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Methylene tetrahydrofolate reductase and methionine synthase gene polymorphisms as genetic determinants of pre-eclampsia

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ABSTRACT

Background: Pre-eclampsia (PE) is a leading cause of maternal and neonatal mortality in Africa; and has been associated with the interplay of genetic, metabolic and environmental factors. Polymorphisms of methylene tetrahydrofolate reductase (*MTHFR*) and methionine synthase (*MTR*) folate cycle genes, have been controversially associated with pre-eclampsia in studies from different human populations.

Objectives: To determine the distribution of *MTHFR* C677T and *MTR* A2756G polymorphisms in a Nigerian population and evaluate possible associations with the occurrence of pre-eclampsia and homocysteine metabolic derangement.

Materials and Methods: This study was a hospital based study carried out in Lagos, South-western Nigeria. Two hundred pregnant women clinically diagnosed with pre-eclampsia (study group) and 200 apparently healthy non-pre-eclamptic pregnant women (control group) were recruited for the study after written informed consent. Pre-eclampsia was diagnosed based on the International Society for the Study of Hypertension in Pregnancy re-classification of 2013. MTHFR C677T and MTR A2756G polymorphisms were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.

Statistical analyzes were performed using SPSS version 23. Hardy-Weinberg distribution were tested with $\chi 2$ test. Logistic regression model was used to evaluate the relationship of variables with pre-eclampsia. A value of $p < 0.05$ was considered statistically significant.

Results: *MTHFR* genotype frequencies of CC, CT and TT were 59.8%; 31.2% and 9.0% in study group and 76.6%; 22.3% and 1.0% in the control group respectively. *MTR* A2756G genotype frequencies of AA, AG and GG genotypes were 71.9%; 20.1% and 8.0% for the study group and 81.5%; 16.4% and 2.1% for the control group.

Occurrence of pre-eclampsia was significantly associated with presence of T allele of *MTHFR* (*OR* = 1.855; $p < 0.05$) and G allele of *MTR* genes (OR = 1.269; $p < 0.05$), Homozygosity of TG haplotype significantly increased the occurrence of pre-eclampsia among Nigerian women (OR = 2.252; $p < 0.05$). Population attributable risk fraction percent for the T and G alleles were 16.4% and 11.5% respectively. Mean plasma Hcy level was not, however, significantly affected by *MTHFR/MTR* haplotypes ($F = 1.54$; $p = 0.157$).

Conclusion: *MTHFR* C677T and *MTR* A2756G polymorphisms were associated with pre-eclampsia in a population of pregnant women in Lagos, Nigeria.

1. Introduction

Impairments of the 1 carbon metabolism cycle have increasingly been implicated in numerous disease states including cardiovascular disorders, cancers, neurodegenerative diseases and adverse reproductive outcomes including hypertensive disorders of pregnancy [1]. As revised by the International Society for the Study of Hypertension in Pregnancy (ISSHP) in 2013, preeclampsia (PE) is characterized by high blood pressure and proteinuria, which may include generalized fluid retention, with a range of organ and system disturbances including a neurological disorder, all of which occur after 20 weeks gestation in a previously normotensive pregnant female [2–4]. Preeclampsia remains a leading cause of morbidity and mortality in Nigeria, West Africa [5].

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Disorders of folate and other B vitamins, are known to affect vital one-carbon transfer reactions in the body. Folate deficiencies may arise either from nutritional deficiencies or from disorders affecting rate limiting enzymes in the folate-methionine metabolic pathway. Such derangement in enzymatic activities have been described in genetic polymorphisms of methylene tetrahydrofolate reductase gene and methionine synthase gene. Methylene tetrahydrofolate reductase (MTHFR) enzyme catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5 methyltetrahydrofolate in a recycling process that can be used in the methionine cycle to generate S-adenosylmethionine (SAM) [6]. S-adenosylmethionine is the most important methyl group donor in the body for the methylation of lipids, proteins and DNA [6]. S-adenosylmethionine is generated by the methionine cycle in which 5-methyltetrahydrofolate transfers single methyl groups to homocysteine in a reaction catalyzed by methionine synthase (with vitamin B12 as cofactor) to produce methionine. After donating the methyl group, 5-methyltetrahydrofolate is converted to tetrahydrofolate and then to 5,10-methylenetetrahydrofolate by serine hydroxymethyltransferase. 5,10-methylenetetrahydrofolate is a key substrate that can be directed towards nucleotide biosynthesis or methionine regeneration.

In the *MTHFR* C677Tgene polymorphism, cytosine is replaced by thymine at position 677 of the nucleotide sequence, and consequently, a protein substitution of alanine by valine at position 222 of amino acid sequence. [7]. This amino acid substitution has been associated with marked increase in the thermolability of MTHFR enzyme and subsequently resulting in reduced enzyme activity. The methionine synthase gene (*MTR*) mutation is an alanine to guanine nucleotide substitution, A2756G, which converts an aspartate to a glycine codon at position 919 of the amino acid sequence [8]. The functional significance of this polymorphism has been associated with defects in DNA synthesis resulting in defective epigenetic modification in the genome and elevated levels of toxic intermediate products such as homocysteine (Hcy) and sometimes oxidative stress in the hypertensive disorders of pregnancy [9,10].

The major aim of this study is to provide additional knowledge on the aetio-pathogenesis and role of genetic biomarkers in pre-eclampsia among Nigerian women. The study would, therefore, afford clinicians the opportunity to review current protocol for folate supplementation in pregnant women in line with their varying genetic profiles and physiological peculiarities.

2. Materials and methods

2.1. Study population and location

This study included a total of 200 pregnant women with clinical and laboratory evidence of PE (study group) according to the ISSHP classification of hypertensive disorders of pregnancy [4] and 200 apparently healthy pregnant women as the control group. Thus, a total of n = 400 participated in the study. The study was carried out at the Lagos University Teaching Hospital (LUTH), Lagos Island Maternity, General hospital, Lagos; Alimosho General Hospital; AB10 specialist Hospital, Ifako-Ijaiye, Lagos. Patients that where in the age group of 18 to 44 years of age were duly registered for antenatal care at the Obstetrics and Gynaecology clinics and pregnant women at gestation age above 20 weeks, and between the same age group range were included in the study. All women with chronic hypertension were excluded from the study, including women with a history of systemic disorders such as kidney disease, diabetes mellitus, and connective tissue disorders, a history of thromboembolism, repeated miscarriage, abruption placenta and preterm labour. Women with history of alcohol use, smokers or use of herbal concoctions were also excluded.

2.2. Data and sample collection

Interviewer administered questionnaire was used to obtain demographic data, obstetric history, health and nutritional history. Blood pressure (BP) readings were taken twice in sitting position with a mercury sphygmomanometer 30 min apart and mean blood pressure readings above 140/90 mmHg were considered as elevated BP. Urinalysis was done using the reagent strips for urinalysis by DFI Company Ltd, Republic of Korea. Subjects with detectable proteinuria (≥2 + on dipstick) and elevated BP with and without complications were classified as PE according to ISSHP guideline recommendation. Each recruited pre-eclamptic subject was matched with a normotensive subject as controls for age, estimated gestational age, and parity. Venous blood (9 mL) was collected into two separate EDTA bottles for analysis of plasma homocysteine, MTHFR enzyme level and oxidative stress parameters in the participants.

2.3. Ethical consideration

Ethical approval for the study was obtained from the Lagos University Teaching Hospital Health Research and Ethical Committee, and the Lagos State Health Service Commission. Before inclusion in the study, oral and written informed consent was obtained from participants after due explanation of research objectives and procedures.

2.4. DNA extraction

Extraction of DNA from whole blood was done by spin column method using Jena Bioscience Blood-Animal-Plant DNA preparation kit (Thuringia, Germany). The concentration, purity and yield of the DNA samples were checked using Unico 2100 Spectrophotometer (Unico, USA) at 260 nm and 280 nm respectively. Eluted DNA samples were stored at −20 °C till they were used.

2.5. PCR amplification and restriction digestion

Polymerase chain reaction (PCR) was performed in a 25 μL reaction volume containing 50 μg of DNA, 0.2 mM dNTP mix, 1X Complete Buffer (Jena Biosciences, Germany), 0.04U/μl High Yield taq polymerase (Invitrogen, USA) and 0.5 μM of each target primer pair. Thermal cycling was done at 94 °C for 2 min; 94 °C for 30 s; 56 °C for 30 s; 72 °C for 30 s; and 72 °C for 2 min; steps 2 to 4 were repeated 35 times. Primer pairs used for *MTHFR* gene exon 4 amplification in this study are listed below as described by Ghaffari *et al.* [11]. *Beta actin* gene was used as the internal control (endogenous gene).

MTHFR 677 locus: forward primer- 5′-TGAAGGAGAAGGTGTCTGC GGGA-3′

MTHFR 677 locus: reverse primer — 5′- AGGACGGTGCGGTGAGA GTG-3′

Similarly, *MTR* A2756G polymorphic variants located on exon 25 of the *MTR* gene were identified after amplification with the following primers as described by Al Farra [12]:

MTR locus: - forward primer- 5′ CATGGAAGAATATGAAGATATTA GAC3′

MTR locus: - reverse primer- 5′ GAACTAGAAGACAGAAATTCT CTA3′

Restriction digestion of the PCR products for the *MTHFR* C677T and *MTR* A2756G amplicons were done using HinfI and Hae III restriction enzymes respectively.

2.6. Genotyping

Gel electrophoresis was by 2% agarose gel prepared by dissolving 2 g of Invitrogen Agarose in 100 mL of 1X Tris-Acetate-EDTA (TAE) buffer in a beaker. The resultant mixture was placed in a microwave for 2 min till all the agarose had dissolved. It was then allowed to cool at room temperature, following the addition of 10 μL of ethidium bromide (EtBr). The gel was poured into a gel plate containing a comb, required to form the wells into which the amplified DNA (PCR product) would be

loaded. The gel plate together with the gel block, were placed in an electrophoretic tank and 1X TAE Buffer was poured into the tank to cover the gel. A mid-range ladder by Jenna Biosciences, Germany containing DNA fragments from 100 bp to 1 kb was loaded into the first well of the 2% agarose gel block, while 10 μL of each PCR product obtained was loaded into the remaining wells of the gel. They were subjected to electrophoresis at 80v for 90 min. After which, the DNA bands were observed using Ultraviolet light from a TRANS-illuminator.

Polymorphism in *MTHFR* C677T created a recognition sequence for the restriction enzyme HinfI and this was detected by digestion of the 198 bp PCR product to yield recognizable undigested 198 bp fragment (genotype CC), presence of both 175 bp and 198 bp fragments (genotype CT), while genotype TT is represented by the presence of only 175 bp fragment. The *MTR* A2756G amplicon digestion with Hae III restriction enzyme yielded the following visible fragments: genotype AA (189 bp), genotype AG (189 bp and 159 bp), and genotype GG with only 159 bp.

2.7. DATA ANALYSIS

Statistical analyses were performed using SPSS 23.0 software (Armonk NY: IBM Corp. USA) Kolmogorov-Smirnov test was used to assess normality of data distribution for continuous variables, and Box plots for identification of outliers. Continuous variables were presented as mean ± standard deviation (SD) for normally distributed data. Categorical variables were expressed as percentages and Chi-square test was used to compare proportions for categorical variables as well as to assess Hardy-Weinberg equilibrium of genotype distribution.

3. Results

3.1. Genotypic AND ALLELIC distribution of MTHFR C677T single nucleotide polymorphism (SNP) AMONG study AND control groups

The *MTHFR* genotype frequencies for CC, CT and TT among the study group were 119 (59.8%); 62 (31.2%) and 18(9%) respectively, while *MTHFR* genotype frequencies for CC, CT and TT among control subjects were 151 (76.6%); 44 (22.3%) and 2 (1%) respectively (Table1). Genotype distribution differed significantly between study and control group ($\chi^2 = 19.6$, $p = 0.001$). Genotypes CT and TT were associated with significant increase in occurrence of PE (OR = 1.788, 95% CI: 1.134–2.812; and OR = 11.420, 95% CI: 2.598–50.194; $p = 0.001$) respectively. The distribution of CC, CT and TT genotypes for both study and control groups were in Hardy Weinberg equilibrium ($p > 0.05$).

The allelic frequencies for *MTHFR* 677C and T among study group were 75.5% and 24.5% respectively (Table 1). Among control group, *MTHFR* 677C and T allelic frequencies were 88.0% and 12.0% respectively ($\chi^2 = 19.6$, $p = 0.001$). The T allele was associated with increased risk of PE (OR = 2.355, 95%CI: 1.613–3.438; p =0.001) and a population attributable risk fraction of 16.4%.

Table 2
Distribution of *MTR* A2765G genotypes and allelic frequencies among the study and control groups.

		Subject category		OR (95%)	p
		Control group N = 199 n (%)	Study group N = 195 n (%)		
Genotype *MTR* A2756G	AA	143(71.9)	159(81.5)	0.720(0.429–1.206)	0.21
	AG	40(20.1)	32(16.4)	1.390(0.829–2.330)	0.21
	GG	16(8.0)	4(2.1)	4.445(1.453–13.614)	0.005
Allele					
Wild type (A)		350(90.0)	326(82.0)	0.518(0.342–0.784)	0.002
Mutant/risk (G)		40(10.0)	72(18.0)	1.933(1.276–2.927)	0.002
PAR(%) for T allele		11.5%			
HWE		$\chi^2 = 0.71$, $P > 0.05$	$\chi^2 = 0.17$, $P > 0.05$		

Key: HWE (Hardy Weinberg equilibrium); PAR (population attributable risk)

3.2. Genotype AND ALLELE distribution of MTR A2765G single nucleotide polymorphism (SNP) AMONG study AND control groups

The *MTR* genotype frequencies for AA, AG and GG polymorphic variants among study group was 143 (71.9%); 40 (20.1%) and 16 (8.0%) respectively (Table 2). The genotype frequencies of AA, AG and GG for control group were 159(81.5%); 32(16.4%) and 4(2.1%) respectively ($\chi^2 = 8.9$, $p = 0.012$; Cramer's V = 0.150, $p = 0.001$). Genotypes AG and GG had odd ratios of 1.390 (95% CI: 0.829–2.330; $p = 0.012$) and 5.560 (95% CI: 1.856–16.652; $p = 0.012$) respectively for associations with PE. Genotypes of *MTR* were in Hardy Weinberg equilibrium for both categories of participants ($p > 0.05$).

The *MTR* gene allele frequency for A and G polymorphic variants among study group was 82.0% and 18% respectively (Table 2). Allelic frequencies of A and G were 90.0% and 10.0% respectively for control group ($\chi^2 = 19.6$, $p = 0.019$; with significant effect size as Cramer's V = 0.224, $p = 0.001$). Subjects with the mutant G allele have increased risk for PE compared to individuals with the wild type (A) allele (OR = 1.933, 95%CI: 1.276–2.926; $p = 0.019$). The population attributable risk fraction for G allele was 11.5%.

3.3. ASSOCIATION of COVARIATES AND risk of PE

In a logistic regression analysis, the presence of mutant alleles of the *MTHFR* gene (T- allele), and the co-inheritance of *MTHFR/MTR* mutant haplotypes (combined homozygous and heterozygous) were significantly associated with the occurrence of PE (Table 3) with Odds

Table 1
Distribution of *MTHFR* C677T genotypes and allelic frequencies among the Study and control Groups.

		Subject category		OR (95%CI)	p
		N = 197 n(%)	Study group N = 199 n(%)		
MTHFR genotype	CC	151(76.6)	119(59.8)	0.559(0.355–0.882)	0.0110
	CT	44(22.3)	62(31.2)	1.788(1.134–2.812)	0.0110
	TT	2(1.0)	18(9.0)	11.420 (2.598–50.194)	< 0.0001
Allele					
Wide type (C)		346(88.0)	300(75.5)	0.424(0.291–0.620)	< 0.0001
Mutant Allele (T)		48(12.0)	98(24.5)	2.355(1.613–3.438)	< 0.0001
PAR(%) for T allele			16.4%		
HWE		$\chi^2 = 0.37$, $P > 0.05$	$\chi^2 = 0.81$, $P > 0.05$		

Key: HWE (Hardy Weinberg equilibrium); PAR (population attributable risk fraction)

Table 3
Association of Covariates with Risk of Pre-eclampsia.

Variables	β	OR(CI)	p
MTHFR gene (T allele)	0.618	1.855 (1.074–3.482)	0.047
MTR gene (G allele)	0.238	1.269 (0.664–2.424)	0.330
Mutant MTHFR/MTR genotypes	0.812	2.252 (1.278–3.967)	0.005

ratios = 1.855 (95% CI:1.074–3.482); and 2.252 (95%CI:1.278–3.967) respectively ($p < 0.05$). However, association of mutant allele of *MTR* genes (G alleles), with occurrence of PE was lost after Bonferroni adjustment (OR = 1.269, 95% CI: 0.664–2.424; $p = 0.330$).

3.4. *MTHFR/MTR* haplotype distribution

MTHFR/MTR gene haplotypes across participant groups were assessed as shown in Fig. 1. Homozygous wild type haplotypes CC/AA (representing the occurrence of wild type *MTHFR* and *MTR* genotypes in an individual) was significantly ($p < 0.05$) higher among control population (66.7%) compared to study group (43%). Heterozygous mutant haplotype CC/AG was evenly distributed between both participant groups (11% each). The frequencies of heterozygous mutant haplotypes CC/GG (6%) CT/AA (23%) CT/AG (7%) and TT/AA (4%) among study group, were proportionally higher when compared to control group frequencies of 1.0%; 16%; 4.0% and 1.0% respectively ($p < 0.05$). In this study, heterozygous haplotypes CT/GG and TT/AG were only described among the study group (1% and 2% respectively). However, homozygous mutant haplotype (TT/GG) was not represented in any of the participants.

MTHFR C677T Allelic Distribution and Mean Plasma Homocysteine among Participants across In the control group (Fig. 2), mutant *MTHFR* (T) allele was associated with significantly higher Hcy levels (11.21 ± 1.56 μmol/L) compared with Hcy level in subjects with wild type(C) allele (10.67 ± 1.82 μmol/L; $p = 0.042$). Similarly Hcy was higher in study group participants with *MTHFR* (T) allele (22.51 ± 2.22 μmol/L) compared to study group participants with *MTHFR* (C) allele (20.37 ± 3.03 μmol/L; $p = 0.046$). As described in Fig. 3, mean plasma Hcy level for *MTR* A2756G wild type(A) allele for control group (10.85 ± 1.82 μmol/L) did not differ from the Hcy level for control group participants with the mutant *MTR* A2756G (G) allele (10.51 ± 1.50 μmol/L; $p > 0.05$). Among the study group, Mean Hcy level for participants with *MTR* A2756G wild type (A) allele (20.71 ± 9.28 μmol/L) was not significantly different from mean Hcy levels reported for participants with the *MTR* A2756G mutant(G) allele (22.55 ± 10.91 μmol/L; $p > 0.05$).

Mean plasma Hcy across six different *MTHFR*C677T/*MTR* A2756G haplotypes identified within the study group (Fig. 4). Though there appears to be a peak in the plasma Hcy level among study group participants with the heterozygous mutant haplotypes CT/GG (31.55 ± 1.50 μmol/L) compared to the mean value of plasma Hcy in pre-eclamptic women with homozygous wild type haplotype CC/AA (19.65 μmol/L ± 8.7 μmol/L); the observed differences in plasma Hcy across different *MTHFR*C677T/*MTR* A2756G SNP haplotypes did not differ statistically ($p < 0.05$).

4. Discussion

The role of *MTHFR* as candidate gene in the aetio-pathogenesis of preeclampsia still remains a subject of debate. More so, available data on this subject are mainly from Asian and other Caucasian populations, with dearth of information on studies involving Africans. Several studies have documented significant geographical variation in the distribution of *MTHFR* polymorphism. From this study, genotype frequency distribution for *MTHFR* C677T polymorphism showed low prevalence of 1% among women in Lagos, in agreement with reports of studies from West African populations [13]. Some studies have described low genotype frequencies for *MTHFR* 677TT among African and African-American populations [14], as similarly observed among control subjects in this study. Low prevalence of *MTHFR* 677TT among African populations have been attributed to selection pressure secondary to low folate diets among Africans [13–15]. However, significant increase in *MTHFR* 677TT genotype frequency was observed in women with preeclampsia. A *META*-analysis report by Wang *et al.* [16] have shown significant association between *MTHFR* T allele and preeclampsia among Caucasians and people of Asian descent but not among people of African descent. However, the authors noted that data involving African studies are few, and where they exist, sample size is low and the statistical power is insufficient to make significant inference.

The genotype and allelic frequencies for *MTR* gene in this study indicated low frequency of the mutant (GG) genotype among healthy pregnant women in Nigeria, which is not much different from values documented in the 1000 human genome project for African-American population [17]. However, these genotype and allelic frequencies for

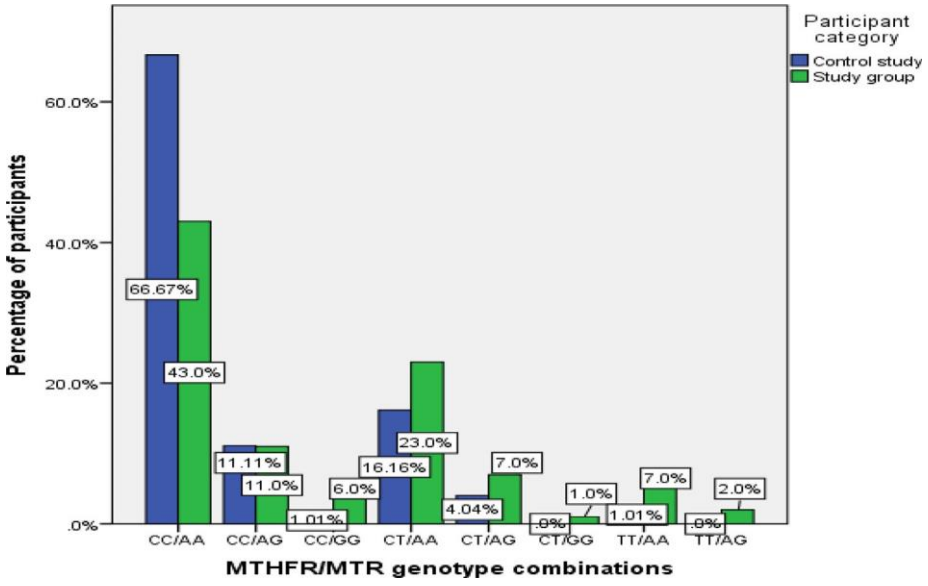

Fig. 1. *MTHFR/MTR* haplotype combination among Participants.

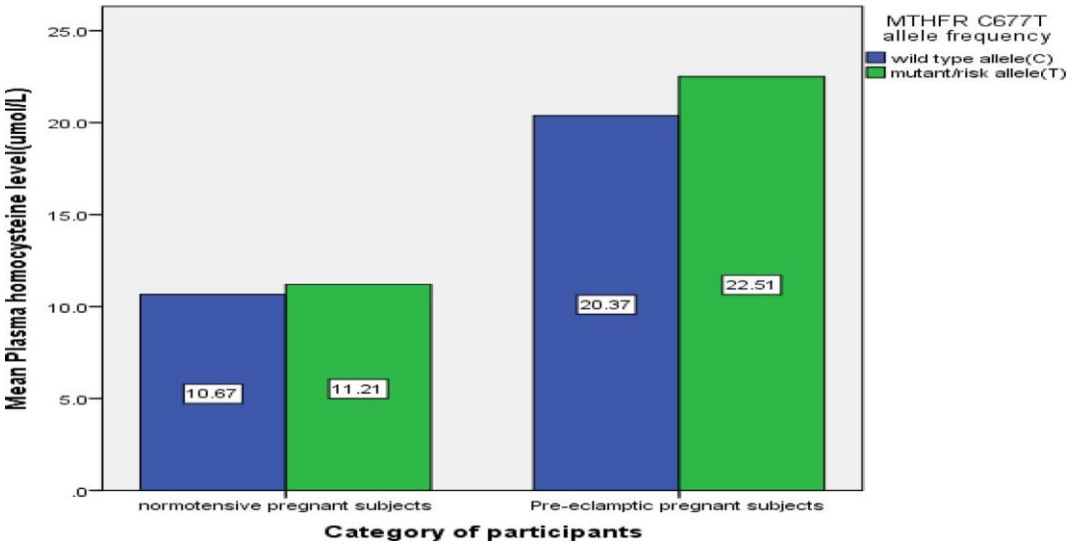

Fig. 2. Mean plasma homocysteine levels stratified by *MTHFR* C677T allelic frequencies across categories of participants.

MTR gene, were higher than the values reported in a study from Burkinafasso [18]. Allelic frequency of *MTR* G-Allele in healthy pregnant women in this study, is only slightly lower than values documented for African-Americans [19]. Though, there was significant increase in frequencies of mutant *MTR* genotypes (AG and GG) in women with PE/E, however, significant association with PE could not be established after statistical adjustment in a regression model. Even though *MTR* A2756G polymorphism has been associated with hyperhomocysteinaemia [20,21], only few literatures are available on the role of *MTR* A2756G polymorphism in PE. Moreover, these few published data are contradictory [22].The inconsistency of the results obtained in various studies may be related to the differences in ethnic and genetic background of the studied populations. However, documented evidence for the role of *MTR* gene polymorphism in pre-eclampsia among African populations are rare.

Though the *MTHFR* 677 T allele was associated with higher values of plasma homocysteine in women with pre-eclampsia, the overall pattern of mean plasma homocysteine in women with pre-eclampsia, was not significantly associated with mutant haplotypes of the studied folate cycle gene SNPs. This is similar to the findings of Komlichenko *et al* in a similar study [23]. This may suggest that *MTHFR* and *MTR* gene mutations alone may not explain the pathological elevation in Hcy observed among women with pre-eclampsia. Other factors such as impaired renal excretion of homocysteine or defects in other pathways involved in homocysteine transulphuration, might be contributory factors in these subjects.

The population attributable risk fraction percent (PAR) is broadly interpreted as the percentage of the disease in a population that can be associated with the risk factor. Documented PAR for *MTHFR* and *MTR* mutant alleles appears to be rare for PE in any population. However, few literatures have documented PAR for *MTHFR* C677T genotypes in subjects with other anomalies such as neural tube defect [24]. This study indicated that Mutant (T) alleles for *MTHFR* gene appear to be more closely associated with PE risk compared to mutant *MTR* (G) allele when PAR is considered. This could have been influenced by the availability of other physiological sources of alternate methylating agents for homocysteine such as betaine which could not be evaluated in this study.

The conclusion from this study is that the *MTR* A2765G and *MTHFR* C677T polymorphism appear to play significant roles in the aetio-pathogenesis of pre-eclampsia in a Nigerian population, but may not explain the variations observed in plasma homocysteine in women with pre-eclampsia.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

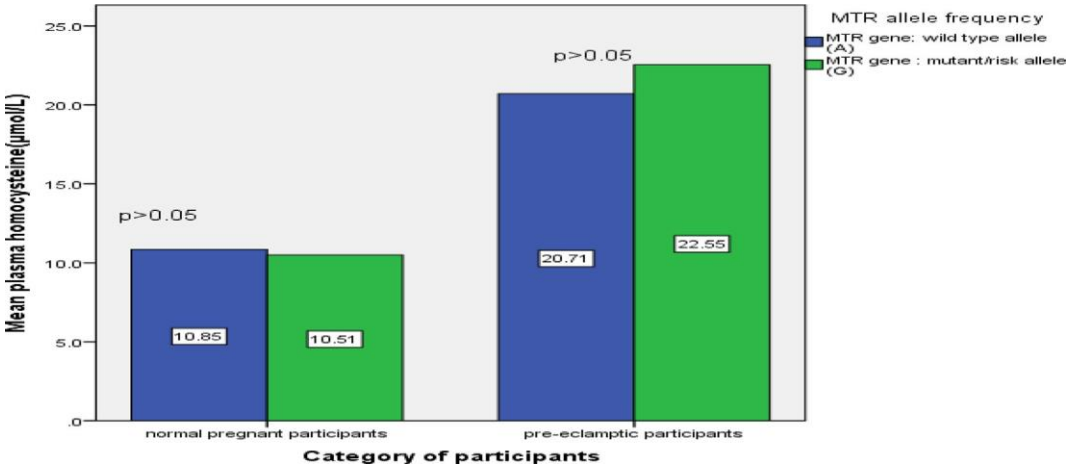

Fig. 3. Mean plasma homocysteine levels with respect to *MTR* A2756G allelic frequencies.

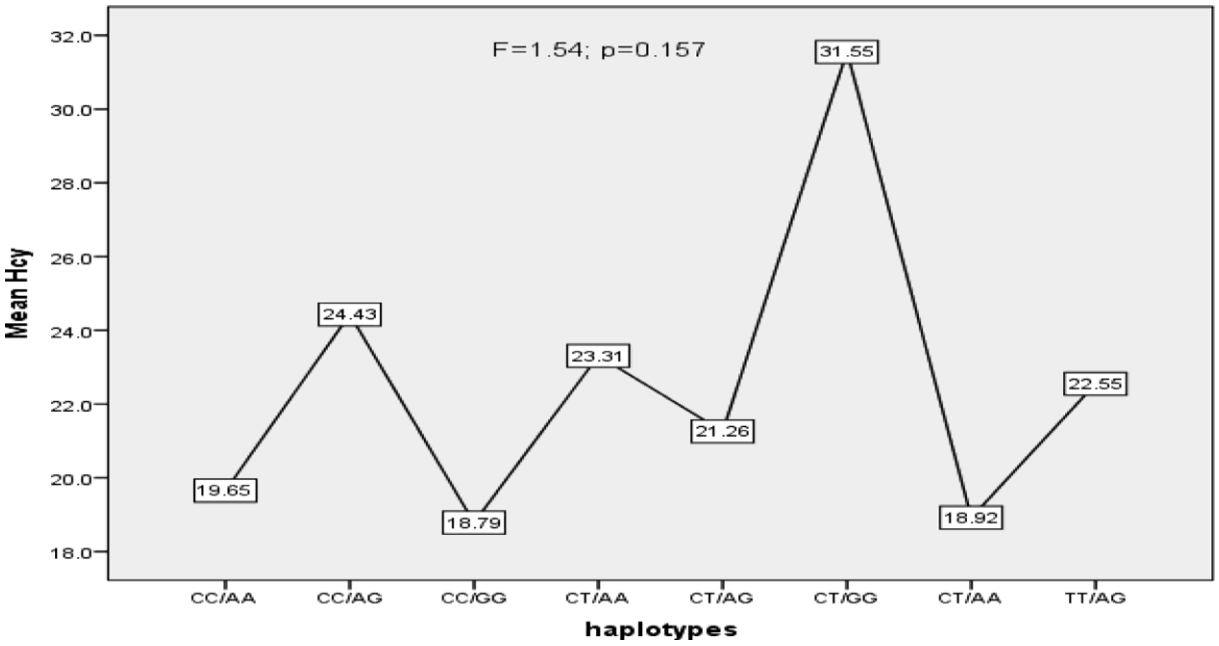

Fig. 4. Mean plasma homocysteine distribution by *MTHFR/MTR* haplotypes in study group.

influence the work reported in this paper.

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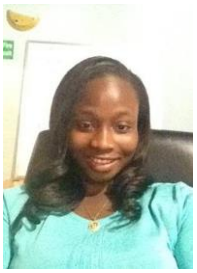

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