

Methylenetetrahydrofolate reductase C677T and A1298C polymorphism and changes in homocysteine concentrations in women with idiopathic recurrent pregnancy losses

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Abstract

Because they have been described as strong risk factors for idiopathic recurrent pregnancy losses (RPLs), we assessed the association between the methylenetetrahydrofolate reductase (MTHFR) single-nucleotide polymorphisms (SNPs) C677T and A1298C and hyperhomocysteinemia in Tunisian women with idiopathic RPL. Study subjects comprised 200 patients with more than three consecutive RPLs, and 200 age-matched parous control women. C677T and A1298C SNPs were analyzed by PCR-RFLP analysis, and fasting serum homocysteine was measured with ELISA. The frequency of MTHFR 677T/T (30.0 vs 7.0%) and 1298C/C (13.5 vs 4.0%) genotypes was significantly higher in patients. While it was similar among patients and controls ($P = 0.095$), higher homocysteine was seen with the T/T (but not 1298A/C and 1298C/C) genotype among patients and controls compared with non-T/T carriers ($P < 0.05$), and in patients vs controls. Higher prevalence of MTHFR 677T/T was seen in late ($P < 0.05$) and early-late ($P < 0.001$) RPL, while higher prevalence of 1298C/C genotype was seen only in early-late RPL ($P < 0.001$), and the prevalence of double heterozygotes was statistically not significant between patients and controls ($P = 0.10$; odds ratio = 2.73). Logistic regression analysis showed that, after adjusting for all variables, homozygosity for MTHFR C677T was associated with late ($P < 0.001$), and combined early-late ($P < 0.001$), while homozygosity for A1298C was associated only with combined early-late ($P = 0.026$), as was secondary-level education, which was associated with early ($P = 0.005$), late ($P = 0.026$) and combined early-late ($P = 0.004$) abortions. Homozygosity for MTHFR C677T (late and early-late) and A1298C (early-late) are risk factor for RPLs, irrespectively of total homocysteine levels.

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Introduction

Idiopathic recurrent pregnancy loss (RPL) is a frequent obstetric complication, and an estimated 1–2% women will present with three or more during their reproductive age, while almost 5% of women will present with two or more RPLs. While the exact cause of RPL remains undetermined in most of the cases, genetic predisposition to venous thrombosis (Finan *et al.* 2002), and elevation in total homocysteine (tHcy) levels (hyperhomocysteinemia) have been described as playing a role in the pathogenesis of RPL. The former was suggested to involve specific single-nucleotide polymorphisms (SNPs) in the genes coding for coagulation factors, including factor V-Leiden and prothrombin (G20210A), while the latter was described to be caused by mutations in methylenetetrahydrofolate reductase (MTHFR), in particular the C677T SNP (Nelen *et al.* 1998, Lissak *et al.* 1999, Wang *et al.* 2004).

MTHFR is critical in homocysteine (Hcy) metabolism, and catalyzes the NADPH-linked reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, and subsequently the vitamin B12-dependent methylation of Hcy to methionine (Jacques *et al.* 1996). A reduction in MTHFR level or activity by specific gene mutations induces hyperhomocysteinemia, characterized by mild-moderate increased plasma tHcy levels, and has been shown to be a risk factor for vascular thrombotic events, including coronary artery disease (Graham *et al.* 1997, Almawi *et al.* 2004a). While several mutations within it were described, the best-characterized MTHFR gene polymorphisms are the alanine-to-valine C677T (Frosst *et al.* 1995), and the glutamate-to-alanine A1298C (Van der Put *et al.* 1998) missense mutations. While both SNPs induce milder forms of MTHFR deficiency (Frosst *et al.* 1995, Chango *et al.* 2000), the A1298C SNP, located in the

enzyme regulatory domain, unlike the C677T SNP which is found within the enzyme catalytic domain, does not result in either a thermolabile protein or increased tHcy (Hanson *et al.* 2001, Friso *et al.* 2002). Interestingly, 677CT/1298AC compound heterozygosity reportedly has similar clinical impact as C677T homozygosity (Chango *et al.* 2000, Chen *et al.* 2005).

Through their effect on tHcy levels, MTHFR mutations have been implicated as risk factors in the pathogenesis of RPL. This was highlighted by the findings that the prevalence of homozygous variants of both SNPs was higher among women with more than three idiopathic RPLs (Nelen *et al.* 1997, Quere *et al.* 1998, Sarig *et al.* 2002). Hyperhomocysteinemia caused by MTHFR mutations or folate deficiency was associated with placental abruption or infarction (Van der Molen *et al.* 2000), pre-eclampsia (Lachmeijer *et al.* 2001, Murakami *et al.* 2001), pregnancy-associated hypertension (Kosmas *et al.* 2004) and RPL (Nelen *et al.* 2000a, Unfried *et al.* 2002). Others failed to demonstrate any association between MTHFR SNPs, hyperhomocysteinemia and RPL (Foka *et al.* 2000, Makino *et al.* 2004), as similar (Holmes *et al.* 1999) or even lower (Makino *et al.* 2004) prevalence rates of MTHFR C677T SNP were seen in patients compared with women with uneventful pregnancies. This prompted us to assess the relationship between RPL, MTHFR C677T and A1298C SNPs and tHcy in 200 Tunisian women with three or more consecutive idiopathic miscarriages, compared with 200 parous women of similar ethnic background with uncomplicated pregnancies and deliveries.

Subjects and Methods

Study subjects

This was a retrospective case-control study conducted in the maternity service of CHU Farhat Hached in Sousse,

central Tunisia in the period 2001–2004. Cases comprised 200 women who had had three or more consecutive RPLs at 5–30 weeks of gestation (Table 1). RPLs were classified as early (5–10 weeks) or late (11–30 weeks). Exclusion criteria included induced abortions, infection, systemic diseases, uterine structural abnormalities and personal or family history of thrombosis. The control group consisted of 200 women who were matched for ethnic origin, with no spontaneous miscarriages and uncomplicated pregnancy (Table 1). Patients and controls were asked to fill in a questionnaire detailing their age, educational status, area of residence, number and pregnancy outcome, and risk factors for venous thromboembolism, including smoking and oral contraceptive use, and to sign an informed consent form indicating their acceptance to participate. The study was conducted after institutional ethics requirements were met. EDTA-anticoagulated blood (5 ml sample) was obtained from participants and was processed within 30 min of collection; plasma aliquots were then immediately frozen for tHcy measurements.

MTHFR genotyping

Total genomic DNA was isolated by the phenol–chloroform method, as is standard, and was dissolved in sterile nuclease-free water. MTHFR C677T genotype analysis was performed by PCR-RFLP analysis using HinfI (Frosst *et al.* 1995) and MbolI (Friedman *et al.* 1999) digestion for C677T and A1298C detection respectively. The C677T mutation introduces a new HinfI restriction site, which results in the digestion of the 198 bp amplicon into 175 and 23 bp fragments. By abolishing an MbolI restriction site, the A1298C mutation results in the digestion of the 163 bp amplicon into 84, 31, 30 and 18 bp fragments, while the wild-type variant is digested into 56, 31, 30, 28 and 18 bp fragments. Digested DNA fragments were then separated on a 3% agarose-1000 (Invitrogen, Paisley, UK) or 1% agarose-1000/2.5% NuSieve agarose (FMC

Table 1 Characteristics of study participants.

	Patients ¹	Controls ¹	P	OR	95% CI
Age	28.68 ± 5.61	28.24 ± 5.51	0.434 ²		
Regional	49:50:41:60	42:43:43:72	0.531 ³		
Smokers	9/191	9/191	1.000 ³	1.000	0.389–2.574
Education			<0.001 ³		
Intermediate	41	69			
Secondary	81	92			
Univeristy	78	39			
Alcohol	5	5	1.000 ³	1.000	0.285–3.509
BMI	25.78 ± 4.01	24.62 ± 3.75	0.360 ²	1.159	0.395–1.923
Oral contraceptives	51	46	0.641 ³	1.146	0.725–1.811
Pregnancy outcomes					
Live	0.51 ± 0.72	3.81 ± 1.40	<0.001 ²	3.300	3.081–3.519
Early loss	2.29 ± 1.30	0.02 ± 0.14	<0.001 ²	2.270	2.088–2.452
Late loss	1.34 ± 1.30	0.04 ± 0.184	<0.001 ²	1.305	1.122–1.488

¹ Study subjects comprised 200 patients and 200 controls.

² Fisher's exact test.

³ Pearson's chi-square test.

Bioproducts, Rockland, ME, USA) prepared in $0.5 \times$ TBE buffer for C677T and A1298C respectively.

Hcy level measurement

Blood was drawn from fasting subjects and placed in plain vacutainers and tubes containing EDTA. For further analysis of plasma total Hcy, the sum of protein-bound and free Hcy was determined by ELISA using a commercial kit according to the manufacturer's instructions (Diazyme Laboratories, San Diego, CA, USA). The assay sensitivity was $2.5\text{--}60.0\text{ }\mu\text{M}$, and tHcy levels $>15\text{ }\mu\text{M}$ were considered elevated.

Statistical analysis

Data were expressed as percentages of the mean or as frequency of the allele. Allelic frequencies were calculated by a gene-counting method. Statistical analysis was performed on SPSS version 11.5 statistics software, which also computed the odd ratios (ORs) and 95% confidence intervals (95% CIs). Data were expressed as percentages of the mean or allele frequency; Pearson's chi-square test was used to assess intergroup significance and Student's *t*-test was used to determine differences in means. Regression multinomial logistic regression analysis was also determined, and results were expressed as beta and *P* values. Statistical significance was set at $P < 0.05$.

Results

Patients and controls

The demographics of study participants are summarized in Table 1. Patients and control subjects were of similar age (at first pregnancy; $P = 0.434$), body mass index (BMI; $P = 0.360$), and residence/regional distribution ($P = 0.531$). Comparable numbers of smokers ($P = 1.000$), alcohol consumers ($P = 1.000$) and oral contraceptive users ($P = 0.641$) were seen in both groups. While the

pregnancy rate was similar between patients and controls, the mean number of live births per woman was significantly higher among controls ($P < 0.001$), patients reporting 726 pregnancy losses in the first and second trimesters, compared with single-case pregnancy losses among controls, which were mainly induced/therapeutic (four in early, and seven in late pregnancy).

MTHFR C677T and A1298C genotype analysis

The distribution of MTHFR C677T and A1298C genotypes was in Hardy–Weinberg equilibrium among patients and controls. The frequency of MTHFR C677T mutant ('T') allele was significantly higher among patients than controls ($P < 0.001$) (Table 2). Higher frequencies of the C/T and the T/T ($P < 0.001$) genotypes of C677T SNP were seen in patients vs controls respectively (Table 2). Similarly, the frequency of the A1298C mutant ('C') allele was significantly higher among patients than controls ($P < 0.001$) (Table 2), and higher frequencies of the C/C but not the A/C genotypes of A1298C SNP were seen in patients vs controls respectively (Table 2), whereas a comparable frequency of double heterozygosity (677C/T and 1298A/C) was seen in 14 patients vs 5 control subjects ($P = 0.10$), and no double-homozygous case was seen in either group (Table 3).

Hcy proportion

Results in Table 4 show that tHcy levels were comparable between patients and controls ($P = 0.095$; Table 4). Significantly elevated tHcy was noted in 677T/T carriers among patients and control subjects, as opposed to C/T or C/C genotype carriers. In contrast, tHcy levels were comparable among all A1298C genotype carriers, both in patients and in controls. Furthermore, the proportion of individuals with hyperhomocysteinemia was comparable between patients (27.7%) and controls (20.6%) ($P = 0.249$, OR = 1.477, 95% CI = 0.759–2.876).

Table 2 Distribution of MTHFR C677T and A1298C genotypes¹.

MTHFR	Genotype frequency			Allele frequency	
	C/C	C/T	T/T	C	T
C677T					
Controls	156 (78.00) ²	30 (15.00)	14 (7.00)	0.855 ± 0.018^3	0.145 ± 0.018
Patients	92 (46.00)	47 (23.50)	61 (30.00)	0.578 ± 0.025	0.423 ± 0.025
<i>P</i> ⁴		<0.001		<0.001	
A1298C					
Controls	130 (65.00)	62 (31.00)	8 (4.00)	0.805 ± 0.020	0.195 ± 0.020
Patients	108 (54.00)	65 (32.50)	27 (13.50)	0.703 ± 0.023	0.298 ± 0.023
<i>P</i>		0.002		<0.001	

¹ Study subjects comprised 200 patients and 200 age-matched healthy controls.

² Number of individuals (percent of total) carrying the indicated genotype.

³ Allele frequency $\pm$ S.E.

⁴ Person's chi-square test.

Table 3 MTHFR C677T and A1298C genotype analysis.

C677T ¹	A1298C ¹	Patients ²	Controls ²	P ³	OR	95% CI
C/T	A/A	20 (20.4) ⁴	23 (26.4)	0.63	0.71	0.36–1.41
C/C	A/C	26 (26.5)	51 (58.6)	<0.001	0.25	0.14–0.48
C/T	A/C	14 (14.3)	5 (9.2)	0.10	2.73	0.92–7.19
C/T	C/C	13 (13.3)	2 (2.3)	0.01	6.50	1.36–21.52
T/T	A/C	25 (25.5)	6 (6.9)	0.001	4.62	1.74–10.88

¹ Determined by PCR and restriction with HinfI (C677T) or MboII (A1298C).

² Subjects include 200 patients and 200 controls.

³ Determined by Fisher's exact test.

⁴ Percent of total within patients or control groups.

Distribution of MTHFR genotypes among patients

Increased prevalence of the MTHFR 677T/T genotype was seen in late ($P < 0.05$) and combined early-late ($P < 0.001$) abortions, but not in women with only early abortions (Table 5). Similarly, a higher proportion of the MTHFR 1298A/C genotype was seen in combined early-late abortions ($P < 0.001$), but not in women with only early or late abortions (Table 5). In addition, the prevalence of the heterozygous variant of MTHFR 677C/T ($P < 0.05$), but not 1298A/C, was seen only in combined early-late RPL (Table 5).

Risk factors for pregnancy loss

Predictors of early, late and early-late abortions were determined by performing three logistic regression analysis models with the dependent variable being early, late, and early-late RPL, and the independent potentially confounding variables being age (categorized according to a cut-off age of 35 years), smoking, Hcy levels, use of oral contraceptives, education, BMI and smoking (Table 6). The only variable that was selected using this technique for the first outcome (early RPL) was education ($P = 0.005$), whereas for the second outcome (late RPL) variables were education ($P = 0.026$) and homozygosity for C677T ($P < 0.001$), and for the third outcome (early-late), they were education ($P = 0.004$) and homozygosity for both C677T ($P < 0.001$) and A1298C ($P = 0.026$). Adjusting for the variables selected, Hcy levels was not associated with any of the stages of idiopathic RPL.

Table 4 Homocysteine levels in patients and controls.

Mutation	Genotype	Patients ¹	Controls ¹	P ²
C677T	C/C	7.08 ± 5.71	7.38 ± 5.39	0.752
	C/T	11.90 ± 8.98	11.51 ± 8.91	0.889
	T/T	15.58 ± 6.99	18.18 ± 2.61	0.465
A1298C	A/A	9.71 ± 7.57	9.93 ± 6.32	0.171
	A/C	12.46 ± 7.38	9.84 ± 7.56	0.165
	C/C	11.24 ± 9.52	10.00 ± 5.40	0.728

¹ Study subjects comprised 200 patients and 200 healthy controls.

² Student's *t*-test.

Table 5 Distribution of MTHFR genotypes among patients.

MTHFR SNP	Controls ¹ (none)	Patients ¹			Total ²
		Early	Late	Early-rate	
C677T					
CC	156	22	18**	52**	248
CT	30	6	9	32*	77
TT	14	5	16**	40**	75
Total ²	200	33	43	124	400
A1288C					
AA	130	16	22	70	238
AC	62	15	17	33	127
CC	8	2	4	21**	35
Total ²	200	33	43	124	400

¹ A total of 200 patients and 200 controls were included.

² Total number of subjects within each category.

* $P < 0.05$; ** $P < 0.001$; comparisons as per the frequency of genotype carrier in patients vs. controls.

Discussion

Insofar as they were implicated in pregnancy complications, including recurrent idiopathic RPLs (Nelen *et al.* 1997, Quere *et al.* 1998, Sarig *et al.* 2002), we investigated the association of MTHFR C677T and A1298C SNPs and hyperhomocysteinemia in women with idiopathic RPL. As patients and controls were matched for several risk factors (older age, smoking, obesity and use of oral contraceptives), results obtained showed that RPL patients had a higher prevalence of both MTHFR SNPs as compared with otherwise healthy control parous women. The allele frequency of MTHFR C677T and A1298C SNPs among control primigravid women was similar to that of healthy Tunisians (data not shown), thereby ruling out probability bias in control selection, and was comparable with rates established for European (Sacchi *et al.* 1997) and Mediterranean (Almawi *et al.* 2004a) communities. In view of their presence among control women, and as not all MTHFR C677T and A1298C mutant genotype carriers experienced RPLs, this indicated that the concerted participation of multiple inherited and non-inherited prothrombotic defects placed women at greatest risk.

Pregnancy losses were categorized into early (5–12 weeks), late (13–30 weeks) and combined early-late. The risk of pregnancy failure was significantly elevated only in carriers of the homozygous variant of both SNPs (677T/T and 1298C/C) and, interestingly, was seen in patients experiencing late (C677T) and combined early-late (C677T and A1298C), but not exclusively early miscarriages. This was in accord with earlier studies (Nelen *et al.* 1997, 1998, Lissak *et al.* 1999), which reported on the association of MTHFR 677T/T (acting through elevation of tHcy) with idiopathic RPL (Gris *et al.* 1999), more so with late than for first trimester abortions. Others failed to establish an association between MTHFR 677T/T genotype and idiopathic RPL (Kutteh *et al.* 1999, Carp *et al.* 2002), while others suggested that homozygosity for MTHFR

Table 6 Regression analysis.

Variable	Stage of pregnancy loss		
	Early	Late	Early + late
Education ¹			
Intermediate	2.33 (0.68–8.03) ²	1.12 (0.39–3.21)	1.32 (0.58–3.00)
Secondary	6.35 (1.73–23.26)	4.08 (1.19–14.03)	3.89 (1.53–9.87)
MTHFR C677T ³			
C/T	—	2.93 (0.97–8.81)	1.99 (0.86–4.63)
T/T	—	16.81 (4.51–62.73)	13.82 (4.42–43.24)
MTHFR A1298C ³			
A/C	—	—	0.68 (0.31–1.48)
C/C	—	—	4.52 (1.20–17.06)

¹ Reference being university education.² OR (95% CI).³ Reference being mutation non-carrier (C/C genotype for C677T and A/A genotype for A1298C).

C677T induced pregnancy loss only if present with other prothrombotic factors (Sarig *et al.* 2002).

Whereas hyperhomocysteinemia was notable among MTHFR 677T/T but not 1298C/C carriers in both groups, tHcy concentrations were comparable between patients and controls. It remains to be seen whether tHcy levels are indeed causally related to idiopathic pregnancy loss, with previous reports implicating hyperhomocysteinemia (Nelen *et al.* 2000a) and the Hcy-lowering agent folate (Nelen *et al.* 2000b) with RPL as risk factors for placental abruption or infarction, pre-eclampsia and early idiopathic RPL (Aubard *et al.* 2000, Steegers-Theunissen *et al.* 2004). While not tested here, it is possible that the association of the MTHFR 677T/T genotype with RPL, independently of hyperhomocysteinemia, was due to interference with red blood cell folate metabolism, as has been suggested (Alessio *et al.* 2004, Golbahar *et al.* 2005). Mechanistically, it was suggested that hyperhomocysteinemia precipitated thrombophilia by inducing the expression of tissue factor (TF), an initiator of blood coagulation *in vivo* (Fryer *et al.* 1993), by circulating monocytes, which apparently acted independently of peroxide and superoxides, since scavengers of both did not block the expression of Hcy-induced TF (Khajuria & Houston 2000). Hyperhomocysteinemia may also act by altering endothelial cell function through upregulation of the expression and secretion of monocyte chemoattractant protein-1 and interleukin-8, which by promoting leukocyte recruitment may contribute to the initiation and progression of vascular disease (Poddar *et al.* 2001).

While MTHFR C677T and A1298C SNPs were implicated in several pregnancy-related complications, including placental anomalies (Van der Molen *et al.* 2000), pre-eclampsia (Lachmeijer *et al.* 2001, Murakami *et al.* 2001) and RPL (Nelen *et al.* 1997, Quere *et al.* 1998 Sarig *et al.* 2002), the association of a particular mutation in diseased populations may be influenced by a number of environmental or genetic risk factors. Accordingly, the significance of the mutation may be overestimated.

Regression analysis results clearly demonstrated that homozygosity for C677T was associated with late and combined early-late RPL, while homozygosity for A1298C was associated with combined early-late idiopathic RPL. Interestingly, education was the only non-inherited risk factor associated with RPL in the population examined. While explanation for this remains speculative, it is tempting to hypothesize that accompanying environmental factors related to maternal socio-economic status may influence RPL, as was suggested elsewhere (Manchester *et al.* 1995).

In conclusion, while MTHFR C677T and A1298C were more prevalent in women with idiopathic RPL, homozygosity for MTHFR C677T and A1298C were implicated in RPL after adjusting for inherited and non-inherited variables. It should be noted here that the contribution of inherited and environmental prothrombotic risk factors may vary significantly according to ethnicity. Further studies, including meta-analysis, are needed for a thorough understanding of the contribution of MTHFR mutations and hyperhomocysteinemia in idiopathic RPL.

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