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Methylenetetrahydrofolate reductase (*MTHFR-677* and *MTHFR-1298*) genotypes and haplotypes and plasma homocysteine levels in patients with occlusive artery disease and deep venous thrombosis

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The aim was to investigate different genotypes and haplotypes of methylenetetrahydrofolate reductase (*MTHFR-677, -1298*) and plasma concentration of total homocysteine (tHcy) in Macedonian patients with occlusive artery disease (OAD) and deep venous thrombosis (DVT). Investigated groups consists of 80 healthy, 74 patients with OAD, and 63 patients with DVT. Plasma tHcy was measured with Microplate Enzyme Immunoassay. Identification of *MTHFR* genotypes and haplotypes was done with CVD StripAssay. The probability level (*P*-value) was evaluated by the Student's *t*-test. Plasma concentration of tHcy in *CC* and *CT* genotypes of *MTHFR C677T* was significantly increased in patients with OAD and in patients with DVT. Plasma concentration of tHcy in *AC* genotype of *MTHFR A1298C* was increased in patients with OAD and in patients with DVT. Plasma concentration of tHcy was significantly increased in *AA* genotype of patients with OAD, but not in patients with DVT. We found a significant increase of plasma tHcy in patients with OAD in comparison with healthy respondents for normal:heterozygote (*CC:AC*), heterozygote:normal (*CT:AA*), and heterozygote:heterozygote (*CT:AC*) haplotypes. Plasma concentration of tHcy in patients with DVT in comparison with healthy respondents was significantly increased for normal:normal (*CC:AA*), normal heterozygote (*CC:AC*), and heterozygote:heterozygote (*CT:AC*) haplotypes. We conclude that *MTHFR C677T* and *MTHFR A1289C* genotypes and haplotypes are connected with tHcy plasma levels in Macedonian patients with OAD and DVT.

Keywords: *MTHFR-677, MTHFR-1298*, plasma total homocysteine, occlusive artery disease, deep venous thrombosis, Macedonians

INTRODUCTION

Total homocysteine (tHcy) plasma level is an independent risk marker for venous thrombosis, myocardial infarction, stroke, congestive heart failure, osteoporotic fractures, and Alzheimer disease. tHcy levels are determined by the interaction of genetic and environmental factors. The *677C-T* polymor-

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Abbreviations: COMT, catechol-*O*-methyltransferase; CVD, cardiovascular disease; DVT, deep venous thrombosis; EIA, enzyme immunoassay; MTHFR, methylenetetrahydrofolate reductase; NNMT, nicotinamide *N*-methyltransferase; OAD, occlusive artery disese; PCR, polymerase chain reaction; tHcy, total homocysteine.

phism in the gene encoding 5,10-methylenetetrahydrofolate reductase (*MTHFR*; 607093.0003) has consistently been associated with plasma tHcy levels. Methylenetetrahydrofolate reductase (EC 1.5.1.20) catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate for homocysteine remethylation to methionine.

The human *MTHFR* gene (MIM *607093) has been localized to chromosome 1p36.3 (Goyette *et al.,* 1994) and is composed of 11 exons (Goyette *et al.,* 1998). *MTHFR* thermolabıle polymorphısms [*MTHFR, C677T, ALA222VAL* and *MTHFR, A1298C, 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. Individuals homozygous for the mutation had significantly elevated plasma tHcy levels. There have been indications that even a slight excess of homocysteine in blood can result in increased risk of cardiovascular diseases (Refsum & Ueland, 1998), therefore a successful method of plasma homocysteine measurement must be characterised by high accuracy and precision. An improved chromatographic method of total plasma homocysteine measurements was developed (Sawuła *et al.,* 2008) in order to obtain higher sensitivity, reliability and reproducibility. But according to others, there is no rationale for measuring the *MTHFR* C677T variant for clinical purposes (Bezemer *et al.,* 2007). There are a lot of published papers connecting the *MTHFR* mutations, mostly *MTHFR C677T,* with plasma tHcy levels. Several meta-analyses showed positive association with vascular diseases (Klerk *et al.,* 2002; Cronin *et al.,* 2005), however, in other meta-analyses associations were not found (Lewis *et al.,* 2005; Ariyaratnam *et al.,* 2007; Keijzer *et al.,* 2007).

Genomewide linkage scan for genes affecting plasma Hcy levels have shown the strongest linkage signal on 11q23 in the vicinity of the *NNMT* (nicotinamide *N*-methyltransferase) gene, which is involved in the metabolism of homocysteine. Haplotype analyses of ten SNPs within this gene identified one haplotype associated with plasma Hcy levels ($p = 0.0003$). It was concluded that the *NNMT* gene may be a major genetic determinant of plasma homocysteine levels in Spanish families and that since this gene encodes an enzyme involved in homocysteine synthesis, the finding would be consistent with known biochemical pathways (Souto *et al.,* 2005).

We published a distribution of the total homocysteine values in female Macedonian population and found normal distribution with mean value (±S.D.) of 7.4±2.8 μmol/L (Krstevska *et al.,* 2001). Plasma tHcy concentrations were not significantly higher in postmenopausal than in premenopausal women (Krstevska, 2001). We analyzed association of methylenetetrahydrofolate reductase (*MTHFR-677, -1298*) polymorphisms with occlusive artery disease and deep venous thrombosis in Macedonians, and concluded that significant association of *MTHFR-677*, and *-1289* polymorphisms with occlusive artery disease or venous thrombosis in Macedonians was not found, except for the protective association between the *MTHFR/CA:CC* diplotype and occlusive artery disease (Spiroski *et al.,* 2008). The aim of this study was to investigate total homocysteine plasma concentration in different genotypes and haplotypes of methylenetetrahydrofolate reductase (*MTHFR-677, -1298*) in Macedonian patients with occlusive artery disease (OAD) and deep venous thrombosis (DVT).

MATERIALS AND METHODS

Investigated groups. The total studied sample consists of 217 individuals composed of three different groups: healthy individuals, patients with occlusive artery disease, and patients with deep venous thrombosis. *a) Healthy individuals* (n=80), 40 female and 40 male, aged 40.7±11.3 years, born in different parts of Macedonia attending the Institute for Transfusion for blood donation. Inclusion of healthy individuals was random, if medical doctor declared their health as acceptable (on the basis of medical documentation, completed interview, and physical examination). From the investigation were excluded individuals with family history of vascular diseases. *b) Occlusive artery disease* (n=74), 28 female and 46 male patients with proved and documented myocardial infarct (n=2), brain infarct (n=20), or peripheral artery thrombosis (n=2), aged 63.3±9.6 years hospitalized at the Institute of Heart Diseases, Faculty of Medicine, and Institute for Transfusion, Skopje for outpatient treatment. *c) Deep venous thrombosis* (n=63), 43 female and 20 male patients (diagnosed by ultrasonography and/or venography), aged 57.7±11.8 years, attending the Institute of Heart Diseases, Faculty of Medicine, and Institute for Transfusion, Skopje for outpatient treatment.

All individuals are 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 Committee of the Ministry of Education and Science from the Republic of Macedonia (No. 13-1672/4-02).

Genomic DNA isolation and storage. Blood samples were collected after written consent; DNA was isolated from peripheral blood leukocytes by the phenol-chlorophorm extraction method or with BioRobot EZ1 workstation (QIAGEN) (Towner,

1995). The quality and quantity of DNA was analyzed by GeneQuant (Pharmacia). Isolated DNA samples were stored in Macedonian Human DNA Bank (hDNAMKD) (Spiroski *et al.,* 2005).

Typing methods. Assay for the identification of *MTHFR* mutations is based on polymerase chain reaction (PCR) and reverse-hybridization with CVD StripAssay (ViennaLab Labordiagnostica GmbH, Austria). The procedure includes three steps: 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 are detected using streptavidin-alkaline phosphatase and colour substrates. The assay covers two mutations: *MTHFR C677T* and *MTHFR A1298C*. The genotype of a sample is determined using the enclosed Collector™ sheet or using the software StripAssay Evaluator, ver. 2.0, ViennaLab Diagnostics GmbH.

Total plasma homocysteine determination. Blood samples were taken after 12 h fasting from the antecubital vein. After about 45 min the samples were centrifuged at 3000×*g* for 10 min. Plasma fractions were aspirated and transferred to plastic tubes and were stored at –20°C until analyzed. Total plasma homocysteine was measured with a Microplate Enzyme Immunoassay, EIA, method (Bio Rad Laboratories, USA) (Frantzen *et al.,* 1998).

Statistical methods. The population genetics analysis package, PyPop, (Lancaster *et al.,* 2003; 2007; Single *et al.,* 2007) was used for analysis of the *MTHFR* data for this report. Plasma concentration of total homocysteine data were analyzed using standard statistical program Statgraphics Plus for Windows ver. 2.1 (Microsoft Corp., Redmond, WA, USA). The probability level (*P*-value) was evaluated by the Student's *t*-test. The results are presented as the arithmetic mean±standard deviation (S.D.). *P* values of 0.05 or less were considered significant.

RESULTS

Genotypes of *MTHFR* and total homocysteine

Plasma concentration of total homocysteine (mean value±standard deviation, in μmol/L) in different genotypes of *MTHFR C677T* in healthy respondents, patients with occlusive artery disease (OAD), and patients with deep venous thrombosis (DVT) is given in Fig. 1.

We can see that the lowest plasma concentration of total homocysteine was found in healthy respondents with *CC* genotype of *MTHFR C677T* (10.78±2.59 μmol/L), with small (insignificant) increase in *CT* genotype (11.44±2.81 μmol/L, $P=0.314$), and significant increase in *TT* genotype of *MTHFR C677T* (14.83±5.49 μmol/L, $P=0.001$). Plasma concentration of total homocysteine in *CC* genotype of *MTHFR C677T* was additionally increased in patients with occlusive artery disease (13.47±3.18 μmol/L, $P<0.001$), and in patients with deep venous thrombosis (14.36±3.42, $P<0.001$). Plasma concentration of total homocysteine was significantly increased in *CT* genotype of patients with occlusive artery disease (16.13±6.41 μmol/L, $P<0.001$), and patients with deep venous thrombosis (13.24±3.24 μmol/L, $P<0.001$). Plasma concentration of total homocysteine in *TT* genotype of *MTHFR C677T* was not significantly different between healthy respondents and patients with occlusive artery disease, and patients with deep venous thrombosis. There was not significant difference in plasma concentration of total homocysteine between genotypes of *MTHFR C677T* in patients with occlusive artery disease and deep venous thrombosis.

Plasma concentration of total homocysteine in different genotypes of *MTHFR A1298C* in healthy respondents, patients with occlusive artery disease (OAD), and patients with deep venous thrombosis (DVT) is given in Table 1.

As shown in Table 1, the lowest plasma concentration of total homocysteine was found in healthy respondents with *AC* (heterozygote) genotype of *MTHFR A1298C* (10.79±2.65 μmol/L), with small (insignificant) increase in *CC* genotype (11.37±2.14 μmol/L, $P=0.714$), and significant increase in *AA* genotype (12.50±4.02 μmol/L, $P=0.030$). Plasma concentration of total homocysteine in *AC* genotype of *MTHFR A1298C* was

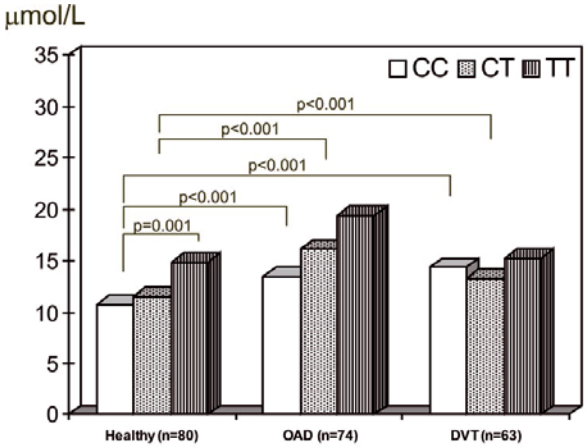

Figure 1. Different genotypes of *MTHFR C677T* and plasma concentration of total homocysteine (mmol/L) in healthy respondents, patients with occlusive artery disease (OAD), and patients with deep venous thromobosis (DVT).

Table 1. Different genotypes of *MTHFR A1298C* and plasma concentration of total homocysteine in healthy respondents, patients with occlusive artery disease, and patients with deep venous thromobosis

MTHFR A1298C genotype	Homocystein (μmol/L±standard deviation)			*P1**	*P2*
	Healthy (n=80)	OAD (n=74)	DVT (n=63)		
AA (wild)	12.50±4.02 (38)	15.68±8.52 (45)	13.75±3.36 (32)	0.038	0.167
AC (heterozygote)	10.79±2.65 (39)	15.86±5.86 (25)	14.45±3.08 (29)	0.0001	0.0001
CC (homozygote)	11.37±2.14 (3)	14.50±2.24 (4)	8.40±1.70 (2)	0.121	0.203
P (AA/AC)	0.030	0.925	0.401		
P (AA/CC)	0.635	0.785	0.034		
P (AC/CC)	0.714	0.654	0.011		

*Abbreviations: *P1*, significant difference between healthy respondents and patients with occlusive artery disease; *P2*, significant difference between healthy respondents and patients with deep venous thrombosis; *P*, significant difference between genotypes; OAD, occlusive artery disease; DVT, deep venous thromobosis.

additionally increased in patients with occlusive artery disease (15.86±5.86 μmol/L, $P<0.001$), and in patients with deep venous thrombosis (14.45±3.08, $P<0.001$). Plasma concentration of total homocysteine was significantly increased in *AA* genotype of patients with occlusive artery disease (15.68±8.52 μmol/L, $P=0.038$), but not in patients with deep venous thrombosis (13.75±3.36 μmol/L, $P=0.167$). Plasma concentration of total homocysteine in *CC* genotype of *MTHFR A1298C* was not significantly different between healthy respondents and patients with occlusive artery disease, and patients with deep venous thrombosis. There was not significant difference in plasma concentration of total homocysteine between genotypes of *MTHFR A1298C* in patients with occlusive artery disease. Patients with deep venous thrombosis with *CC* genotype of *MTHFR A1298C* had lowest plasma concentration of total homocysteine (8.40±1.70 μmol/L), while it was significantly increased in *AA* (13.75±3.36 μmol/L, $P=0.034$) and *AC* genotype (14.45± 3.08 μmol/L, $P=0.011$).

Haplotypes of *MTHFR* and total homocysteine

Plasma concentration of total homocysteine in different haplotypes of *MTHFR C677T:A1298C* in healthy respondents, patients with occlusive artery disease, and patients with deep venous thrombosis is given in Table 2.

We can see that plasma concentration of total homocysteine in different haplotypes of *MTHFR C677T:A1298C* is lowest in normal:normal (*CC:AA*) haplotype in healthy respondents (10.94±2.06 μmol/L) and in patients with occlusive artery disease (12.24±2.91 μmol/L), and is increased in the haplotypes with combinations of heterozygotes and homozygotes. Statistically significant increase was found only for the haplotype heterozygote:heterozygote (*CT:AC*) in patients with occlusive artery disease ($P=0.049$). The lowest plasma concentration of total homocysteine in patients with deep venous thromobosis was found in the normal:homozygote (*CC:CC*) haplotype (8.40±1.70, $P=0.021$), but the number of samples is too small to be accepted.

Table 2. Different haplotypes of *MTHFR C677T:A1298C* and plasma concentration of total homocysteine in healthy respondents, patients with occlusive artery disease, and patients with deep venous thromobosis.

MTHFR haplotype C677T:A1298C	Healthy (n=80)		Occlusive artery disease (n=74)		Deep venous thrombosis (n=63)		*P1*	*P2*
	Mean±S.D. (n)*	*P*	Mean±S.D. (n)	*P*	Mean±S.D. (n)	*P*		
Normal:Normal (*CC:AA*)	10.94±2.06 (7)		12.24±2.91 (9)		14.85±2.68 (6)		0.334	0.013
Normal:Heterozygote (*CC:AC*)	10.66±2.84 (24)	0.817	14.05±3.54 (12)	0.227	15.11±3.09 (12)	0.863	0.004	<0.001
Normal:Homozygote (*CC:CC*)	11.37±2.14 (3)	0.772	14.50±2.24 (4)	0.198	8.40±1.70 (2)	0.021	0.122	0.203
Heterozygote:Normal (*CT:AA*)	11.77±3.10 (20)	0.518	15.43±6.05 (26)	0.140	12.44±3.30 (16)	0.126	0.018	0.535
Heterozygote:Heterozygote (*CT:AC*)	10.99±2.39 (15)	0.962	17.53±7.13 (13)	0.049	13.99±3.08 (17)	0.507	0.002	0.005
Homozygote:Normal (*TT:AA*)	14.83±5.49 (11)	0.094	19.42 ±14.78 (10)	0.171	15.19±3.26 (10)	0.833	0.348	0.859

*Abbreviations: *P*, significant difference with Normal:Normal (*CC:AA*) haplotype; *P1*, significant difference between healthy respondents and patients with occlusive artery disease; *P2*, significant difference between healthy respondents and patients with deep venous thrombosis.

We found a significant increase of plasma total homocysteine in patients with occlusive artery disease in comparison with healthy respondents for normal:heterozygote (*CC:AC*) (*P1*=0.004), heterozygote:normal (*CT:AA*) (*P1*=0.018), and heterozygote: heterozygote (*CT:AC*) (*P1*=0.002) haplotypes. Plasma concentration of total homocysteine in patients with deep venous thromobosis in comparison with healthy respondents was significantly increased for normal:normal (*CC:AA*) (P2=0.013), normal heterozygote (*CC:AC*) (P2<0.001), and heterozygote:heterozygote (*CT:AC*) (P2=0.005) haplotypes (Table 2).

Examining the effect of the combination of both mutations (Table 2), the *677TT* genotype was always associated with the normal *1298AA* genotype in all individuals, and the *1298CC* genotype was always associated with the normal *677CC* genotype. Double mutant homozygous individuals were not observed.

DISCUSSION

In this manuscript we report plasma concentration of total homocysteine (tHcy) in different genotypes and haplotypes of methylenetetrahydrofolate reductase gene (*MTHFR-677, -1298*) in Macedonian patients with occlusive artery disease and deep venous thrombosis.

We found that plasma concentration of tHcy in *CC* and *CT* genotypes of *MTHFR C677T* was significantly increased in patients with OAD and in patients with DVT. Plasma concentration of tHcy in *AC* genotype of *MTHFR A1298C* was also increased in patients with OAD and DVT. Plasma concentration of tHcy was significantly increased in *AA* genotype of patients with OAD, but not in patients with DVT. Statistically significant increase was found only for the haplotype *CT:AC* in patients with OAD. We found a significant increase of plasma concentration of tHcy in patients with OAD for *CC:AC, CT:AA,* and *CT:AC* haplotypes, as well as in patients with DVT for *CC:AA, CC:AC*, and *CT:AC* haplotypes.

Our results in patients with occlusive artery disease are in agreement with those of others where the most important genetic determinant of tHcy in the general population is the common *C677T* variant of methylenetetrahydrofolate reductase gene (*MTHFR*) that results in higher tHcy. Individuals with the homozygous mutant (*TT*) genotype have a significantly higher (14–21%) risk of heart disease. Plasma tHcy is very responsive to intervention with the B vitamins required for its metabolism, in particular folic acid, and to a lesser extent vitamins B_{12} and B_6. Thus, although primarily aimed at reducing neural-tube defects (van der Linden *et al.,* 2006), folic acid fortification may have an important role in the primary prevention of CVD *via* tHcy lowering (Sazci *et al.,* 2006; Kothekar, 2007; Freitas *et al.,* 2008; Ghazouani *et al.,* 2008; McNulty *et al.,* 2008; Poduri *et al.,* 2008).

Increased tHcy levels in our patients with deep venous thromobosis are similar with several published results (den Heijer *et al.,* 1998; Wald *et al.,* 2002; Hotoleanu *et al.,* 2007). Meta-analysis of prospective and retrospective studies demonstrates a modest association of homocysteine with venous thrombosis (den Heijer *et al.,* 2005). In a single large study, *MTHFR* C677T was not associated with the risk of venous thrombosis, and the narrow confidence interval excludes even a small effect. Therefore, mildly elevated homocysteine levels as a result of *MTHFR* 677TT do not seem to cause venous thrombosis.

In several papers the polymorphisms *C677T* and *A1298C* of *MTHFR* and fasting plasma homocysteine levels do not seem to be significant risk factors for venous thromboembolic disease (Salamon *et al.,* 2001; Domagala *et al.,* 2002). At present, the status of homocysteine as a target for intervention in the prevention of atherothrombotic arterial and venous disease is uncertain. Current evidence does not support the use of B vitamin supplements to reduce vascular risk (den Heijer *et al.,* 2005).

Homocysteine level is not dependent on *MTHFR* gene mutations. There is a report that nicotinamide *N*-methyltransferase (*NNMT*) gene may be a major genetic determinant of plasma homocysteine levels and that since this gene encodes an enzyme involved in homocysteine synthesis, the finding would be consistent with known biochemical pathways (Souto *et al.,* 2005). Plasma homocysteine was negatively associated with plasma vitamin B_{12} concentration and plasma folate, with the degree of correlation between plasma vitamin B_{12} and homocysteine concentrations dependent on *MTHFR* genotype (Bailey *et al.,* 2002). The results showed that tHcy at baseline was significantly higher for the *677TT* genotype group compared to the *677CC* genotype group and that this group responded with a significantly larger increase in tHcy upon coffee exposure than the *677CC* and *677CT* genotype groups (Strandhagen *et al.,* 2004). Catechol-*O*-methyltransferase (*COMT) Val(158)* carriers had significantly higher tHcy than *Met(158)* homozygotes. The effect was limited to individuals homozygous for the *MTHFR T(677)* allele. In addition, individuals homozygous for the *COMT G(-287)* allele tended to have lower tHcy levels. High activity variants of *COMT* interact with the low activity variant of *MTHFR* to increase tHcy levels (Tunbridge *et al.,* 2008).

We reported results from *MTHFR-677*, and *-1289* polymorphisms on the same cohort (identical healthy participants and patients as in this study) in

which association with occlusive artery disease and deep venous thrombosis was not found, except for the protective *MTHFR/CA:CC* diplotype with artery occlusive disease (Spiroski *et al.*, 2008). The most frequent *MTHFR-677* genotype in healthy participants was *CT* with observed frequency of 44.6%, lower frequency was found for *CC* genotype (42.2%), and the lowest frequency was found for *TT* genotype, 13.2%. The frequencies of *MTHFR-677 CT* and *TT* genotypes were slightly increased in patients with occlusive artery disease and deep venous thrombosis, with a consecutive decrease of *CC* genotype. All genotypes in healthy participants and patients with blood vessel disease showed a good fit with Hardy-Weinberg equilibrium. The most frequent *MTHFR-1298* genotype in healthy participants was *AA* with observed frequency of 49.4%, lower frequency was found for *CA* genotype, and the lowest frequency was found for *CC* genotype, 3.6%. The frequency of *MTHFR-1298* genotypes *AA* and *CC* were slightly increased in patients with occlusive artery disease and deep venous thrombosis, with a consecutive decrease of *CA* genotype. All genotypes in healthy participants and patients with blood vessel disease showed a good fit with Hardy-Weinberg equilibrium (Spiroski *et al.*, 2008).

The significant differences of plasma total homocysteine levels in healthy participants and patients with OAD and DVT with different genotypes and haplotypes of *MTHFR-677* and *-1289*, in spite of a lack of association with *MTHFR-677* and *-1289* polymorphisms, can be explained with additional genetic and/or external interactions. DNA samples from 6793 participants in the third National Health and Nutrition Examination Survey (NHANES III) during 1991–1994 were genotyped for polymorphisms of genes coding for folate pathway enzymes 5,10-methylenetetrahydrofolate reductase (MTHFR) 677C3T and 1298A3C, methionine synthase reductase (MTRR) 66A3G, and cystathionine-β-synthase 844ins68 (Yang *et al.*, 2008). The results suggest that dietary intake of folic acid can attenuate significantly the negative impact on serum folate and homocysteine of selected polymorphisms of folate-related genes, which could be one of the explanations for our data.

We noticed that the *677TT* genotype was always associated with the normal *1298AA* genotype in all individuals, and the *1298CC* genotype was always associated with the normal *677CC* genotype. Double mutant homozygous individuals were not observed in this study. Similar data were reported by others (Freitas *et al.*, 2008; Spiroski *et al.*, 2008). In the meta-analysis with reliable data on combined *MTHFR* genotypes in general populations (n=5389) the combined data comprised the following totals for each genotype at nucleotide positions *677* and *1298: 838 CC/AA* (i.e., *677CC/1298AA*), 1225 *CC/AC*, 489 *CC/CC*, 1120 *CT/AA*, 1093 *CT/AC*, 8 *CT/CC*, 606 *TT/AA*, 10 *TT/AC*, and 0 *TT/CC*. The estimated haplotype frequencies, and the fractional contribution of each, were *677C/1298A*, 0.37; *677C/1298C*, 0.31; *677T/1298A*, 0.32; and *677T/1298C*, 0.0023 to 0.0034. Thus, a vast majority of *677T* alleles and *1298C* alleles are associated with *1298A* alleles and *677C* alleles, respectively (Ogino & Wilson, 2003). The absence of *MTHFR* diplotypes in Macedonians could be as a result of selective pressures or of the small frequencies in the investigated groups.

In summary, *MTHFR C677T* and *MTHFR A1289C* genotypes and haplotypes are connected with tHcy plasma levels in Macedonian patients with occlusive artery disease and deep venous thrombosis. The results can be part of the complex interaction between candidate genes and external factors responsible for cardiovascular diseases in Macedonians.

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