Association of Methylenetetrahydrofolate Reductase (*MTHFR-677* and *MTHFR-1298*) Genetic Polymorphisms with Occlusive Artery Disease and Deep Venous Thrombosis in Macedonians

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> **Received:** July 12, 2007
> **Accepted:** January 11, 2008

> **Croat Med J. 2008;49:39-49**
> doi:10.3325/cmj.2008.1.39

Aim To analyze the association of methylenetetrahydrofolate reductase polymorphisms (*MTHFR-677* and *MTHFR-1298*) with occlusive artery disease and deep venous thrombosis in Macedonians.

Methods We examined 83 healthy respondents, 76 patients with occlusive artery disease, and 67 patients with deep venous thrombosis. Blood samples were collected and DNA was isolated from peripheral blood leukocytes. Identification of *MTHFR* mutations was done with CVD StripAssay (ViennaLab, Labordiagnostika GmbH, Vienna, Austria) and the population genetics analysis package, PyPop, was used for the analysis. Pearson *P* values, crude odds ratio, and Wald's 95% confidence intervals were calculated.

Results The frequency of *C* alleles of *MTHFR-677* was 0.575 in patients with deep venous thrombosis, 0.612 in patients with occlusive artery disease, and 0.645 in healthy participants. The frequency of *T* allele of *MTHFR-677* was lower in healthy participants (0.355) than in patients with occlusive artery disease (0.388) and deep venous thrombosis (0.425). The frequency of *A* allele for *MTHFR-1298* was 0.729 in healthy participants, 0.770 in patients with occlusive artery disease, and 0.746 in patients with deep venous thrombosis. The frequency of *C* allele of *MTHFR-1298* was 0.271 in healthy participants, 0.230 in patients with occlusive artery disease, and 0.425 in patients with deep venous thrombosis. No association of *MTHFR-677* and *MTHFR-1289* polymorphisms with occlusive artery disease and deep venous thrombosis was found, except for the protective effect of *MTHFR/CA:CC* diplotype for occlusive artery disease.

Conclusion We could not confirm a significant association of *MTHFR-677* and *MTHFR-1289* polymorphisms with occlusive artery disease or deep venous thrombosis in Macedonians, except for the protective effect of *MTHFR/CA:CC* diplotype against occlusive artery disease.

Methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20) catalyzes the conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a cosubstrate for homocysteine remethylation to methionine. The human *MTHFR* gene (MIM *607093) has been localized at chromosome 1p36.3 (1), and is composed of 11 exons (2).

Twelve alleles of human *MTHRF* gene (0001-0012) have been identified so far (3). *MTHFR* thermolabile polymorphisms (*MTHFR, 677C-T, ALA222VAL* and *MTHFR, 1298A-C, GLU429ALA*) were investigated in several diseases. The mutation in the heterozygous or homozygous state correlated with reduced enzyme activity and increased thermolability in lymphocyte extracts; in vitro expression of the mutagenized cDNA containing the mutation confirmed its effect on the thermolability of *MTHFR*. Individuals homozygous for the mutation had significantly elevated plasma homocysteine levels. Thus, the *677C-T* mutation may represent an important genetic risk factor for vascular disease (4-6). There are many articles connecting *MTHFR* mutations, mostly *MTHFR 677C-T,* with plasma homocysteine levels. Several meta-analyses showed a positive association of *MTHFR* mutations with vascular diseases (7-9), although several did not (10-12).

Since both mutations (*677C-T* and *1298A-C MTHFR*), when homozygous, were associated with a decreased DNA methylation status (although the effect was slightly less pronounced for the *1298A-C* transversion), it was suggested that *1298CC MTHFR* genotype, independently of folate availability, and *677TT MTHFR* genotype with concomitant low folate levels, might be potential risk factors for diseases associated with decreased DNA methylation status (13).

We believe that *MTHFR* mutations influence the homocysteine metabolism, but are in weak association with vascular diseases and could be analyzed in combination with other candidate genes for vascular diseases. There are no data on *MTHFR-677* and *MTHFR-1298* polymorphisms in Macedonian population and their possible associations with different diseases. The aim of this study was to analyze the association of methylenetetrahydrofolate reductase polymorphisms (*MTHFR-677 and MTHFR-1298*) with occlusive artery disease and deep venous thrombosis in order to investigate the role of *MTHFR* mutations as candidate genes in different vascular diseases in Macedonians.

Participants and methods

Participants

The total studied sample consisted of 226 participants, divided into three different groups as follows: healthy individuals, patients with occlusive artery disease, and patients with deep venous thrombosis.

Healthy individuals (n = 83). There were 40 women and 43 men, aged 40.7 ± 11.3 years, born in different parts of Macedonia. They were age and sex non-matched healthy individuals who attended the Institute for Transfusion for blood donation between May 5, 2003 and April 25, 2004 and agreed to take part in this study as a control group if a medical doctor declared their health as acceptable (on the basis of medical documentation, an interview, and physical examination). Individuals with family history of blood vessel diseases were excluded from the investigation.

Occlusive artery disease (n = 76). There were 29 women and 47 men with diagnosed and documented myocardial infarction (n = 52), brain infarction (n = 22), and peripheral artery thrombosis (n = 2). They were 63.3 ± 9.6 years old consecutive patients hospitalized at the Institute of Heart Diseases, University School of Medicine, or attended the Institute for Trans-

fusion for outpatient treatment between May 5, 2003 and April 25, 2004.

Deep venous thrombosis (n = 67). There were 45 women and 22 men with a diagnosis of deep venous thrombosis made by ultrasonography and/or venography. They were 57.7 ± 11.8 years old consecutive patients who attended the Institute of Heart Diseases, University School of Medicine and the Institute for Transfusion for outpatient treatment between May 5, 2003 and April 25, 2004.

All individuals were of Macedonian origin and residents of different geographical areas of the Republic of Macedonia. All patients and healthy individuals included in this study signed a written consent to participate in the study, which was approved by the Ethics Committee of the Ministry of Education and Science of the Republic of Macedonia (No. 13-1672/4-02).

Genomic DNA isolation and storage

DNA was isolated from peripheral blood leukocytes by phenol-chlorophorm extraction method or with BioRobot EZ1 workstation (QIAGEN) (14). The quality and quantity of DNA was analyzed by GeneQuant (Pharmacia Biotech, Uppsala, Sweden). Isolated DNA samples were stored in the Macedonian Human DNA Bank (15).

Typing methods

Assay for the identification of *MTHFR* mutations was based on polymerase chain reaction (PCR) and reverse-hybridization with CVD StripAssay (ViennaLab Labordiagnostika GmbH, Vienna, Austria). The procedure included three steps as follows: 1) DNA isolation; 2) PCR amplification using biotinylated primers; 3) hybridization of amplification products to a test strip containing allele-specific oligonucleotide probes immobilized as an array of parallel lines. Bound biotinylated sequences were detected using streptavidin-alkaline phosphatase and color substrates (16). The assay covered 2 mutations – *MTHFR C677T* and *MTHFR A1298C*. The genotype of the sample was determined using the enclosed Collector sheet or StripAssay Evaluator software, version 2.0 (ViennaLab Diagnostics GmbH).

Statistical analysis

The population genetics analysis package, PyPop, developed by the Biostatistics Core for the Workshop (17-19), was used for analysis of the *MTHFR* data. Allele frequencies and expected Hardy Weinberg proportions (HWP) for each *MTHFR* mutation were determined (20). The exact test for genotype frequency deviation from HWP was performed, using the Arlequin implementation accessed via PyPop (21). The single nucleotide polymorphisms that deviated from HWP were evaluated to determine whether there was an excess of homozygotes or heterozygotes. χ^2 test was used to determine if any particular genotypes were significantly different from the expected frequencies. The Ewens-Watterson homozygosity test of neutrality (22) with Slatkin's exact *P* values (23,24) was used to indicate deviations from the hypothesis of neutral selection for each locus. Linkage disequilibrium was calculated, where *D'* weights the contribution to linkage disequilibrium of specific allele pairs by the product of their allele frequencies; W_n is a re-expression of the χ^2 statistic for deviations between the observed and expected haplotype frequencies; and *S* is defined as twice the difference between log-likelihood of obtaining the observed data given the inferred haplotype frequencies [ln(L_1)], and the likelihood of the data under the null hypothesis of linkage equilibrium [ln(L_0)]) (25). Pearson *P* values, crude odds ratio (OR), and Wald's 95% confidence interval (CI) were calculated to test the associations between *MTHFR* mutations and blood vessel disease with GraphPad Quick-

Calcs – free statistical calculators (*http://www.graphpad.com/quickcalcs/*). The level of statistical significance was set at $P<0.05$.

Results

MTHFR alleles

Frequencies of *MTHFR* alleles, test of neutrality with F_{nd} statistic (Ewens-Watterson test of neutrality), and Slatkin's Exact *P* Value with *P* of F statistics in Macedonians are shown in Table 1.

The frequency of *C* alleles for *MTHFR-677* varied between 0.575 for deep venous thrombosis, 0.612 for occlusive artery disease, and 0.645 for healthy participants, indicating a common wild type allele. The frequency of *T* allele was lower in healthy participants (0.355) than in patients with occlusive artery disease (0.388) or deep venous thrombosis (0.425). The frequency of *A* alleles for *MTHFR-1298* varied from 0.729 for healthy participants, 0.770 for patients with occlusive artery disease, and 0.746 for patients with deep venous thrombosis, indicating a common wild type allele. The frequency of *C* allele was 0.271 in healthy participants, 0.230 in patients with occlusive artery disease, and 0.254 in patients with deep venous thrombosis. For all the *MTHFR* alleles, the test of neutrality showed negative value for F_{nd} statistics, without significant *P* of F statistics, which indicated balancing selection operating on the alleles at that locus in all the groups.

MTHFR genotypes

The most frequent *MTHFR-677* genotype in healthy participants was *CT*, with the observed frequency of 44.6%. A lower frequency was found for *CC* (42.2%) and the lowest (13.2%) for *TT*. The frequencies of *MTHFR-677 CT* and *TT* genotypes were slightly increased in patients with occlusive artery disease and deep venous thrombosis, with a decrease in *CC* genotype (Table 2). All genotypes in healthy participants and patients with blood vessel disease showed no deviation from HWP. The most frequent *MTHFR-1298* genotype in healthy participants was *AA* (49.4%). A lower frequency was found for *CA* genotype (47.0%) and the lowest for *CC* (3.6%). The frequency of *MTHFR-1298* genotypes *AA* and *CC* was slightly increased in patients with occlusive artery disease and deep venous thrombosis, with a consecutive decrease in *CA* genotype. In some instances, χ^2 test could not be performed because the expected frequency

Table 1. Frequencies of *MTHFR-677* and *MTHFR-1298* alleles, test of neutrality with F_{nd} statistic (Ewens-Watterson test of neutrality), and Slatkin's Exact *P* Value with *P* of F statistics in Macedonians*

					Test of neutrality (F)	
		Alleles			EWN	SEPV
MTHFR mutation	No. of patients	allele	number	frequency	F_{nd}	*P* of F
MTHFR-677						
Healthy	83	*C*	107	0.645	-1.696	0.106
		T	59	0.355		
Occlusive artery disease	76	*C*	93	0.612	-1.781	0.083
		T	59	0.388		
Deep venous thrombosis	67	*C*	77	0.575	-1.842	0.059
		T	57	0.425		
MTHFR-1298						
Healthy	83	*A*	121	0.729	-1.320	0.176
		C	45	0.271		
Occlusive artery disease	76	*A*	117	0.770	-1.061	0.220
		C	35	0.230		
Deep venous thrombosis	67	*A*	100	0.746	-1.184	0.203
		C	34	0.254		

*Abbreviations: MTHFR – methylenetetrahydrofolate reductase; EWN – Ewens-Watterson test of neutrality; SEPV – Slatkin's Exact *P* Value.

Table 2. Observed vs expected *MTHFR-677* and *MTHFR-1298* genotypes for each group, Hardy Weinberg proportions, and Guo and Thompson Hardy Weinberg Output in Macedonians*

Investigated group	Genotype	Observed number	Observed frequency (%)	Expected number	*P*	HWP *P*	GTHWO *P*
	MTHFR-677						
Healthy						0.805	0.812
	CC	35	42.2	34.5	0.930		
	CT	37	44.6	38.0	0.867		
	TT	11	13.2	10.5	0.874		
Occlusive artery disease						0.483	0.630
	CC	27	35.5	28.5	0.786		
	CT	39	51.3	36.1	0.629		
	TT	10	13.2	11.5	0.668		
Deep venous thrombosis						0.575	0.803
	CC	21	31.3	22.1	0.811		
	CT	35	52.2	32.8	0.695		
	TT	11	16.4	12.1	0.747		
	MTHFR-1298						
Healthy						0.085	0.161
	AA	41	49.4	44.1	0.641		
	CA	39	47.0	32.8	0.279		
	CC	3	3.6	6.1	0.209		
Occlusive artery disease						0.688	0.522
	AA	46	60.5	45.0	0.885		
	CA	25	32.9	26.9	0.708		
	CC	5	6.6	4.0	†		
Deep venous thrombosis						0.320	0.203
	AA	35	52.2	37.3	0.705		
	CA	30	44.8	25.4	0.358		
	CC	2	3.0	4.3	†		

*Abbreviations: MTHFR – methylenetetrahydrofolate reductase; HWP – Hardy Weinberg proportions; GTHWO – Guo and Thompson Hardy Weinberg Output.
†Cannot be calculated because expected number was <5, χ^2 test.

was smaller than 5 (*MTHFR-1298/CC* in patients with occlusive artery disease and *MTHFR-1298/CC* in patients with deep venous thrombosis). All genotypes in healthy participants and patients with blood vessel disease showed no deviations from HWP (Table 2).

MTHFR haplotypes and linkage disequilibrium

The most frequent haplotype of *MTHFR-677:MTHFR-1298* in healthy Macedonians was *CA*, followed by *TA*, *CC*, and *TC* (Table 3). Similar haplotype frequencies were found patients with occlusive artery disease and deep venous thrombosis. Haplotypes of *MTHFR-677:MTHFR-1298* in healthy Macedonians, patients with occlusive artery disease, and in patients with deep venous thrombosis deviated from HWP (*P*<0.001 for all groups; Table 3).

Observed vs expected *MTHFR-677* and *MTHFR-1298* diplotypes for each investigated group, χ^2, and HWP in Macedonians are shown on Table 4. We observed 6 of possible 10 diplotypes in all groups. Four expected diplotypes (*CC:TA, TC:TA, TC:CC*, and *TC:TC*) were not found in any of the groups. Be-

Table 3. Observed *MTHFR-677* and *MTHFR-1298* haplotypes for each group, χ^2, and Hardy Weinberg proportions in Macedonians. The first nucleotide from haplotypes (*C* or *T*) belongs to *MTHFR-677* and the second nucleotide (*A* or *C*) belongs to *MTFHR-1298*

Investigated group	Haplotype	Observed number	Observed frequency	χ^2	HWP* *P*
Healthy				34.70	<0.001
	CA	77	0.464		
	CC	30	0.181		
	TA	44	0.265		
	TC	15	0.090		
Occlusive artery disease				19.74	<0.001
	CA	71	0.467		
	CC	22	0.145		
	TA	46	0.303		
	TC	13	0.085		
Deep venous thrombosis				33.89	<0.001
	CA	60	0.448		
	CC	17	0.127		
	TA	40	0.298		
	TC	17	0.127		

*MTHFR – methylenetetrahydrofolate reductase; HWP – Hardy Weinberg proportions.

Table 4. Observed vs expected *MTHFR-677* and *MTHFR-1298* diplotypes for each investigated group, χ^2, and Hardy Weinberg proportions in Macedonians. The first nucleotide from haplotypes (*C* or *T*) belongs to *MTHFR-677* and the second nucleotide (*A* or *C*) belongs to *MTFHR-1298*

Investigated group	Diplotype	Observed number	Observed frequency (%)	Expected Number	χ^2	HWP* *P*
Healthy						
	CA:CA	8	9.6	17.0	5.44	0.020
	CA:TA	22	26.5	20.4	0.12	0.725
	CA:CC	24	28.9	13.9	7.31	0.007
	CA:TC	15	18.1	7.0	9.30	0.002
	TA:TA	11	13.3	5.8	4.58	0.032
	CC:TA	0	0	8.0	7.95	0.005
	CC:CC	3	3.6	2.7	†	†
	TC:TA	0	0	4.0	†	†
	TC:CC	0	0	2.7	†	†
	TC:TC	0	0	0.7	†	†
Occlusive artery disease						
	CA:CA	10	13.05	16.6	2.61	0.106
	CA:TA	26	34.2	21.5	0.95	0.330
	CA:CC	12	15.8	10.3	0.29	0.591
	CA:TC	13	17.1	6.1	7.90	0.005
	TA:TA	10	13.05	7.0	1.33	0.249
	CC:TA	0	0	6.7	6.66	0.010
	CC:CC	5	6.6	1.6	†	†
	TC:TA	0	0	3.9	†	†
	TC:CC	0	0	1.9	†	†
	TC:TC	0	0	0.6	†	†
Deep venous thrombosis						
	CA:CA	6	8.9	13.4	4.11	0.043
	CA:TA	18	26.9	17.9	0.00	0.983
	CA:CC	13	19.4	7.6	3.81	0.051
	CA:TC	17	25.4	7.6	11.58	<0.001
	TA:TA	11	16.4	6.0	4.24	0.039
	CC:TA	0	0	5.1	5.07	0.024
	CC:CC	2	3.0	1.1	†	†
	TC:TA	0	0	5.1	5.07	0.024
	TC:CC	0	0	2.2	†	†
	TC:TC	0	0	1.1	†	†

*MTHFR – methylenetetrahydrofolate reductase; HWP – Hardy Weinberg proportions.
†Cannot be calculated because expected number <5, χ^2 test.

Table 5. Linkage disequilibrium for the loci *MTHFR-677:MTHFR-1298**

Group	D	D'	Wn	ln(L_1)	ln(L_0)	S	*P*
Healthy	0.096	1.000	0.453	-138.55	-152.34	27.58	<0.001†
Occlusive artery disease	0.089	1.000	0.436	-127.76	-139.18	22.84	<0.001†
Deep venous thrombosis	0.108	1.000	0.502	-110.96	-122.23	22.54	<0.001†

**D'* weights the contribution to linkage disequilibrium of specific allele pairs by the product of their allele frequencies (25); W_n is a re-expression of the χ^2 statistic for deviations between observed and expected haplotype frequencies; *S* is defined as twice the difference between log-likelihood of obtaining the observed data given the inferred haplotype frequencies [ln(L_1)], and the likelihood of the data under the null hypothesis of linkage equilibrium [ln(L_0)]); *P* value is the fraction of permutations that results in values of *S* greater or equal to that observed. *P* <0.05 is indicative of overall significant linkage disequilibrium.
†Significant values.

cause of that, most of the observed diplotypes in healthy Macedonians and in patients with deep venous thrombosis, except for *MTHFR/CA:TA* diplotype, deviated from HWP (P<0.05). In patients with occlusive artery disease, all diplotypes did not deviate from HWP, except for *CA:TC* and *CC:TA* diplotype (P<0.010; Table 4).

Linkage disequilibrium for the loci *MTHFR-677:MTHFR-1298* was significant in healthy participants, participants with occlusive artery disease, and participants with deep venous thrombosis (P<0.001; Table 5).

Association between MTHFR mutations and blood vessels diseases

We did not find a significant association between *MTHFR-677* and *MTHFR-1298* alleles or genotypes and occlusive artery disease (Pearson P>0.05; Table 6). There was also no

Table 6. Association between *MTHFR-677* and *MTHFR-1298* alleles and genotypes with occlusive artery disease, Pearsons *P* value, crude odds ratio, and Wald's 95% confidence interval (CI) in Macedonians*

Allele or genotype	No. (%) of respondents with disease	No. (%) of respondents healthy (n = 83)	Pearson *P* value	Odds ratio	Wald's 95% CI
MTHFR-677:	OAD (n = 76)				
C	93 (61.18)	107 (64.50)	0.563	1.151	0.730-1.812
T	59 (38.82)	59 (35.50)	0.563	0.869	0.552-1.369
CC	27 (35.52)	35 (42.20)	0.419	1.323	0.699-2.503
CT	39 (51.32)	37 (44.60)	0.429	0.763	0.410-1.421
TT	10 (13.16)	11 (13.20)	1.000	1.008	0.410-2.476
MTHFR-1298:	OAD (n = 76)				
A	117 (77.0)	121 (72.9)	0.402	1.243	0.747-2.069
C	35 (33.0)	45 (27.1)	0.402	0.804	0.483-1.339
AA	46 (60.5)	41 (49.4)	0.159	1.571	0.837-2.949
CA	25 (32.9)	39 (47.0)	0.070	0.553	0.290-1.053
CC	5 (6.6)	3 (3.6)	0.393	1.878	0.433-8.140
MTHFR-677:	DVT (n = 67)				
C	77 (57.46)	107 (64.50)	0.234	1.343	0.842-2.139
T	57 (42.54)	59 (35.50)	0.234	0.745	0.467-1.187
CC	21 (31.34)	35 (42.20)	0.180	1.597	0.816-3.124
CT	35 (52.24)	37 (44.60)	0.412	0.735	0.387-1.399
TT	11 (16.42)	11 (13.20)	0.646	0.778	0.320-1.888
MTHFR-1298:	DVT (n = 67)				
A	100 (74.6)	121 (72.9)	0.734	1.094	0.651-1.836
C	34 (25.4)	45 (27.1)	0.734	0.914	0.544-1.535
AA	35 (52.2)	41 (49.4)	0.739	1.120	0.588-2.134
CA	30 (44.8)	39 (47.0)	0.787	0.915	0.479-1.746
CC	2 (3.0)	3 (3.6)	0.830	0.820	0.133-5.059

*Abbreviations: MTHFR – methylenetetrahydrofolate reductase; OAD – occlusive artery disease; DVT – deep venous thrombosis.

significant association between *MTHFR-667* and *MTHFR-1298* alleles or genotypes with deep venous thrombosis (Pearson $P>0.05$; Table 6).

We did not find a significant association between *MTHFR* haplotypes with occlusive artery disease (Pearson $P>0.05$; Table 7). We found a significant negative association between *MTHFR/CA:CC* diplotype and artery occlusive disease ($P=0.048$; OR, 0.461; CI, 0.212-1.003). The rest of *MTHFR* diplotypes were not significantly associated with occlusive artery disease. We did not find a significant association between *MTHFR* haplotypes or diplotypes with deep venous thrombosis (Pearson $P>0.05$; Table 7).

Discussion

Our study confirmed the presence of *MTHFR-677*, and *MTHFR-1298* polymorphisms in Macedonian population, and their possible association with occlusive artery disease and deep venous thrombosis. There was no significant association of *MTHFR-677* and *MTHFR-1298* polymorphisms with occlusive artery disease or deep venous thrombosis, except the protective effect of *MTHFR/CA:CC* diplotype against occlusive artery disease.

We found negative F_{nd} for *MTHFR-677* and *MTHFR-1298*, but no significant *P* of F value, which indicates that balancing selection is operating on the alleles at that cluster. In all groups *MTHFR-677* and *MTHFR-1298* did not deviate from HWP, while *MTHFR-677:MTHFR-1298* haplotypes did. The same was true for *MTHFR-677:MTHFR-1298* diplotypes in all groups (with few exclusions). We observed 6 of 10 possible diplotypes in all the groups. Four expected diplotypes (*CC:TA, TC:TA, TC:CC*, and *TC:TC*) were not found in any of the groups. We found a significant linkage disequilibrium between the pair of *MTHFR-677:MTHFR-1298* loci in healthy population, occlusive artery disease, and deep venous thrombosis. The absence of diplo-

Table 7. Association between *MTHFR-677:MTHFR-1298* haplotypes and diplotypes with occlusive artery disease and deep venous thrombosis, Pearsons *P*-value, crude odds ratio (OR), and Wald's 95% confidence interval (CI) in Macedonians*

	No. of patients				Wald's
	with disease	healthy	Pearson *P*	OR	95% CI
Haplotypes:	OAD (n = 152)	(n = 166)			
CA	71	77	0.954	1.013	0.652-1.575
CC	22	30	0.386	0.767	0.421-1.398
TA	46	44	0.457	1.203	0.738-1.961
TC	13	15	0.879	0.941	0.433-2.049
Diplotypes:	OAD (n = 76)	(n = 83)			
CA:CA	10	8	0.484	1.420	0.529-3.811
CA:TA	26	22	0.290	1.442	0.731-2.845
CA:CC	12	24	0.048	0.461	0.212-1.003
CA:TC	13	15	0.873	0.935	0.413-2.120
TA:TA	10	11	0.986	0.992	0.395-2.487
CC:CC	5	3	0.393	1.878	0.433-8.14
Haplotypes:	DVT (n = 134)	(n = 166)			
CA	60	77	0.781	0.937	0.593-1.480
CC	17	30	0.202	0.659	0.346-1.255
TA	40	44	0.521	1.180	0.712-1.956
TC	17	15	0.308	1.463	0.701-3.051
Diplotypes:	DVT (n = 67)	(n = 83)			
CA:CA	6	8	0.886	0.922	0.303-2.801
CA:TA	18	22	0.960	1.019	0.492-2.108
CA:CC	13	24	0.179	0.592	0.274-1.277
CA:TC	17	15	0.278	1.541	0.703-3.377
TA:TA	11	11	0.586	1.286	0.520-3.181
CC:CC	2	3	0.831	0.820	0.133-5.059

*Abbreviations: MTHFR – methylenetetrahydrofolate reductase; OAD – occlusive artery disease; DVT – deep venous thrombosis.

types and significant linkage disequilibrium in Macedonians could be a result of selective pressures or low frequencies in the groups.

High frequency of *MTHFR-677/TT* genotype (18-19%) was found in several studies conducted in Italy (4,26-28), while the lowest frequency (6.2%) was found in Germany (29) and Croatia (6%) (30). The frequency of *MTHFR-677/TT* genotype in Greece was reported in 16.7% of the population (31). Allele and genotype frequencies of *MTHFR-677* in Macedonia were high compared with other European populations. Unfortunately, there are not enough data on the *MTHFR-677* and *MTHFR-1298* haplotypes and diplotypes to make reliable comparisons between populations, as well as association analysis with different diseases.

Geographic and ethnic distribution of the *677C-T* polymorphism in the *MTHFR* gene was studied in more than 7000 newborns from 16 areas in Europe, Asia, the Americas, the Middle East, and Australia. The *TT* genotype was particularly common in northern China (20%), southern Italy (26%), and Mexico (32%). There was also some evidence for geographic gradients in Europe (north to south increase) and China (north to south decrease). The *TT* genotype frequency was low among newborns of African origin, medium among newborns of European origin, and high among newborns of American Hispanic origin. Areas with extreme frequencies showed deviations from HWP (Helsinki, southern Italy, and southern China). The findings suggested the existence of selective pressures leading to a marked variation (32).

Several studies found a significant association of *MTHFR-677* and/or *MTHFR-1298* with blood vessel diseases, which is in discordance with our data (7-9).

Non-significant association between *MTHFR-677* and *MTHFR-1298* alleles, genotypes, haplotypes, and diplotypes with occlusive artery disease and deep venous thrombosis found in our study is in agreement with most of the studies, especially on European populations (33-39). Caucasian patients are the most convenient for examining disease associations due to their greater genotype variability and larger number of patients with coronary artery disease. Our results suggest that neither *677CT* heterozygotes nor mutant homozygotes have an increased or decreased risk for coronary artery disease, compared with *677CC* genotype. Likewise, *1793GA* genotype did not demonstrate a significant association with coronary artery disease, compared with *1793GG* patients (40).

A meta-analysis of the risk of coronary heart disease related to the *677C-T* polymorphism showed that individuals with *677TT* genotype have a significantly higher risk of coronary heart disease, particularly if they have a low folate status. These results supported the hypothesis that impaired folate metabolism,

resulting in high homocysteine levels, is causally related to an increased risk of coronary heart disease (7-9). However, another meta-analysis, which included case-control and prospective studies, found no association of *MTHFR 677 C→T* polymorphism and coronary heart disease in Europe, North America, or Australia (10). In another study, eight candidate gene variants were analyzed in 32 431 individuals, comprising mainly Chinese, Japanese, and Korean individuals. Of eight candidate genes, the following three were associated with ischemic stroke: angiotensin I converting enzyme insertion/deletion polymorphism in the Chinese and Japanese; *C677T* variant of 5,10- *MTHFR* in the Chinese and Koreans; and apolipoprotein E gene in the Chinese and Japanese (11).

Cardiovascular system diseases are complex genetic traits, which include hundreds of associated candidate genes (41). Our study of two mutations of a single gene (*MTHFR-677* and *MTHFR-1298*) can function as a beginning of a complex investigation of candidate genes for cardiovascular diseases in Macedonians. There is a possibility that positive results might be spurious and negative results a consequence of low statistical power of our study. This could be due to the small sample size or methodological shortcomings, such as possible selection of an inappropriate control group. In order to have more precise conclusions for genetic background of cardiovascular diseases in Macedonians, it is necessary to investigate as many candidate genes as possible, in well-defined subgroups of phenotypes, and with a larger number of participants.

In summary, the association of *MTHFR-677*, and *MTHFR-1289* polymorphisms with occlusive artery disease and deep venous thrombosis in Macedonians was not found, except for the protective effect of *MTHFR/CA:CC* diplotype on artery occlusive disease. The results can be used for population meta-analysis, as well as for association studies with different diseases.

Acknowledgments

This research is part of the project "Blood Homocysteine Level and Prevalence of *C677T* Mutation of Enzyme Methylentetrahydropholate Reductase (*MTHFR*) as a Risk Factors for Blood Vessel Diseases", supported by the Ministry of Education and Science from Republic of Macedonia (No 13-1672/4-02). For sample collection, technical support, and laboratory direction, we thank Elena Zaharieva.

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