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Two polymorphisms in methylenetetrahydrofolate reductase gene (C677T and A1298C) frequently associated with recurrent spontaneous abortion show no association in Saudi women

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Abstract: Methylenetetrahydrofolate reductase (MTHFR) deficiency is the most common genetic cause of hyperhomocysteinemia, which has been implicated in the etiology of recurrent spontaneous abortion (RSA). This study was designed to investigate the association between two single nucleotide polymorphisms (SNP) (rs1801133 [C677T] and rs1801131 [A1298C]) in the MTHFR gene and RSA, in Saudis. These two SNPs were selected as these polymorphisms have a different effect on the activity and stability of the enzyme, and significantly diverse effects have been reported in relation to the association with RSA. Ethical approval was acquired from the IRB at King Saud University (KKUH), Saudi Arabia, and written informed consent was obtained from each participant. The study group comprised of 100 Saudi women with unexplained RSA and 100 age-matched controls, both attending KKUH for a routine checkup. Blood was drawn in EDTA tubes, and DNA was extracted. Genotyping was conducted using TaqMan SNP genotyping assay kits. The frequency of the T allele of C677T was 0.165 in patients and 0.17 in controls. Genotype frequencies for CC, CT and TT genotypes were 70%, 27% and 3%, respectively in RSA, and 71%, 24% and 5%, respectively, in the controls ($p > 0.05$). For the A1298C polymorphism, the C allele frequencies were 0.345 in patients and 0.28 in controls, while genotype frequencies for AA, AC and CC genotypes were 44%, 43%, and 13%, respectively, in patients, and 54%, 36%, and 10%, respectively, in controls ($p > 0.05$). The frequency of CC genotype and C allele of A1298C were higher in the patients with RSA, but not significantly, while C677T genotypes and allele frequencies did not differ between patients and controls. The results suggested that MTHFR gene polymorphisms are population-specific and may not associate with RSA in Saudi women.

Introduction

Approximately 15% of all clinically recognized pregnancies result in a spontaneous loss (Meka and Reddy, 2006), and this can be physically, emotionally, and financially taxing for the couples. Recurrent spontaneous abortion (RSA) is historically defined as three consecutive pregnancy losses before 20 weeks of gestation (Reiss, 1998). According to the WHO reports, there are an estimated 40–50 million abortions every year in the world (http://www.worldometers.info/abortions/), and this corresponds to approximately 125,000 abortions per day.

Recurrent spontaneous abortion (RSA) is a multifactorial disorder, and both genetic and environmental factors are implicated in its etiology. These include uterine or anatomic anomalies, infections, and endocrine, genetic, immunologic, and thrombophilic abnormalities (Kaur and Gupta, 2016). Environmental influences, such as consumption of alcohol, caffeine, or cocaine, as well as cigarette smoking, have been reported to contribute to sporadic miscarriages. However, accurate data on toxic doses or exposure are difficult to

obtain (Forray, 2016). In addition, 25–40% of RSA are of unknown etiology (von Steinburg and Schneider, 2009).

One of the factors implicated in RSA development is abnormal folate metabolism (Ray and Laskin, 1999). Folate, a member of the vitamin B complex group, plays an essential role in the synthesis of deoxyribonucleic acid (DNA) and the maintenance of methylation reactions in the cells. Anomalies in folate metabolism influence both these reactions and have been implicated in the development of several diseases, including developmental defects (Nazki et al., 2014). Methylenetetrahydrofolate reductase (MTHFR) is a pivotal enzyme within the folate methionine pathway. It is responsible for the conversion of 5,10-methylene-THF to 5-methyl-THF, the main circulating form of folate that donates a methyl group to homocysteine (Hcy) and re-methylates it into methionine (Nazki et al., 2014).

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Homocysteine is methylated, and forms S-adenosyl methionine (SAM), which plays a vital role as a methyl donor required for the maintenance of genomic methylation patterns that determine gene expression, DNA conformation, and is necessary for the synthesis of myelin, neurotransmitters, and membrane phospholipids. Folate deficiency reduces SAM levels resulting in lower DNA cytosine methylation and elevated levels of Hcy.

Several factors influence MTHFR activity. These include variations within the gene sequence that alter the affinity of the enzyme for either substrate or its cofactor, flavin adenine dinucleotide (FAD) (Frosst et al., 1995; Marini et al., 2008). Under conditions of reduced MTHFR activity, 5, 0-methylenetetrahydrofolate concentration increases with a resultant subsequent lowering of 5-methyltetrahydrofolate concentration, which leads to hyperhomocysteinemia and low folate level in the plasma. Homocysteine has a cytotoxic effect, induces apoptosis of trophoblasts, and reduces the secretion of the human chorionic gonadotropin (Di-Simone et al., 2003).

Many polymorphisms within the MTHFR gene have been reported in the literature; however, very few have conclusive results due to the disease and population dynamics. The two most widely studied of these are the common C677T and A1298C single nucleotide polymorphisms (SNP) (Frosst et al., 1995; Hubacek et al., 2015). These polymorphisms (C677T [rs1801133] and A1298C [rs1801131]) in the MTHFR gene result in enzyme thermolability and decrease its activity by up to 40% (Nursal et al., 2018).

Several studies have shown a close association between the C677T and A1298C and the development of RSA (Hubacek et al., 2015; Poursadegh-Zonouzi et al., 2012; Kamali et al., 2018; Behjati et al., 2006; Parveen et al., 2013; Farahmand et al., 2016; El Achi et al., 2018; Al-Achkar et al., 2017; Sah et al., 2018; Chatzidimitriou et al., 2017). However, others have failed to do so (Dell'Edera et al., 2018; Dissanayake et al., 2012; Settin et al., 2011; Hwang et al., 2017;

Gonçalves et al., 2016; López-Jiménez et al., 2016; Rai, 2014). There appear to be substantial population differences in this association, where among the Iranians, Indians, Caucasians, Lebanese, Syrians, and Greeks, a significant association has been demonstrated (Hubacek et al., 2015; Poursadegh-Zonouzi et al., 2012; Kamali et al., 2018; Behjati et al., 2006; Parveen et al., 2013; Farahmand et al., 2016; El Achi et al., 2018; Al-Achkar et al., 2017; Sah et al., 2018; Chatzidimitriou et al., 2017), while in Mexicans, Brazilians, Sinhalese, Koreans, Roman, Spanish, Italians, and Egyptians (Dell'Edera et al., 2018; Dissanayake et al., 2012; Settin et al., 2011; Hwang et al., 2017; Dell'Edera et al., 2018; Gonçalves et al., 2016; López-Jiménez et al., 2016; Rai, 2014). No significant association was reported. This augmented our interest to investigate, the role of these two polymorphisms in RSA development among the Saudis. To the best of our knowledge only one study from Jeddah, Saudi Arabia, has investigated the prevalence of C677T polymorphism in recurrent miscarriage but has not documented the frequencies of the CC, CT, and TT genotypes in the patients. The authors only refer to homozygous and heterozygous as occurring at a frequency of 23.75% and 1.75%, respectively, and there is no indication of which homozygosity they mean (Turki et al., 2016). In their study, they have not included healthy controls to compare the frequencies. Furthermore, no study has so far explored the association between A1298C and RSA. Hence, this is the first study reporting detailed genotype and allele frequencies of the two SNPs in Saudi RSA patients and controls.

This study was designed to determine the association between MTHFR polymorphisms (C677T [rs1801133] and A1298C [rs1801131]) and RSA in Saudi women and to compare the results obtained with results in women with no previous history of abortion.

Materials and Methods

The study was carried out at the Recurrent Abortion clinic at King Khalid University Hospital, Riyadh, Saudi Arabia, between 1/6/2010 and 1/10/2011. The study was approved by the Institutional Review Board (IRB), College of Medicine, King Saud University (No. E-10-132).

The sample size was calculated using http://osse.bii.a-star.edu.sg/calculation1.php from OSSE (an Online Sample Size Estimator). Power desired was kept at 80%, significance level desired =5%, minor allele frequency assumed in cases 35%, and in control 15%. The sample size obtained was 96.

The study group comprised of 100 Saudi women with RSA, consecutively referred to the clinic of ZB. The patient's group (RSA group [cases]) in the study included women who were suffering from three or more consecutive spontaneous miscarriages and were of Saudi nationality. Non-Saudi females were excluded. In addition, the exclusion also depended on associated illnesses, such as congenital heart disease, insulin-dependent diabetes mellitus, renal disease, cardiovascular disease, systemic lupus erythematosus (SLE), and women with any risk factor for recurrent abortions such as inborn errors of metabolism, congenital heart disease, kidney disease, or cervical

incompetence. As a routine procedure at KKUH, all miscarriage cases were subjected to cytogenetic studies, and women with a congenital malformation or chromosomal abnormality were excluded from the study. The controls consisted of 100 Saudi women who had at least two children and were without known pregnancy losses or any known medical illnesses. The controls were matched in age and body mass index with the patients and were attending the out-patients clinics of ZB for a routine checkup. The cases and controls were explained the nature of the study, and those who volunteered were requested to sign an informed consent form. Routine analysis at the Hospital laboratory was performed to exclude known causes of abortion. Demographic data and clinical data of the patients were collected, including parental karyotypes, hormone levels, toxoplasmosis, cytomegalovirus, rubella, antiphospholipid antibodies, protein C, protein S, glucose level, hysteroscopy, hysterosalpingography, and serial ultrasound. Only women with normal values were included.

Blood samples (8 mL) were drawn, by venipuncture, from both the RSA and control groups, in tubes containing anti-coagulant, EDTA. The blood was centrifuged at 3500 rpm for 10 min, and the plasma, cells, and the buffy coat were carefully separated. DNA was extracted from blood using the Gentra Puregene DNA purification kit (Qiagen).

The concentration of DNA in each sample and its purity were determined using NanoDrop 2000 Spectrophotometer. The DNA was diluted to 50 ng/μL, stored at –20°C in the Laboratory at the Department of Zoology, College of Science, KSU. Riyadh, until required for analysis. Using TaqMan genotyping assay kits (Applied Biosystems), the samples were genotyped for the two SNPs (rs1801133 and rs1801131). All probes used were those designed by Applied Biosystem (C___1202883_20, C____850486_20) following the protocol provided by the manufacturer. The genotyping was carried out on LightCycler 480 Instrument II Real-Time PCR System, following the protocol: initial denaturation at 94°C for 15 min, followed by 45 cycles of 94°C for 15 s and 60°C for 1 min.

Statistical analysis
The information was filled on Microsoft Excel spreadsheets and entered on a personal computer. Data analysis was carried out using the Statistical Package for the Social Sciences (SPSS version 20, SPSS Inc., Chicago, IL). Mean and standard deviation (SD) were calculated, and the results in the two groups were compared using the Student's *t*-test. In each group, genotypes and allele frequencies were manually calculated and compared between the normal and the patient group using http://ihg.gsf.de/cgi-bin/hw/hwa1.pl. Odds ratio (OR), 95% confidence interval (CI), Chi-square (χ^2), and the *p*-value were obtained. $p < 0.05$ was considered statistically significant. Hardy–Weinberg Equilibrium was tested using both the above website. Haplotype analysis was carried out, and the frequency of the constructed haplotypes was compared in the RSA and controls.

Results

The demographic and clinical characteristics of the participants in this study are presented in Tab. 1. The

patient and the control group were age and BMI-matched. The mean age of the RSA patient was 33.20 ± 6.291 years (range 18–45 years), and that of the control was 33.10 ± 0.733 years (range 18–45 years; *p* = 0.981).

The prevalence of consanguinity was calculated and is presented in Tab. 2. Of the RSA patients, 55% of the females were married to their cousins compared to 35% of the control group. The prevalence of consanguinity was significantly higher in the patient's group compared to the controls (*p* = 0.015).

The genotype and allele frequencies were calculated for the two studied SNPs. Both SNPs obeyed Hardy–Weinberg Equilibrium (*p* > 0.1). Tab. 3 presents the genotype and allele frequency of C677T MTHFR mutation. The frequencies of T alleles were 0.165 in patients and 0.17 in controls. The frequencies of CC, CT and TT genotypes were 70%, 27% and 3% in patients, and 71%, 24% and 5% in controls. Overall, the distribution of the various genotypes of C677T did not differ significantly among RSA patients (*p* = 0.893).

The genotype and allele frequencies of A1298C in the MTHFR gene in the RSA patients and controls are presented Tab. 4. The frequencies of C alleles were 0.345 in patients and 0.28 in controls. The frequencies of AA, AC and CC genotypes were 44%, 43% and 13% in patients, and 54%, 36% and 10% in controls. The genotypes and allele frequencies of mutant C allele and genotype CC of MTHFR A1298C were slightly higher in the RSA patients compared to the control group but failed to reach significance (*p* = 0.160). Four main haplotypes were identified (Tab. 5). Three of the haplotypes did not differ significantly between the patients and controls. However, the CT haplotype, which was absent in the controls, occurred at a frequency of 2.79% in the patients, and the difference in the frequency between the two groups was statistically significant.

Discussion

Methylenetetrahydrofolate reductase (MTHFR) is a vital enzyme in 'one-carbon metabolism,' and due to the involvement of folic acid and its metabolites in this pathway, any abnormality in the level or amount of the MTHFR may lead to serious consequences (Nazki *et al.,* 2014). These may occur in the form of cardiovascular disease, neural tube defects, preeclampsia, cleft lip/palate, hypertension, thrombosis, complications of pregnancy, e.g., RSA, osteoporosis, dementia, schizophrenia, depression, Alzheimer's disease, migraine, Down syndrome, certain types of cancer (glaucoma), and epilepsy (https://omim.org/entry/236250). The two polymorphisms, we included in this study, have been extensively investigated in RSA. The C677T is a cytosine (C) to thymine (T) transition at position 677, within exon 4 of the gene, and results in an alanine to valine substitution. The mutant protein has reduced enzyme activity at 37°C and has higher thermolability. The enzyme activity in the CT heterozygote and the TT homozygotes is reduced by 35% and 70%, respectively, when compared to the normal CC genotype. Homozygosity for the T allele is associated with reduced

TABLE 1

Demographic data of RSA patients and control subjects included in this study

Parameter	Group	Mean	SD	t	p
Age (years)	C	33.10	7.330	−0.104	0.981
	P	33.20	6.291		
Height (cm)	C	158.49	5.452	1.512	0.132
	P	157.02	8.076		
Weight (kg)	C	70.50	15.168	−1.015	0.311
	P	72.91	18.218		
BMI (kg/m^2)	C	28.28	6.140	−1.425	0.156
	P	29.63	7.215		
No of children	C	3.61	1.693	6.313	0.0001
	P	1.95	2.012		
No. of pregnancies	C	3.62	1.692	−7.630	0.0001
	P	6.41	3.241		
No. of abortion	C	0 (None)	0	−11.025	
	P	4.51	2.414		

p, Significance

TABLE 2

Prevalence of consanguinity (1st and 2nd degree) in RSA patients and controls

Group	No. of samples (No.)	Consanguinity % No.	1st % No.	2nd % No.	None % No.	χ^2 (p) Three way	χ^2 (p) Binary
RSA Patients	100	55%	33%	22 %	45%	8.35	8.08
		55	33	22	45		
Controls	100	35%	19%	16%	65%		
		35	19	16	65	0.015	0.004

Binary: total consanguineous vs non-consanguineous (None).
Three way: 1st cousin, and 2nd cousin vs non-consanguineous (None).
χ^2: Chi-squared statistic, p: Significance.

TABLE 3

The genotype and allele frequencies of C677T in the RSA patients compared to the control group

Genotype of C677T	Control No. (%)	Patients (%)	Control *vs.* Patients*			
			OR	CI	χ^2	*p*-value
CC	71 (71%)	70 (70%)	Ref.			
CT	24 (24%)	27 (27%)	1.14	0.60–2.17	0.16	0.687
TT	5 (5%)	3 (3%)	0.61	0.14–2.64	0.45	0.504
Total	100	100				
Allele	**Control (Frequency)**	**Patients (Frequency)**	**Control *vs.* Patients****			
			OR	CI	χ^2	*p*-value
C	0.83	0.835	Ref.			
T	0.17	0.165	1.04	0.61–1.75	0.02	0.893

No: Number of individuals; OR: Odds Ratio; CI: Confidence Interval; χ^2: Chi-squared statistic.
*Using 3 × 2 Contingency Table: The Chi-squared statistic is 0.6836. The p-value is 0.710504. The result is not significant ($p > 0.05$).

TABLE 4

The genotype and allele frequencies of A1298C in the RSA patients compared to the control group

Genotype of A1298C	Control* No. (%)	Patients* No. (%)	Control *vs.* Patients			
			OR	CI	χ^2	*p*-value
AA	54 (54%)	44 (44%)	Ref.			
AC	36 (36%)	43 (43%)	1.87	0.40–8.69	0.66	0.41
CC	10 (10%)	13 (13%)	1.59	0.64–3.98	1.01	0.315
Total	100	100				
Allele	**Control (Frequency)****	**Patients (Frequency)****	**Control *vs.* Patients**			
			OR	CI	χ^2	*p*-value
A	0.72	0.655	Ref.			
C	0.28	0.345	1.35	0.88–2.07	1.97	0.160

No: Number of individuals; OR: Odds Ratio, CI: Confidence Interval; χ^2: Chi-squared statistic.
*Using 3 × 2 contingency tables: The Chi-squared statistic is 2.032. The *p*-value is 0.362046. The result is not significant ($p > 0.05$).
**Using 2 × 2 Contingency tables: The Chi-squared statistic is 1.9665. The *p*-value is 0.160815. The result is not significant ($p > 0.05$).

TABLE 5

Haplotype analysis to determine association with RSA

	Haplotype association					
	A1298C	C677T	Frequency Control	Frequency Patients	OR (95% CI)	*p*-value
1	A	C	0.56	0.5229	1.00	–
2	C	C	0.27	0.3171	1.27 (0.81–1.99)	0.29
3	A	T	0.17	0.1321	0.83 (0.46–1.48)	0.53
4	C	T	0	0.0279	Very high values	<0.0001

enzyme activity resulting in mild to moderately elevated Hcy levels (Frosst *et al.*, 1995).

The second polymorphism in the *MTHFR* gene involves an A to C transition at position 1298 within exon 7, which results in a change from glutamate to an alanine residue. The activity of the enzyme is decreased but not to the same extent as in the C677T allele (Weisberg *et al.*, 1998; Poursadegh-Zonouzi *et al.*, 2012). It has been shown that neither the homozygous nor the heterozygous state of A1298C leads to an elevation in plasma Hcy or lower plasma folate concentration, which is evident for the homozygous state for C677T. However, compound heterozygosity for the C677T and the A1298C allele is associated with reduced MTHFR enzyme activity, higher plasma Hcy, and lower plasma folate concentrations (Van der Put *et al.*, 1998). The combined association of these two alleles produces a similar biochemical profile to those individuals who are homozygous for the T allele of C677T. The population frequency for A1298C homozygosity is not as well documented as for the C677T allele and is thought to have a prevalence of about 10% (Zetterberg *et al.*, 2002). To date, over 30 other mutations have been identified which, are associated with severe MTHFR deficiency (Sibani *et al.*, 2003).

Several interesting features came to light during this investigation. Firstly, our results showed that consanguinity could be a factor contributing to fetal loss in the form of RSA among Saudi females since the RSA patient had a higher frequency of cousin marriages compared to the control group. Secondly, our findings show that the two studied SNPs do not play a role in influencing susceptibility to RSA in the Saudi population. The genotyping for the C677T polymorphism showed that the frequencies of T alleles were 0.165 in the patients and 0.17 in the controls. The prevalence of the genotypes CC, CT, and TT did not differ in the Saudi RSA patients and controls ($p = 0.924$). Turki *et al.* (2016) also reported no association in a group of women suffering from RSA (recurrent miscarriage), their study did not compare the results with controls, and no explanation was given how the association was studied.

Regarding genotypes and alleles of A1298C polymorphism in the present study, though the frequencies of CC was slightly higher in the patients' group, it did not reach a significant difference between the RSA patients and healthy controls ($p = 0.160$). No studies were found on Saudis during our extensive literature review, and we are confident that this is the first report exploring the association between A1298C and RSA. Our results are in agreement with the results in several populations, including Mexican, Brazilian, Sinhalese, Egyptian, Korean, Italians, Romans, Slovaks, and Spanish and contradict the results reported in Iranian, Indian, Caucasians, Lebanese, Syrian, Nepalese, Slavonic, Bulgarians, and Greek (Tab. 6).

TABLE 6

A summary of some studies that investigated association between MTHFR (C677T and A1298C) gene polymorphisms and RSA in different populations

SNP	Number of samples	Population	Clinical implication	References
No association				
C677T and A1298C	IRPL = 56, N = 50	Mexican	The most frequent prothrombotic factor in the RPL group	López-Jiménez et al. (2016)
C677T and A1298C	RM = 137, N = 100	Brazilian	No significant differences between recurrent miscarriage case and control groups	Gonçalves et al. (2016)
C677T and A1298C	RPL = 200, N = 200	Sinhalese	Not significantly associated with recurrent pregnancy loss	Dissanayake et al. (2012)
C677T and A1298C	RPL = 70, N = 136	Egyptian	RPL increased but not statistically significant	Settin et al. (2011)
C677T and A1298C	MS = 89, N = 150	Brazilian	Polymorphisms in the MTHFR, genes were not associated with recurrent miscarriage	Boas et al. (2015)
C677T and A1298C	Pregnant = 797	Italy	No association	Dell'Edera et al. (2018)
C677T and A1298C	RPL = 302; C = 315	Korean	No association	Hwang et al. (2017)
C677T and A1298C	Obstetric complications: 50; C = 79	Roman	No association	Bozikova et al. (2015)
C677T and A1298C	RM:60; C = 30	Spanish	No association	Creus et al. (2013)
A1298C	Meta-analysis	Indians	No association	Rai (2014)
Association				
C677T and A1298C	Meta-analysis	Iranian	Significant strong association	Kamali et al. (2018); Behjati et al. (2006); Farahmand et al. (2016)
C677T	Obstetric complications:120; C = 105	Slovak	Strong association	Bozikova et al. (2015).
C677T and A1298C	RM = 200, N = 300	Indian	Significant association	Parveen et al. (2013)
A1298C	Meta-analysis	Indian	Significant association	Nair et al. (2013)
C677T and A1298C	RPL = 171, N = 144	Lebanese	Strong relationship with RPL and CVD	El Achi et al. (2018)
C677T and A1298C	RPL = 100; N = 106	Syrian	Strong association	Al-Achkar et al. (2017)
C677T	RPL = 35 couples; C = 35 couples	Nepal.	Significant association	Sah et al. (2018)
C677T	RPL = 133; C = 74	Austria, Middle European white	C677T associated with elevated serum levels of homocysteine and idiopathic recurrent miscarriage.	Unfried et al. (2002)
C677T and A1298C	RA = 464	Slavonic	Increased the risk of abortion	Hubacek et al. (2015)
A1298C	Healthy nulliparous women = 2,034	Australia	Protection against pregnancy complications	Said et al. (2010)

RM, recurrent miscarriage; RPL, recurrent pregnancy loss; RA, recurrent abortion; IRPL, idiopathic recurrent pregnancy loss. N = Normal; C = Control.

These two polymorphisms have been extensively investigated in RSA in different populations, but the reports are contradictory. Meta-analyses have been used to clarify the status of MTHFR polymorphism and have shown significant population differences (Kamali et al., 2018; López-Jiménez et al., 2016; Nefic et al., 2018; Nair et al., 2013; Bozikova et al., 2015). It was shown in the Middle European white population, that polymorphism of MTHFR gene is associated with idiopathic recurrent miscarriage, and

homozygosity for MTHFR C677T polymorphism confers a 3.7 fold increase in its risk (OR 3.7, 95% CI 1.2–11.8) (Unfried et al., 2002). From the Czech Republic, a study on the Slavonic population (Hubacek et al., 2015), showed that both C677T and A1298C were more frequent in spontaneous abortion, and their combination significantly increased the risk of abortion (OR 1.28; 95% CI 1.05–1.57; p = 0.017). Studies on the Indian population showed that MTHFR A1298C polymorphism is a genetic risk factor for

pregnancy loss (Nair et al., 2013). However, a meta-analysis failed to show any association between this polymorphism and recurrent pregnancy loss (Rai, 2014). Said and coworkers (Said et al., 2010) reported from Melbourne, Australia, that homozygosity for MTHFR A1298C might protect against pregnancy complications.

In a group of Roman women, Bozikova et al. (2015) reported that none of the polymorphisms studied were significantly associated with pregnancy complications. Creus et al. (2013) report on Spanish women failed to show any variation in the level of plasma Hcy levels, RBC folate, and vitamin B12 serum levels or the prevalence of homozygous and heterozygous C677T mutation in MTHFR gene in the patients and controls groups. Later, Boas et al. (2015) also reported that the investigated gene polymorphisms and serum homocysteine, vitamin B12, and folate levels were not associated with idiopathic RSA.

Interestingly, contradictions are also reported from the same population. The example of Iranians is worth mentioning. Yousefian et al. (2014) and Bagheri et al.'s (2010) findings suggested that MTHFR mutations might not be associated with RSA in the Iranians. While other studies have shown a strong association between these polymorphisms and RSA (Kamali et al., 2018; Behjati et al., 2006; Farahmand et al., 2016).

The reports that show hyperhomocysteinemia and low folate level, in association with the two polymorphisms (C677T and A1298C), suggest that these two abnormalities will influence fetal development and hence will lead to premature birth. This should be the situation in every population. However, there are contradictions, and the different possibilities that cause variable results may be as follows: (i) Study population size differences in the different studies, (ii) The influence of other genetic factors on the gene expression of the MTHFR gene with different polymorphic forms of the studied SNPs, (iii) The influence of specific environmental factors on the gene expression and protein stability of the expressed proteins, and, finally, (iv) Possible epigenetic variations that influence the MTHFR gene expression and function of the expressed protein.

Hence, in conclusion, these reports further ascertain the need for association studies in each population, as they point out to the significance of other genetic, environmental and epigenetic factors that are modulating the role played by a specific SNP on the causation of a particular disorder.

Finally, as mentioned above, our results also showed that consanguinity could be a factor contributing to fetal loss in the form of RSA among Saudi females since the RSA patient had a higher frequency of cousin marriages compared to the control group. Other studies have shown an association between cousin marriages and RSA (Assaf and Khawaja, 2009; Mokhtar and Abdel-Fattah, 2001; Bener and Hussain, 2006), though others have failed to do so. (Khlat, 1988; Abdulrazzaq et al., 1997; Al-Awadi et al., 1986). It has been suggested that there are possibly recessive inherited genes or SNPs that, when inherited in the homozygous state, influence the development of RSA. Further studies are warranted to identify these genes.

In conclusion, this study has highlighted extensive contradictions in the nature of the association between the two studied SNPs (C677T and A1298C) in the MTHFR gene in different populations. In Saudis, the results show that there is no association between the studied SNPs with RSA. However, there is a limitation of this study, i.e., the small sample size. Further studies on larger sample size are warranted to confirm the findings of this study. Particularly the significant association of the haplotype CT of A1298C and C677T with RSA needs further investigation on larger sample size. These findings have opened avenues for further research on the possible association between MTHFR gene polymorphisms and RSA in different populations and have highlighted a need to investigate the mechanisms leading to an association in the different populations. A multicenter trial may identify factors influencing the MTHFR gene polymorphisms and their role in RSA development.

Data Availability: The authors declare that they have no conflicts of interest to report regarding the present study.

Authors Contributions: AA, AAMA and AAA designed the study and conducted the experimental work, ZB recruited the patients, collected the patient's data, and contributed to the finalization of the manuscript. AA, ZB, ASW, FBA and AT, analyzed the data, wrote the manuscript. The manuscript was finalized for publication by ASW, AA, and ZB.

Conflicts of Interest: The authors declare that there is no conflict of interest.

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