Upper Left (UL), 1.1-A}: Box-plot analysis showing that the plasma homocysteine levels were significantly higher in Preeclampsia (PE) cases compared to normal term full delivery (NFTD) cases ($p = 0.004$). {Upper Centre (UC), 1.1-B}: Box-plot analysis showing that the homocysteine level were significantly higher in severe PE cases compared to mild PE cases ($p = 0.088$) within PE group. {Upper Right (UR), 1.1-C}: Box-plot analysis showing that the homocysteine level were significantly higher in PE cases with negative pregnancy outcome ($p = 0.003$). {Lower Left (LL), 1.1-D}: Box-plot analysis within PE group showing that the homocysteine levels were higher in PE associated preterm delivery compared to PE term delivery cases ($p = 0.301$). {Lower Cente (LC), 1.1-E}: Box-plot analysis within PE group indicated that higher homocysteine levels were associated with negative outcome of delivery in both severe and mild PE cases. {Lower Right (LR), 1.1-F}: Box-plot analysis within PE group suggested that higher homocysteine levels were associated with negative outcome of delivery in both term and preterm delivery cases.

(Fig. 1.2).

3.3. Estimation of serum vitamin B12 levels

Individuals with higher levels of Hcy are generally recommended with the folic acid and Vitamin B12 supplementations, as the adequate quantity of these B vitamins plays a major role in balancing the Hcy in blood. Deficiency of vitamin B12 raises the blood Hcy levels, as vitamin B12 acts as a cofactor in the metabolism of Hcy. The increased Hcy levels decrease the Vitamin B12 and folate levels in the body [34]. The serum Vitamin B12 levels were evaluated by commercially available ELISA kit. The Vitamin B12 levels were non-significantly higher in the PE cases (312.550 ± 158.791 pmol/l) compared to the NFTD cases (277.870 ± 108.409 pmol/l) ($p = 0.381$); while the levels were comparable between mild (341.783 ± 153.897 pmol/l) and severe PE cases (263.825 ± 163.622 pmol/l) ($p = 0.739$); which indicates that the Vitamin B12 levels may not be the primary cause of increased Hcy levels (Fig. 2).

The difference in Vitamin B12 levels were not significant between PE-preterm delivery cases (316.30 ± 174.36 pmol/l) and PE-term delivery cases (305.05 ± 132.67 pmol/l) ($p = 0.906$); as well as between pregnancy outcome status in PE cases ($p = 0.341$). No impact of Vitamin B12 levels was observed w.r.t outcome in the PE sub-cohorts as the data obtained by comparing PE outcome cases on the basis of severity showed higher levels of Vitamin B12 in subjects of mild PE cases with negative outcome compared to positive outcome cases, and the pattern was similar in severe PE cases. In the severe PE sub-cohort, the mean Vitamin B12 levels were higher in term delivery (329.425 ± 74.323 pmol/l) compared to preterm delivery (211.35 ± 204.09 pmol/l), while the trend was different in the mild PE cases where the levels were found to be comparable [preterm delivery (349.398 ± 145.64 pmol/l) and term delivery (330.36 ± 179.21 pmol/l)]. The Vitamin B12 levels were non-significantly greater in pregnancy cases with negative outcome compared to baby live status in both PE related term and preterm delivery cases (Fig. 3).

The entire set of Vitamin B12 data is suggestive that its levels may not be a major biochemical issue for the hyper-Hcy levels in the studied cohort and sub-cohorts. Hence forth, the next focus of the study shifted from the biochemical regulations to genetic regulations involved in folate metabolism and Hcy regulation, and is detailed below.

3.4. Distribution of MTHFR C677T and TYMS1494del6 polymorphisms and its association with preeclampsia and associated complications

PCR-RFLP analysis was performed to screen *MTHFR C677T* polymorphism *HinfI* restriction digestion (Fig. 4-A). The *MTHFR* variant genotype distribution was significantly higher in severe PE cases in comparison to mild PE cases ($p = 0.050$), and was linked to increased risk of severe PE {OR = 4.242[0.903–19.932] at 95%CI, $p = 0.072$}. The results didn't show any deviation from Hardy- Weinberg equilibrium.

PCR-RFLP analysis approach was used to screen *TYMS del6* polymorphism using *DraI* restriction enzyme. PCR amplification of *TYMS* gene showed amplification of 152 bp for *6 bp del* allele and 158 bp for *6 bp ins* allele. On restriction digestion using *DraI*, the amplicon yielded either a single uncut band of 152 bp representing variant homozygous 6 bp del allele, or four bands of 152 bp, 158 bp, 88 bp and 70 bp for heterozygote *6 bp ins/del* allele, while the homozygote *6 bp ins* allele yielded two bands of 88 bp and 70 bp (Fig. 4-B). The *TYMS del6* genotype distribution of the enrolled pregnancy cases is given in Table 2B. The data showed that the distribution of *TYMS del/del* variant genotype was higher in PE delivery cases (41.38%) compared to NFTD cases (6.77%) (Table 2). Our results didn't depict any deviation from Hardy-

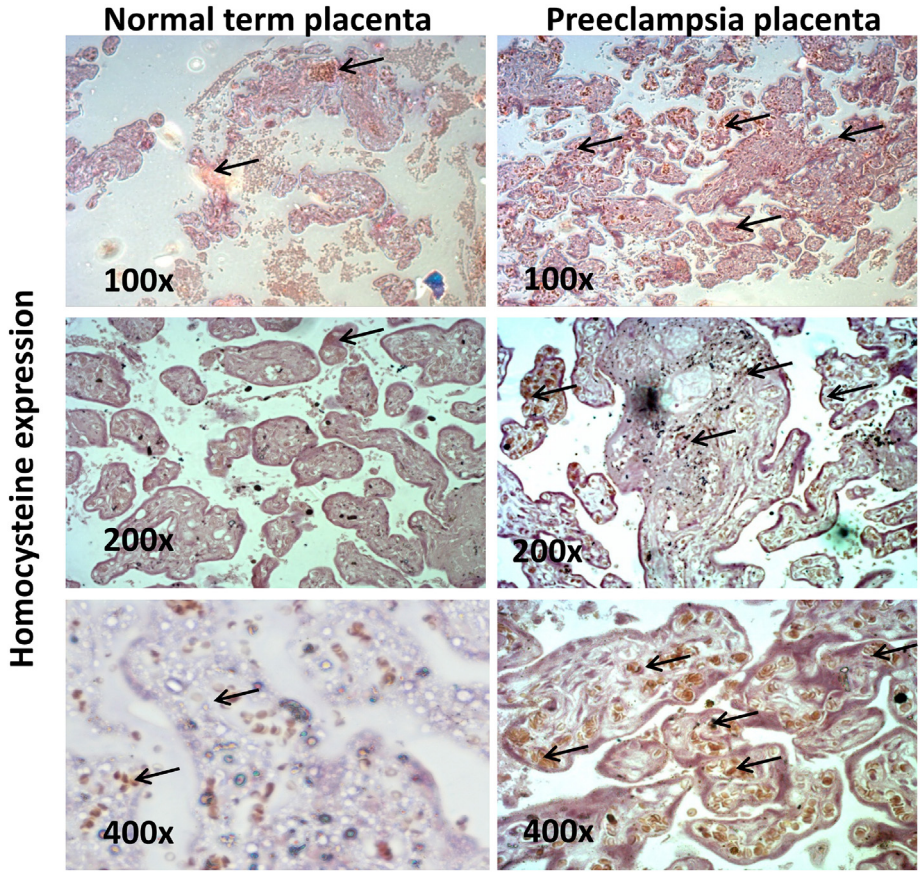

Fig. 1.2. Representative panel of IHC results showing the upregulation of Homocysteine expression in preeclampsia compared to normal term full delivery.

Weinberg equilibrium. The presence of *TYMS del/del* polymorphism was found to significantly increase the risk of preeclampsia by more than nine folds {OR = 9.719[5.060–18.671] at 95%CI, $p < 0.001$}. The presence of *TYMS del/del* polymorphism significantly increased the risk of severe PE compared to mild PE ($p = 0.010$) by more than two folds {OR = 2.702[1.256–5.812] at 95%CI, $p = 0.012$}.

The variant MTHFR genotype was found to be non-significantly associated with increased homocysteine levels in both NFTD and PE cases (Fig. 5-A and B). Further analysis within the PE sub-group showed the association of higher homocysteine levels in severe PE cases with variant *MTHFR* genotype (Fig. 5-C). Within the PE delivery group, the *MTHFR C677T* genotype polymorphism was not associated with changes in homocysteine levels in live birth as well as PE cases with negative pregnancy outcome (Fig. 5-D). No significant association of homocysteine levels was found in term delivery cases with variant *MTHFR* genotype (Fig. 5-E).

When all the enrolled pregnancy cases were considered together, homocysteine levels were found to be significantly higher in *TYMS 6 bp del/del* and *ins/del* compared to both *TYMS 6 bp ins/ins* genotype ($p < 0.001$) (Fig. 6-A); and higher in *TYMS 6 bp del/del* genotype compared to *TYMS ins/del* heterozygote genotype (Fig. 6-A). When both the control term delivery and PE cases were considered as two different cohorts, higher homocysteine levels were found in *TYMS 6 bp del/del* genotype followed by *TYMS 6 bp ins/del* and *TYMS 6 bp ins/ins* genotype in PE cases (Fig. 6-B). In the PE group, *TYMS 6 bp del/del* genotype polymorphism was found to be associated with higher homocysteine levels in severe PE cases compared to *ins/ins* and *ins/del* genotypes (Fig. 6-C). Within the PE delivery group also *TYMS 6 bp del/del and*

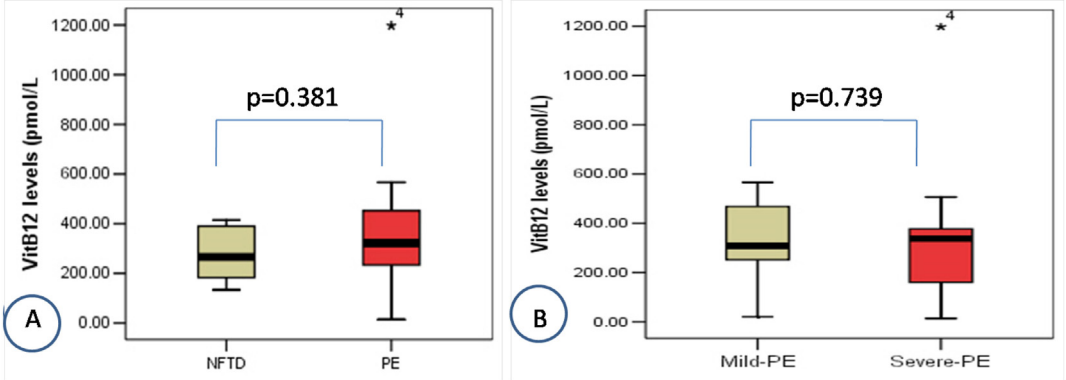

Fig. 2. Box-plot representation of serum vitamin B12 levels. (A) Box-plot representing higher levels of Vitamin B12 in PE cases compared to NFTD and (B) Box-plot representing Vitamin B12 levels in mild and severe PE cases. The values for both the cohorts of PE were comparable.

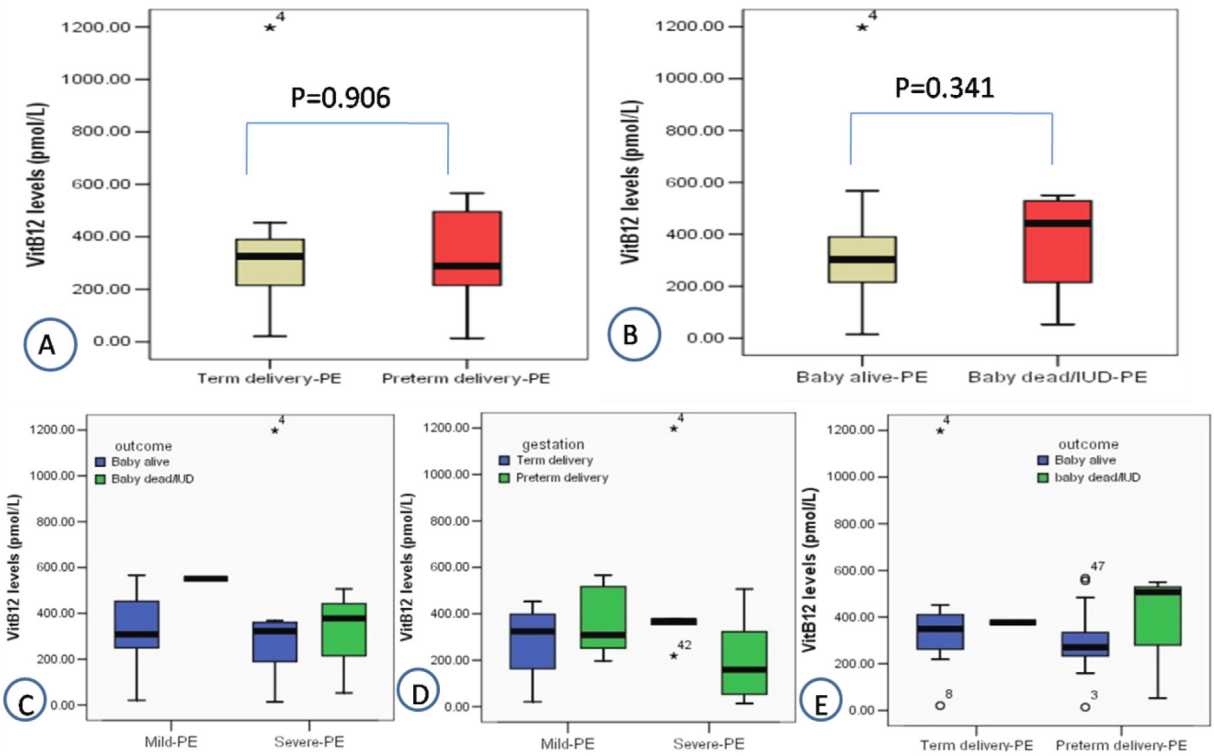

Fig. 3. Box-plot depicting the PE-associated serum Vitamin B12 levels. The first figure (A) showing changes in Vitamin B12 levels on the basis of gestational period; (B) Box-plot showing changes in Vitamin B12 levels on the basis of outcome; (C) Box-plot showing changes in Vitamin B12 levels on the basis of severity with reference to outcome; (D) Box-plot showing changes in Vitamin B12 levels on the basis of severity with reference to gestation; (E) Box-plot showing changes in Vitamin B12 levels on the basis of gestational period with reference to outcome.

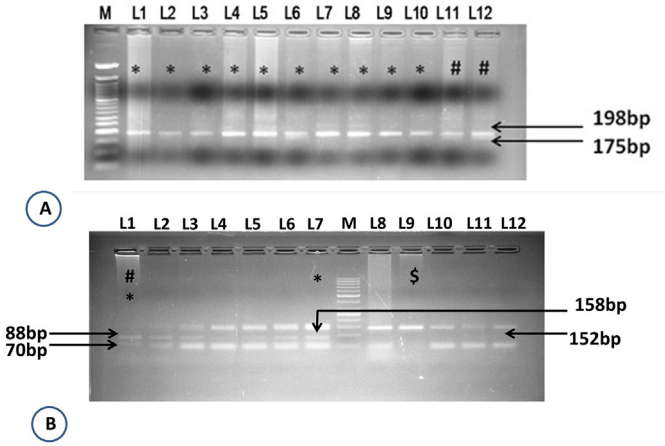

Fig. 4. (Upper panel: 4-A): Representative photograph of agarose gel electrophoresis of the restriction digestion products of *MTHFR* gene showing the presence of either wild type (characterized by presence of 198 bp and marked by *) or heterozygous (characterized by presence of 198 + 175 + 23 bp and marked by #) in the studied cases. (Lower panel: 4-B): Representative agarose gel electrophoresis of RFLP analysis of *TYMS 1494del6 bp* genotyping showing the presence of 6 bp deletion characterized by presence of 152 bp marked as ($), 6 bp insertion/deletion characterized by presence of 152 + 158 + 88 + 70 bp for heterozygous state marked as (*) and 88 + 70 bp for homozygous ins condition marked as (#*).

TYMS 6 bp ins/del genotype polymorphism showed the association of higher homocysteine levels with the negative pregnancy outcome (Fig. 6-D). Further analysis showed that *TYMS 6 bp del/del* and *TYMS 6 bp ins/del* genotype was associated with increased homocysteine levels in both PE related term and preterm delivery cases (Fig. 6-E).

3.5. Combined genotype distributions and its association with preeclampsia

The combined genotype distributions of *MTHFR C677T* and *TYMSdel6* polymorphisms in the enrolled pregnancy cases are tabulated in Table 3. The data showed that the presence of combined variant genotype of *MTHFR* and *TYMS del6* polymorphism resulted in an increased risk of severe PE compared to FTND cases {OR = 2.151[0.718–6.449] at 95% CI, p = 0.181}. Further, the combined variant genotype of *MTHFR* and *TYMSdel6* polymorphism resulted in significant increase (p = 0.014) in the risk of severe PE compared to mild PE {OR = 5.469[1.239–24.146] at 95% CI, p = 0.026}. When all the enrolled pregnancy cases were considered together, homocysteine levels were found to be significantly higher in combined variant genotype compared to combined wild-type genotype (p < 0.001) (Fig. 7-A). When all the NFTD and PE cases were considered as two different groups, higher homocysteine levels were found in combined variant genotypes compared to combined wild-type genotype in both in PE and NFTD cases (Fig. 7-B); and the results were consistent in both mild and severe PE groups (Fig. 7-C). Also within the PE delivery group the combined variant genotypes showed association with higher homocysteine levels both in live birth cases as well in the PE cases with negative pregnancy outcome (Fig. 7-D). Further, analysis also showed that the increased homocysteine levels associated with combined variant genotypes was consistent in both term delivery and preterm delivery in PE cases (Fig. 7-E).

4. Discussion

PE is a pregnancy-induced hypertensive disorder which generally occurs after 20 weeks of gestation and it remains not treated it can lead to eclampsia. In terms of clinical symptoms, PE and eclampsia are distinct from each other. The mildest disorder in this continuum is PIH (pregnancy-induced hypertension). PE is associated with hypertension

Table 2
MTHFR C677T and *TYMS1494del6* genotype distribution between NFTD and PE cases.

Cases	N	*MTHFR* C677T genotype			Less common allele frequency	*p* value	Odds ratio at 95% CI
		Wild-type	Heterozygous	Homozygous			
NFTD	192	172[89.58]	20[10.42]	0[0]	20[10.42]	Ref	Ref
PE	145	138[95.17]	7[4.83]	0[0]	7[4.83]	0.062	1.717[0.897–3.288]
PE-Mild	108	105[97.22]	3[2.78]	0[0]	3[2.78]	0.017	0.246[0.071–0.847
PE-Severe	37	33[89.19]	4[10.41]	0[0]	4[10.41]	0.943	1.042[0.335–3.247]

Cases	N	*TYMS1494del6* genotype			Less common allele frequency	p value	Odds ratio at 95% CI
		6 bp ins/ins	6 bp ins/del	6 bp del/del			
NFTD	192	21[10.94]	158[82.29]	13[6.77]	171[89.06]	Ref	Ref
PE	145	20[13.79]	65[44.83]	60[41.38]	125[86.21]	0.428	0.768[0.399–1.477]
Mild PE	108	15[13.89]	55[50.93]	38[35.18]	93[86.11]	0.451	0.761[0.375–1.547]
Severe PE	37	5[13.51]	10[27.03]	22[59.46]	32[86.49]	0.652	0.786[0.276–2.237]

Cases represented as numbers (%age).
*Statistically significant.

and proteinuria while eclampsia is associated to convulsions in addition to hypertension and proteinuria (Shah, 2009). Globally, PE is an established public health problem contributing to both maternal and perinatal morbidity and mortality (Dolea and AbouZahr, 2000; Shah et al., 2009; McClure et al., 2009). However, the effect of this disease is experienced more severely in developing countries (Igberase and Ebeigbe, 2006) due to late presentation of the disease which makes medical interventions ineffective (Onakewhor and Gharoro, 2008). In India, the incidence of PE is found to be 8–10% of the pregnancy and it is the 3rd leading cause of maternal mortality (Krishna Menon and Palaniappan, 1994; MacKay et al., 2001). Clinical study conducted by the regional hospital in Assam, suggested the higher frequency of maternal and baby death due to untreated condition of PE that progresses to Eclampsia resulting in unexpected seizure risking the life of both the mother and the baby (Doley and Pegu, 2016); but till date there is scarce of available scientific data on the underlying PE associated risk factors and following negative outcome in patients from Assam particularly from the region of Lower Assam. The present study is a step towards understanding the biochemical and molecular genetic basis of PE in lower Assam with special reference to the folate pathway. For the present study, pregnancy cases were stratified as NFTD cases (> 37 weeks) and PE cases. The PE cases were further stratified into two cohorts namely; Mild PE and Severe PE [ACOG 2011]. The majority of the enrolled PE cases belonged to mild PE cohort compare to severe cohort. The majority of the subjects belonged to lower socio-economic background and was more prevalent in young women belonging to age group of 22.59 ± 3.73.

Homocysteine (Hcy) is an endogenous thiol and an intermediate in the methionine cycle that does not participate in protein synthesis. Status of elevated plasma Hcy concentrations referred to as hyperhomocysteinaemia (HHcy) in pregnant women had been associated with PE (Aubard et al., 2000). Additionally, higher maternal Hcy concentrations predicted earlier delivery (Kalhan, 2016). Similar to these reported observations, the data from our study also showed that the

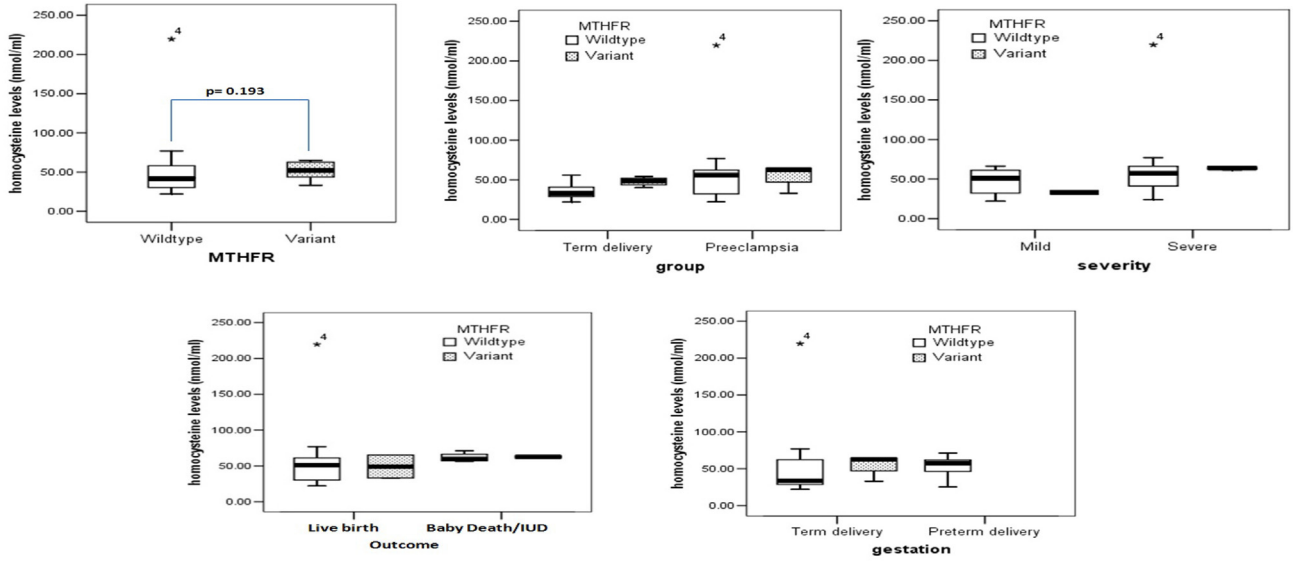

Fig. 5. (Left to Centre to Right), {Upper Left (UL) and Upper Centre (UC), 5-A and 5-B}: Box-plot analysis showing that the variant MTHFR genotype was found to be non-significantly associated with increased homocysteine levels in both NFTD and PE cases. {Upper Right (UR), 5-C}: Box-plot analysis within the PE sub-group showed the association of higher homocysteine levels in severe PE cases with variant *MTHFR* genotype. {Lower Left (UL), 5-D}: Box-plot analysis within the PE delivery group showed that the *MTHFR C677T* genotype polymorphism was not associated with changes in homocysteine levels in live birth as well as PE cases with negative pregnancy outcome {Lower Right (UR), 5-E}: Box-plot analysis within the PE sub-group showed no association of homocysteine levels in term delivery cases with variant *MTHFR* genotype.

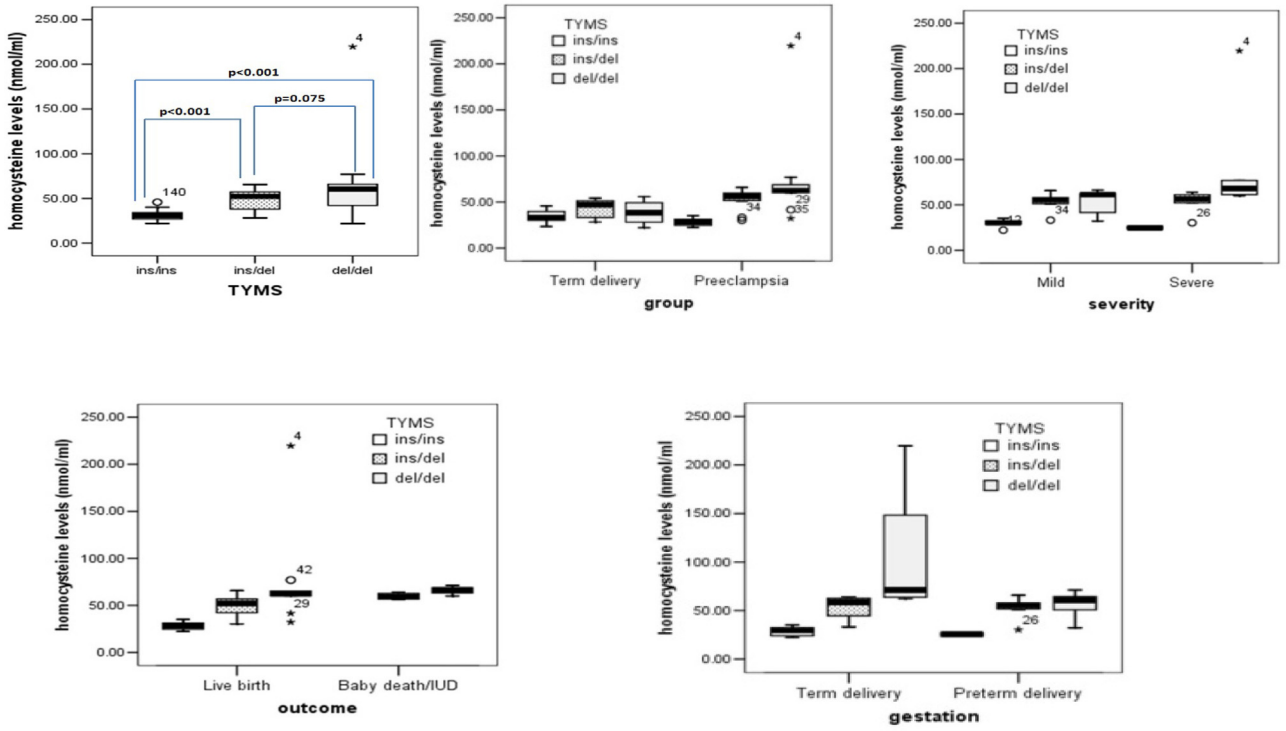

Fig. 6. (Left to Centre to Right), {Upper Left (UL), 6-A}: Box-plot analysis in all enrolled pregnancy cases showing that the homocysteine levels were significantly higher in *TYMS 6 bp del/del* and *ins/del* compared to both *TYMS 6 bp ins/ins* genotype ($p < 0.001$) and higher in *TYMS 6 bp del/del* genotype compared to *TYMS ins/del* heterozygote genotype. {Upper Centre (UC), 6-B}: Box-plot analysis between both FTND and PE cases showed higher homocysteine levels in *TYMS 6 bp del/del* genotype followed by *TYMS 6 bp ins/del* and *TYMS 6 bp ins/ins* genotype in PE cases {Upper Right (UR), 6-C}: Box-plot analysis within the PE sub-group showed that *TYMS 6 bp del/del* genotype polymorphism was associated with higher homocysteine levels in severe PE cases compared to *ins/ins* and *ins/del* genotypes. {Lower Left (UL), 6-D}: Box-plot analysis within the PE delivery group showed that *TYMS 6 bp del/del and TYMS 6 bp ins/del* genotype polymorphism was associated with higher homocysteine levels in negative pregnancy outcome. {Lower Right (UR), 6-E}: Box-plot analysis within the PE sub-group showed that *TYMS 6 bp del/del* and *TYMS 6 bp ins/del* genotype was associated with higher homocysteine levels in both PE related term and preterm delivery cases.

Table 3
Combinatorial genotype distributions in the studied cohorts.

Cases	N	*MTHFR* C677T/ *TYMS1494del6 combined genotype*			*MTHFR* and *TYMS* variant combined genotype	p-value	ODDs ratio at 95% CI
		Combined wild-type Genotype [CC//ins/ins]	*Combined heterozygous genotype* [CC//ins/del Or CC// del/del Or CT//ins/ins Or TT//ins/ins]	*Combined homozygous variant genotype* [CT//ins/del Or TT//ins/del Or CT//del/del Or TT//del/del]			
NFTD	192	18[9.38]	161[83.85]	13[6.77]	174[90.63]	Ref	Ref
PE	145	20[13.79]	118[81.38]	7[4.83]	125[86.21]	0.205	0.647 {0.329–1.272}
Mild PE	108	15[13.89]	90[83.33]	3[2.78]	93[86.11]	0.231	0.641[0.309–1.331]
Severe PE	37	5[13.51]	27[72.98]	5[13.51]	32[86.49]	0.444	0.662[0.229–1.911]

Cases represented as Numbers (%age).

tHcy levels were significantly higher in PE cases compared to NFTD cases. Elevated Hcy levels were observed in severe PE cases compared to mild PE cases. The Hcy level was significantly higher in PE cases with negative pregnancy outcome and was higher in PE associated preterm delivery compared to PE term delivery cases. Analysis with PE group clearly indicated that higher homocysteine levels were associated with negative outcome of delivery in both severe and mild PE cases. Analysis within the PE group also suggested that higher homocysteine levels were associated with negative outcome of delivery in both term and preterm delivery cases. The data thereby is clearly suggestive of the importance of HHcy in predisposition to PE and associated complications.

Deficiency of vitamin B12 (cofactor in the metabolism of Hcy) raises Hcy levels (Mukhopadhyay et al., 2017), and hence Vitamin B12 supplementations along with folic acid are used for treatment of HHcy. Hence, we next targeted to evaluate the concentration of serum Vitamin B12 in the subjects enrolled for the present study. The findings from our study suggested increased Vitamin B12 levels in PE cases compared to NFTD subjects ($p = 0.381$), and comparable levels between PE sub-cohorts ($p = 0.739$); suggesting that Vitamin B12 levels may not be linked with HHcy in the studied population. Similar data was documented by Makedos et al., 2006 which suggested non significant relation of increased Hcy with Vitamin B12. The Vitamin B12 levels were not found to be associated with pregnancy outcome or gestation period between PE sub-cohorts. The contrasting data obtained from the study by Dhobale et al., 2012 that reported the higher concentration Vitamin B12 is associated with preterm delivery.

Alterations at the genetic level bring changes in the normal functioning of the enzymes responsible for the maintenance of the Hcy metabolism. Therefore, the next major focus of the study was to explore the association of genetic alterations in the critical folate pathway genes and their correlation with elevated Hcy levels in the maternal blood. The gene polymorphism of *MTHFR C677T* and *TYMS 1494del6* were undertaken for the present study in order to check for their association

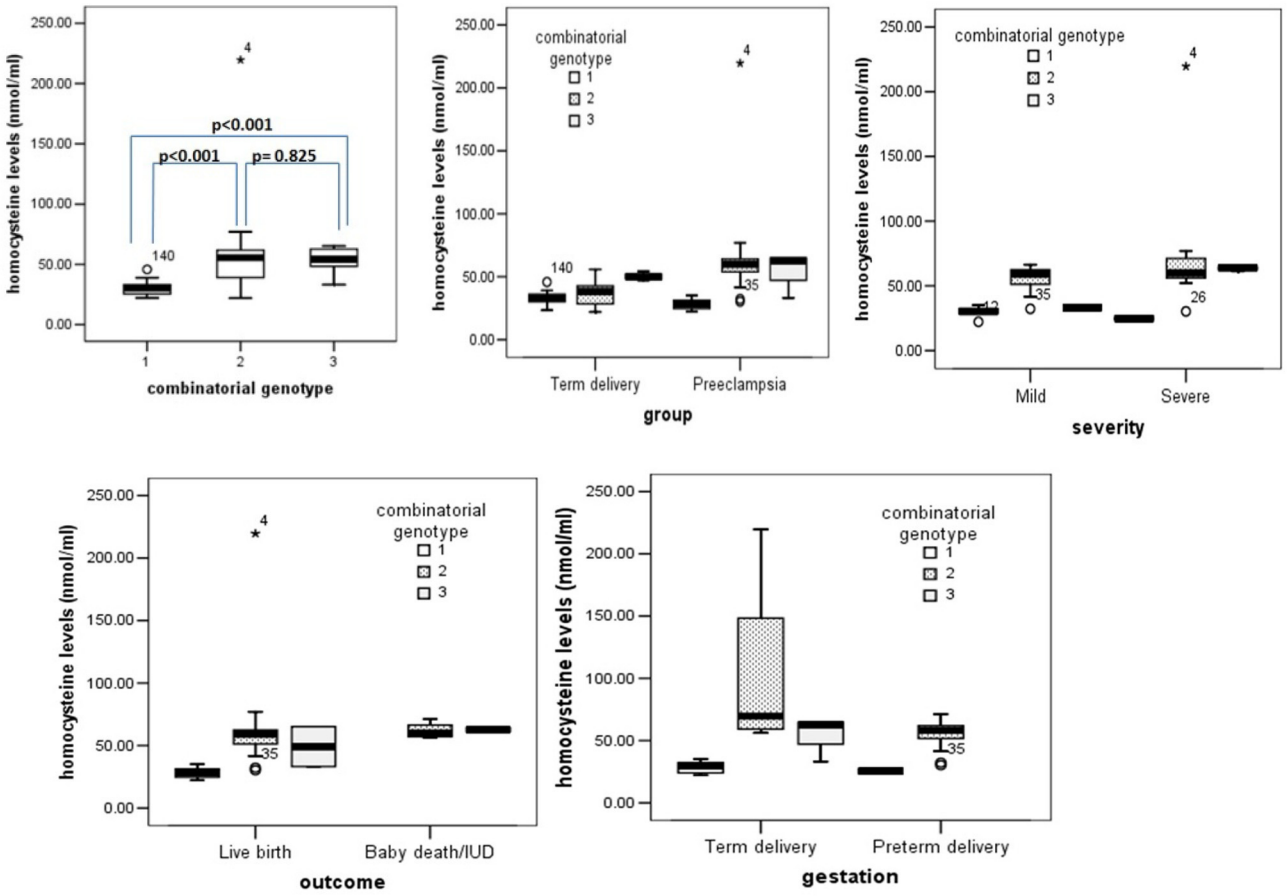

Fig. 7. (Left to Centre to Right), {Upper Left (UL), 7-A}: Box-plot analysis in all enrolled pregnancy cases showing that the homocysteine levels were significantly higher in combined variant genotype compared to combined wild-type genotype ($p < 0.001$). {Upper Centre (UC), 7-B}: Box-plot analysis between both NFTD and PE cases showed higher homocysteine levels in combined variant genotypes compared to combined wild-type genotype in both in PE and NFTD cases. {Upper Right (UR), 7-C}: Box-plot analysis within the PE sub-group showed that consistent results in both mild and severe PE groups. {Lower Left (UL), 7-D}: Box-plot analysis within the PE delivery group showed that the combined variant genotypes was associated with higher homocysteine levels both in live birth cases as well in the PE cases with negative pregnancy outcome. {Lower Right (UR), 7-E}: Box-plot analysis within the PE sub-group showed increased homocysteine levels associated with combined variant genotypes was consistent in both term delivery and preterm delivery in PE cases.

with the elevated Hcy levels in the predisposition of PE. It was observed that the distribution of variant *MTHFR* genotype between NFTD cases and PE cases was statistically non-significant. Similar reports had been published by several other research groups interpreting an insignificant association between the *MTHFR C677T* polymorphism and PE (Mann et al., 2010; Dickerson et al., 2010). However, the distribution of *MTHFR* variant genotype was significantly higher in severe PE cases compared to mild PE cases ($p = 0.050$), and was associated with increased risk of severe PE by more than four folds {OR = 4.242]. This result is in agreement with the results of Youssef et al., 2017, who reported that a variant *MTHFR* genotype in PE patients may be a genetic risk marker for the development of severe hypertension or severe PE.

Our study evidenced the distribution of homozygous *TYMS del/del* variant genotype was associated with PE susceptibility ($p < 0.001$) and severity compared to NFTD subjects, and increased the risk of negative pregnancy outcome in PE cases [OR = 9.719], and was associated with increased levels of tHcy and related complications. The variant TYMS genotypes were associated with preterm delivery in PE cases. There is limited availability of data concerning the associate influence of *TYMS 1494del6* polymorphism with PE based pregnancy; but it has been found that TYMS homozygous *6 bp del/del* has a great significance with the increasing risk of preterm delivery (Tiwari et al., 2017). However, some other scanty reports also suggested no association of *TYMS 1494del6 del/del* genotype in preterm delivery (Wang et al., 2015). Our finding report is against Kealey et al., 2005 study which led to the observation that *TYMS 3′UTR del/del* genotype is well associated with reduced Hcy level. This finding is supported by Trinh et al., 2002 in which it has been documented that TYMS polymorphism (*TYMS 3/3*) represents a newly identified genetic determinant of plasma folate and tHcy status, and is found to be independent of MTHFR genotype.

Under the enrolled pregnancy cases for the present study, the combined genotype distributions of both *MTHFR C677T* and *TYMSdel6* polymorphisms was studied and the obtained result suggested the presence of combined variant genotype of *MTHFR C677T* and *TYMS del6* polymorphism resulted in an increased risk of PE susceptibility and severity. The findings are concurrent to the earlier published reports by other research groups (Kealey et al., 2005; Trinh et al., 2002; Rah et al., 2012; Park et al., 2008; Bae et al., 2007). The variant combinatorial genotype was associated with increased tHcy levels in all cohorts and sub-cohorts, preterm delivery and negative pregnancy outcome.

To conclude, the present study points out the significance of genetic factors and associated hyper-homocysteinemia in predisposition to PE and other pregnancy associated complications. The study provides significant insights about the prognostic significance of *MTHFR C677T, TYMS 1494del6* genotype, tHcy and Vitamin B12 levels as a risk factor for PE, negative pregnancy outcome and preterm delivery. It is suggested from our study that screening of plasma homocysteine levels and *TYMSdel6* polymorphism of the pregnant women may be useful in preventing negative pregnancy outcome for those women who are at

the risk of PE.

Declaration of Competing Interest

There are no conflicts of interest.

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