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The association between Folate metabolism, C677T and A1298C Polymorphisms of Methylene tetrahydrofolate reductase Gene and the genetic susceptibility of Preeclampsia among a sample of Jordanian pregnant women

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ABSTRACT

Background: Preeclampsia (PE) is a pregnancy disorder causes uteroplacental dysfunction resulting in fetal growth restriction. *Aim:* This study aimed to evaluate the association between C677T and A1298C polymorphisms of methylene tetrahydrofolate reductase (*MTHFR*) gene and PE susceptibility in a sample of Jordanian women. *Methodology*: The analysis of *MTHFR* C677T and A1298C polymorphisms was done by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). PCR-RFLP products were digested with *hinfI* and *MboII* enzymes. The digestion products were electrophoresed on 2% agarose gel, stained and visualized under UV light. The levels of homocysteine, folic acid, and B12 were assayed using ELISA. *Results*: The study revealed a significant difference in plasma levels of homocysteine in PE patients versus the controls. TT, CT polymorphisms, AC polymorphism, mutant T, C alleles of *MTHFR* C677T and A1298C polymorphisms, respectively, also, combined polymorphisms 677CT/1298CC, 677CT/1298AA and 677TT/1298AA were shown to be associated with increased PE susceptibility. *Conclusion*: Our data suggest that *MTHFR* C677T and A1298C polymorphisms are most likely to be risk factors for developing PE among Jordanian pregnant women. Also, the two types of polymorphisms may synergize to increase the risk of PE.

Keywords: *MTHFR*, Homocysteine, Folic acid, single nucleotide polymorphisms, Hypertension

1. INTRODUCTION

PE is a pregnancy disorder which likely begins during implantation with or without proteinuria and results in injury to the mother's organs and lasts throughout the remainder of pregnancy until labor (Peres et al., 2018; Derouiche et al. 2020). Globally, 5% to 7% of pregnant women are affected each year, and it is ranked first among the causes of premature births, and can lead to maternal death (Cornelius, 2018). The causes of preeclampsia are widely unknown and the predisposing risk factors are overlapping including different genetic and environmental factors. In Jordan, it is well known that the incidence of consanguinity is high. However, there is a large controversy in the correlation between consanguinity and the incidence of PE (Francis et al., 2001).

According to Genome-wide association studies (GWAS), it was revealed that the susceptible genes for the development of PE are responsible for regulating maternal-fetal interactions on one hand and unfavorable genes' polymorphisms on the other hand (Salonen et al., 2000). These genes include Storkhead Box 1(*STOX1*) gene (Frendo et al., 2003), *MTHFR* C677T polymorphism (Benedetto et al., 2002), Angiotensin-converting enzyme inhibitor (*ACEI*) I/D polymorphism (Verdonk et al., 2014), factor V Leiden mutation (Scholz et al., 2008). The gene encoding for methylene tetrahydrofolate reductase (MTHFR) enzyme (EC 1.5.1.20) is located on chromosome 1p36.3, 2.2 kb in length, and encompassed 11 exons, an important enzyme for folate and homocysteine metabolism (Goyette et al., 1998).

Several studies showed the association between preeclampsia and different single nucleotide polymorphisms (SNPs) including those involved in the gene encoding for MTHFR enzyme particularly C677T and A1298C. *MTHFR* C677T polymorphisms involve the substitution of alanine to valine due to a SNP at position 677 (C→T) in exon 4 causing a decrease in the activity of the enzyme and impairment of folate binding due to the increase in its thermolability. The other variant is (A→C) transversion at position 1298 in exon 7 leading to the substitution of glutamate with alanine which also decreases the enzymatic activity but to a lesser degree (Egan et al., 2004). *MTHFR* C677T is associated with increased folic acid demand for the maintenance of normal homocysteine to methionine remethylation causing low serum levels of folate, and a slight increase in the plasma level of total homocysteine (Waterman et al., 2011). The high level of plasma homocysteine is a good genetic indicator for the contribution of *MTHFR* C677T polymorphism in the pathophysiology of PE (Powers et al., 1998).

So, The present study aimed to evaluate the association between C677T and A1298C polymorphisms in the gene encoding for *MTHFR* as potential risk factors and the susceptibility for PE, also, assess the interrelations between these polymorphisms and homocysteine levels in a sample of Jordanian pregnant women. The conduction of such a study to explore the effects of *MTHFR* gene polymorphisms as risk factors for PE could be considered the first time in Jordan.

2. MATERIALS AND METHODS

248 participants were enrolled in the present study (107 preeclamptic patients and 141 normotensive pregnant women as a control group), they were selected from the attendants of the Obstetrics Clinic, Labor Ward and Care Unit at Al-Karak governmental Hospital and the Islamic Hospital, Amman, Jordan, during the period from January 2017 till April 2019. Each one of the patients was primigravidae, in the second half of her pregnancy and with a single pregnancy.

The approval of the study had been obtained from both the Medical Ethics and the Scientific Committees of the Faculty of Medicine under Mutah University instructions (the reference number of the ethical approval: 201614). Each participant gave a written consent to be enrolled in the study.

Exclusion criteria

Obese patients, patients with a history of previous hypertension, chronic nephritis, or diabetes mellitus were excluded.
All subjects were evaluated according to the following:
- Proper history taking and clinical examination particularly stressing on the exclusion criteria mentioned before.

Blood samples

Venous blood was withdrawn from each participant in two test tubes; one was used for the biochemical analyses and the second was EDTA-treated for the DNA extraction and complete blood count.

***MTHFR* C677T and A1298C polymorphisms analysis**

DNA was extracted from all samples using the method of Sambrook and Russell (2006), PCR-RFLP was applied as described by Van et al., (1998). The sequences of primers for *MTHFR* C677T polymorphisms were as follows: forward-5′- TGA AGG AGA AGG TGT CTG CGG GA -3′ and reverse-5′- AGG ACG GTG CGG TGA GAG TG -3′ and for A1298C polymorphisms were forward-5′-

CTT TGG GGA GCT GAA GGA CTA CTA C -3′ and reverse-5′- CAC TTT GTG ACC ATT CCG GTT TG -3′. These primers were used to amplify a 198 bp and 163 fragments of DNA for *MTHFR* C677T and A1298C polymorphisms, respectively. PCR began with denaturation at 94°C for two minutes, then, 35 cycles of denaturation at 94°C for one minute, annealing at 62°C for one minute, and extension at 72°C for one minute, the final extension was at 72°C for ten minutes. PCR-RFLP products for the two polymorphisms were digested with 5 units of *hinfI* and *MboII* enzymes (Promega, Madison, WI, USA) for *MTHFR* C677T and A1298C polymorphisms, respectively at 37°C for two hours. The electrophoresis of the digested amplicons on a 2% agarose gel followed by ethidium bromide staining revealed a 198 bp band in wild type (677CC); 175 bp and 23 bp bands in heterozygous mutants (C677T), 198, 175 and 23 bp bands for the homozygous mutants (677TT) for *MTHFR* C677T polymorphisms, while, for A1298C polymorphisms, the digested amplicons were 56, 31, 30, 28 and 18 bp bands for the wild type (1298 AA), 84, 56, 31, 30, 28 and 18 bp bands for the heterozygotes (A1298C) and 84, 31, 30 and 18 bp bands for the homozygous mutants (1298CC). Due to the small size, the 23, 31, 30, 28, and 18 bp bands were not seen.

Statistical analyses

Data were analyzed using SPSS Statistics V.21 software (IBM Corp., Armonk, NY). The numerical data were expressed as mean±SD and the difference between groups was assessed using Analysis of variance (ANOVA). The qualitative data were expressed as frequency, percentage, and odds ratio, the possible connection between two variables was assessed using Pearson's $\chi 2$. Allele counting was applied to determine the frequencies of the polymorphisms. Pearson's $\chi 2$ was also used to evaluate the frequencies of the polymorphisms associated with the Hardy-Weinberg equilibrium concordance.

3. RESULTS

To explore the association of PE with MTHFR C677T and A1298C polymorphisms, we included 107 PE subjects with their mean age 24.7 ± 2.81 and 141 healthy normal age matched subjects with their mean age24.0 ± 2.72. Compared to the normotensive pregnant women, the preeclamptic patients had significantly higher levels of plasma homocysteine, systolic, and diastolic blood pressure (p<0.001) and B12 (p<0.05) as summarized in Table 1.

Table 1 The clinical and laboratory data of the studied groups

Parameter	Preeclampsia group (no. 107)	Normotensive pregnant group (no. 141)	*P* value
Systolic BP (mmHg)	150.8 ± 9.43	120.9 ± 5.10	<0.001*
Diastolic BP (mmHg)	105.1 ± 9.42	76.8 ± 4.13	<0.001*
Homocysteine (μmol/l)	19.7 ± 2.088	6.70 ± 0.394	<0.001*
Folic acid (ng/ml)	4.73 ± 0.432	4.72 ± 0.529	0.920
B12 (pg/ml)	394.6 ± 36.0	384.9 ± 27.9	0.0218**

* *P*<0.001 is significant versus the normotensive pregnant women

** *P*<0.05 is significant versus the normotensive pregnant women

Table 2 The association between MTHFR C677T and A1298C polymorphisms versus age, systolic blood pressure, diastolic blood pressure, and plasma levels of homocysteine, folic acid and B12

	C677T Polymorphisms				A1298C Polymorphisms			
	C/C	C/T	T/T	*p*	A/A	A/C	C/C	*P* value
Systolic BP	138.1 ± 15.5	146.5 ± 18.9	137.5 ± 14.2	0.106	138.8 ± 16.8	144.1 ± 16.9	140.8 ± 15.9	0.527
Diastolic BP	92.4 ± 13.2	103.3 ± 19.0	90.8 ± 12.9	0.012*	93.6 ± 15.7	99.1 ± 17.0	95.6 ± 14.8	0.466
Homocysteine (μmol/l)	17.95 ± 1.85	23.84 ± 2.22	21.31 ± 1.86	0.230	18.02 ± 2.03	21.80 ± 1.86	19.47 ± 1.94	0.346
Folic acid (ng/ml)	4.86 ± 0.49	4.65 ± 0.40	4.52 ± 0.41	0.023*	4.71 ± 0.50	4.62 ± 0.45	4.86 ± 0.38	0.321
B12 (pg/ml)	359.9 ± 28.1	410.7 ± 20.6	389.0 ± 32.6	<0.001*	392.2 ± 33.0	393.8 ± 33.7	385.7 ± 36.1	0.745

* Significant *p*-value < 0.05.

Regarding the association between *MTHFR* C677T polymorphisms and the clinical & biochemical data, our results revealed significant differences between the three polymorphisms for the diastolic blood pressure, and plasma levels of folic acid and B12 in preeclamptic patients (p= 0.012, 0.023, and <0.001, respectively), while, for *MTHFR* A1298C polymorphisms, no significant differences were found (Table 2).

Table 3 The frequency of MTHFR C677T and A1298C polymorphisms separately and in combined forms in the pre-eclamptic patients and the controls

	Pre-eclampsia group (no. 107)	Normotensive pregnant group (no. 141)	OR	95% (CI)	*P* value
MTHFR 677 Polymorphisms					
677C/C	49 (45.79%)	75 (53.19%)	0.710	0.75 to 1.58	0.238
677C/T	33 (30.84%)	35 (24.82%)	1.330	0.97 to 1.93	0.116
677T/T	25 (23.36%)	31 (21.99%)	1.290	0.94 to 1.92	0.042 *
T allele	84 (39.25%)	90 (31.91%)	1.400	1.06 to 2.14	0.031*
C allele	130 (60.75%)	192 (68.09%)	0.740	0.80 to 1.87	0.340
MTHFR 1298 Polymorphisms					
1298A/A	56 (52.34%)	90 (63.83%)	0.780	0.92 to1.56	0.212
1298A/C	25 (23.36%)	25 (17.73%)	1.270	1.01 to 1.73	0.027 *
1298C/C	26 (24.30%)	26 (18.44%)	1.190	1.09 to 1.61	0.283
C allele	78 (36.45%)	85 (30.14%)	1.230	0.88 to 1.86	0.280
A allele	136 (63.55%)	197 (69.86%)	0.800	0.73 to 1.54	0.348
Combined polymorphisms					
C677C/A1298A	31 (28.97%)	44 (31.21%)	0.860	0.89 to 1.31	0.039 *
C677C/A1298C	8 (7.48%)	16 (11.35%)	0.690	0.74 to 1.75	0.013 *
C677C/C1298C	11 (10.28%)	16 (11.35%)	0.890	0.2 to 4.02	0.731
C677T/A1298A	13 (12.15%)	35 (24.82%)	1.470	0.95 to 3.5	0.226
C677T/A1298C	8 (7.48%)	0 (0%)	N/A	N/A	N/A
C677T/C1298C	11 (10.28%)	6 (4.26%)	1.560	1.13 to 2.36	0.044 *
T677T/A1298A	11 (10.28%)	8 (5.67%)	1.380	0.25 to 7.63	0.467
T677T/A1298C	8 (7.48%)	8 (5.67%)	1.080	0.19 to 6.32	0.614
T677T/C1298C	6 (5.60%)	8 (5.67%)	0.800	0.12 to 5.07	0.239

* Significant p-value < 0.05.

Hardy–Weinberg equation was used to evaluate the polymorphisms at the C677T and A1298C loci. The frequencies of *MTHFR*C677C, C677T, and T677T polymorphisms in the preeclamptic patients were 45.79%, 30.84% and 23.36%, respectively, and in controls were 53.19%, 24.82% and 21.99% respectively (table 3). While, for A1298C locus, when the preeclamptic patients and the controls were assessed for Hardy–Weinberg equation, the frequencies of *MTHFR*A1298A, A1298C, and C1298C polymorphisms in preeclamptic patients were 52.34%, 23.36%, and 24.30% respectively, while, in the controls, the frequencies were 63.83%, 17.73% and 18.44% respectively. The Odds ratio study for the frequency of C and T alleles in C677T polymorphisms was statistically significant for the T allele in the preeclamptic patients (Odds ratio = 1.400, 95% CI is 1.06 to 2.14, p = 0.031), while, for the C allele, it was statistically insignificant (Odds ratio = 0.740, 95% CI is 0.80 to 1.87, p = 0.640). Also, the frequency of A and C alleles in A1298C polymorphisms, the Odds ratio study showed a statistically insignificant difference for both alleles (Odds ratio= 1.230, 95% CI is 0.88 to 1.86, p=0.280 for the C allele, Odds ratio=0.800, 95% CI is 0.73-1.54, p=0.348 for the A allele).

Studying the frequency of the different polymorphisms among the preeclamptic patients and the controls showed that *MTHFR* C677T and T677T versus C677C were associated with increased risk for preeclampsia (OR=1.330, 95% CI=0.97 to 1.93, p = 0.116, OR=1.290, 95% CI=0.94 to 1.92, p=0.042, OR=0.710, 95% CI=0.75 to 1.58, p = 0.238, respectively). The T allele in *MTHFR* C677T polymorphisms is associated with 1.400 times increase in the risk for PE (Odds ratio = 1.400, 95% CI is 1.06 to 2.44, p = 0.031). Subjects with *MTHFR* polymorphisms (A1298C and C1298C) had been shown to be subjected to an increased risk for preeclampsia (OR = 1.270, 95% CI = 1.01 to 1.73, p = 0.027, OR= 1.190, 95% CI = 1.09 to 1.61, p = 0.283, respectively). The C allele in *MTHFR* A1298C polymorphisms is associated with 1.230 times increase in the risk for preeclampsia progression (Odds ratio= 1.230, 95% CI is 0.88 to 1.86, p =0.280). The combined polymorphism 677CT/1298AC was detected in 7.48% of the preeclamptic patients, while, none of the controls had been detected to have this combined polymorphic form. The highest risk for preeclampsia was found in 677CT/1298CC combined polymorphism (OR =1.560, 95% CI = 1.13 to 2.36, p= 0.044) (figure 1).

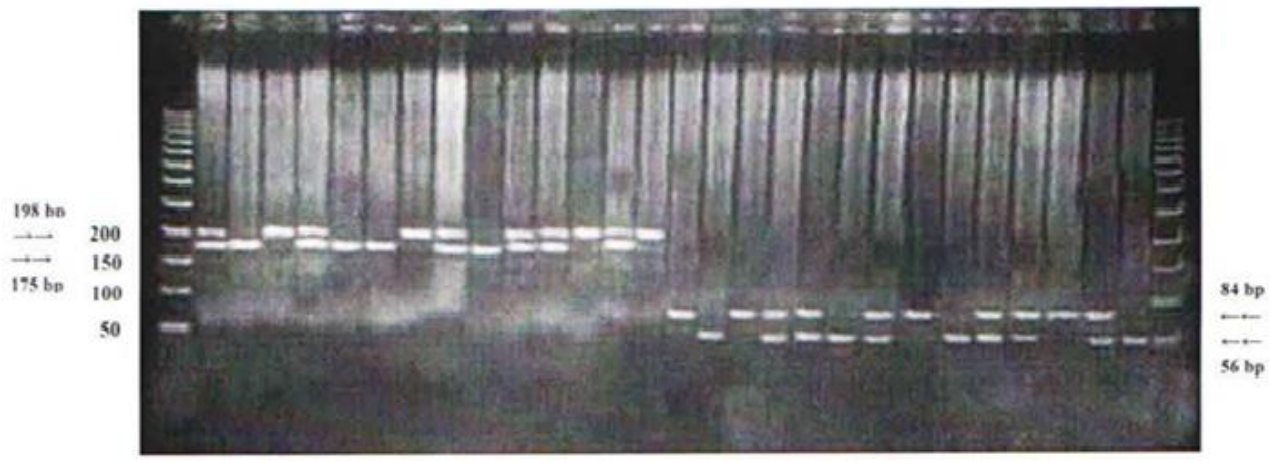

Figure 1 2% agarose gel electrophoresis of the amplicons of PCR-RFLP analysis of *MTHFR* C667T polymorphisms after being digested by *hinfI* showed a 198 bp fragment for the homozygous CC polymorphism, three fragments 198,175 and 23 bp for the heterozygous CT polymorphism, and two fragments 175 and 23 bp for the homozygous TT polymorphism. Also, PCR-RFLP analysis for the amplicons of A1298C polymorphisms after being digested by *MboII* showed the digested amplicons 56, 31, 30, 28, and 18 bp bands for the wild type (1298 AA), 84, 56, 31, 30, 28, and 18 bp bands for the heterozygotes (A1298C) and 84, 31, 30, and 18 bp bands for the homozygous mutants (1298CC). The 100 bp DNA ladder was indicated on both sides of the gel.

4. DISCUSSION

PE is considered a serious specific placental-mediated pregnancy disorder. There are several genes subjected to mutations that may be associated with various severe complications including thromboembolism. Some of these genetic polymorphisms affect the genes encoding for the involved enzymes in homocysteine metabolism resulting in elevation of plasma level of homocysteine which might be associated with placental vascular thrombosis causing PE and recurrent miscarriage (van der Put et al., 1998). *MTHFR* and its C677T and A1298C polymorphisms have been associated with the pathogenesis of different diseases not only PE but also, various cancers such as cervical and prostate cancers (Zhu et al., 2013). Several studies were conducted for the assessment of the association between *MTHFR* polymorphisms and PE in different populations, interestingly; the results were inconsistent in most of them (Fan et al., 2014).

Our results revealed that there is a statistically significant difference regarding systolic, diastolic blood pressure, plasma homocysteine and B12 levels in the preeclamptic women when compared to the normotensive pregnant women, while, there was no significant difference for the plasma levels of folic acid between the two groups. Our findings regarding systolic and diastolic blood pressure are consistent with the results of Pinheiro et al., (2015) and Luo and Li (2018). The hyperhomocysteinemia shown in the present study is in accordance with various studies (De et al., 2000). Previous studies including the study of Lopez-Quesada et al., (2003) had described the association between hyperhomocysteinemia and increased the risk for arterial stiffness as a leading factor for PE (Teles et al., 2011). Our results regarding B12 are consistent with theses obtained by Vander Jag and his colleagues (2011).

The evaluation of *MTHFR* C677T polymorphisms showed that the frequency of CC polymorphism bwas the highest in both the preeclamptic patients and the controls group, and TT genotype was the least in both groups. While, for A1298C locus, the

frequencies of AA and AC polymorphisms were the highest and the least in the preeclamptic patients and the controls, respectively. Regarding the frequency of alleles, T allele in the C677T polymorphisms was not the most frequent among the preeclamptic patients but its frequency was statistically significant, suggesting that it is a risk allele and may be associated with the development of PE. The C allele was considered as a non-risk allele and may play a role in the protection against PE. While, A allele in the A1298C polymorphisms was the highest in frequency in both the preeclamptic and the control groups, but, the frequencies of both A and C alleles were statistically insignificant in the preeclamptic patients when compared to the controls.

Our results are consistent with the findings of Ibrahim et al., (2012) who reported that T677T polymorphism and T allele are among the risk factors for PE, while, *MTHFR* C677C polymorphism and C allele are not risk factors for PE. Also, Kupferminc et al., (1999) reported that women with pregnancy complications as PE have *MTHFR* T677T polymorphism versus those without complications. Also, the positive family history for PE showed a higher frequency for TT polymorphism compared to the controls. In the present study, the frequency of TT polymorphism of *MTHFR* was found to be 23.36% and 21.99% in preeclamptic patients and the normotensive pregnant women, respectively, with a statistically significant difference (p= 0.042) between the two groups.

Previously, it was reported that the frequency of T677T polymorphism was the highest among the different *MTHFR* C67T polymorphisms, while, in the present study C677C polymorphism was the highest in frequency, this controversy in the results could be explained by the difference in ethnicity which was supported by the conclusion of Wu et al. The ethnicity is considered as an effective factor that links the susceptibility with *MTHFR* C677T polymorphisms predisposing to moderate hyperhomocysteinemia. Such a state of hyperhomocysteinemia may damage the vascular endothelium that leads to enhancement of the vasopressor effects followed by elevated blood pressure that is associated with PE (Wu et al., 2015).

Our results showed an apparent significant association between the frequency of C677T polymorphisms in the preeclamptic patients versus the controls (OR= 1.330 and 1.290, for C677T and T677T polymorphisms, respectively). This is in agreement with the study of Wu et al., (2015), Sunkara et al., (2010). On the other hand, other studies reported an insignificant association between *MTHFR* C677T polymorphisms and PE (Kumar et al., 2010). Regarding *MTHFR*A1298C polymorphisms, the frequency of the homozygous forms (A1298A and C1298C) in the preeclamptic patients showed no significant difference when compared to the corresponding forms in the normotensive pregnant women, on the other hand, the comparison of the frequency the heterozygous form (A1298C) in both groups revealed a statistically significant difference which could be associated with an increase in the risk for preeclampsia (OR= 1.27, CI is 1.01 to 1.73, p= 0.027). However, *MTHFR* A1298C polymorphism showed an association with the risk for the development of other diseases such as cardiovascular diseases (Szczeklik et al., 2001).

The stronger association between *MTHFR* C677T polymorphisms and PE was expected to be more than that for *MTHFR* A1298C polymorphisms. This can be explained by the reduction in the activity of the enzyme due to C677T polymorphisms (70%) compared to a reduction of only 30% in the case of A1298C polymorphisms (Weisberg et al., 2001). Those findings are inconsistent with the study of Wu et al., (2015) who stated that no correlation was found between *MTHFR* A1298C and the risk for PE. This inconsistency of the results may be attributed to the small sample size and ethnicity variance. In studying *MTHFR* C677T A1298C combined forms, it was found that these combinations 677CT/1298CC, 677CT/1298AA, and 677TT/1298AA were associated with increased risk of 1.560, 1.470 and 1.380 folds, respectively, for PE. Van der Put et al., (1998) reported that the combined forms of C677T and A1298C polymorphisms can act synergistically, it was suggested that both polymorphisms in heterozygous models can cause more reduction in the activity of *MTHFR* and higher levels of plasma homocysteine than the heterozygous form of each one of them separately.

Furthermore, another study found that the combined heterozygous form of *MTHFR*C677T A1298C polymorphisms has been associated with decreased plasma levels of homocysteine (Friedman et al., 1999). *MTHFR* 677CC/1298AA combined from was significantly higher in the control group compared to the preeclamptic patients and associated with decrease in the risk for PE (OR= 0.860, 95% CI 0.89 to 1.31, p= 0.039), so, it can be considered as a protective factor that may decrease susceptibility for PE.

5. CONCLUSION

Our results suggest that the *MTHFR* T677T genotype and the C allele in A1298C genotype could be risk factors for PE and in Jordanian pregnant women. Also, the data revealed that the two gene polymorphisms may associate together to increase the risk for PE as what has been shown in the combined forms 677CT/1298CC, 677CT/1298AA, and 677TT/1298AA. Those findings can provide a better idea for understanding the correlation between *MTHFR*T677T and A1298C polymorphisms and PE pathogenesis.

Abbreviations

PE	Preeclampsia
MTHFR	Methylenetetrahydrofolate reductase
PCR-RFLP	Polymerase Chain Reaction-Restriction Fragment Length Polymorphism
ELISA	Enzyme-Linked Immunosorbent Assay
UV	Ultraviolet
CD4	Cluster of differentiation 4
STOX1	Storkhead Box 1
ACEI	Angiotensin-converting enzyme inhibitor
EPHX	Epoxide hydrolase
SNP	Single nucleotide polymorphisms
ANOVA	Analysis of variance

Author's contribution

SS, participated in the development of the study protocol, the study design, conducted the data analysis, interpretation of the findings, manuscript writing and revision. OA participated in the assessment of the enrolled subjects clinically, the data collection, conducted the data analysis and manuscript revision. ASM, participated in the clinical assessments of the participants, samples collection, interpretation of the findings and manuscript revision. AAM, participated in the clinical assessments of the participants, samples collection, interpretation of the findings and manuscript revision. HA, participated in the study design, conducted the data analysis, interpretation of the findings, manuscript writing and revision

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Informed consent

Written and oral informed consent was obtained from each participant in the study

Ethics approval

The study has been approved by the Scientific and Ethics Committees of the Faculty of Medicine, Mutah University, Jordan (The reference number of the ethical approval is: 201614).

Declaration of conflicting interest

The authors declare that there are no conflicts of interest.

Data and materials availability

All data associated with this study are present in the paper.

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