# Associations of Serum Total Homocysteine Levels with Various Demographic, Clinical and Genetic Characteristics in Healthy Greek Adults

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## ABSTRACT

Aim: The aim of this study was to investigate the association of serum total Hcy (tHcy) levels with various demographic, clinical and genetic characteristics in healthy Greek adults.

Methods: Anthropometric characteristics (height, weight), systolic and diastolic blood pressure, complete blood count and biochemical assessments, were recorded and measured among 383 Greek adults (199 men). Serum folate, Cobalamin (Cbl) and tHcy levels were determined using immunoassays methods. The MTHFR C677T and A1298C gene polymorphisms were genotyped using polymerase chain reaction and reverse hybridization.

Results: MTHFR C677T gene polymorphism, serum folate and Cbl levels were correlated with serum tHcy levels independently. The individuals with 677TT genotype had significantly higher serum tHcy levels than individuals with 677 CC or CT genotypes. Regarding the MTHFR C677T gene polymorphism, the existence of the T allele was associated with statistically significantly lower serum folate and higher serum tHcy levels than C allele. Regarding the MTHFR A1298C gene polymorphism, the existence of the C allele was associated with statistically significant lower serum tHcy levels than A allele. Furthermore, there was no significant correlation between the serum tHcy levels and demographic (except age) or clinical characteristics (sex, BMI, smoking status, SBP, DBP, HGB, HCT, TC, TG, HDL-C, LDL-C, TC/HDL-C).

Conclusions: Serum tHcy levels are influenced by the existence of MTHFR C677T gene polymorphism (mainly 677TT genotype), serum folate and Cbl levels. Individuals with hyperhomocysteinemia should be further investigated for the existence of MTHFR C677T gene polymorphism, with the aim to determine the suitable treatment.



## KEYWORDS

cobalamin; folate; homocysteine; MTHFR C677T; MTHFR A1298C



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## INTRODUCTION

Homocysteine (Hcy) is a sulfur-containing non-essential amino acid produced as an intermediary of methionine metabolism. It is either metabolized to cysteine by the transsulfuration pathway with *cystathionine β-synthase* (CBS) as the main enzyme with the contribution of vitamin B6, or converted back to methionine by the remethylation pathway with the action of the enzymes methionine synthase (MS) and $N_5$,$N_{10}$-methylene tetrahydrofolate reductase (MTHFR) and the contribution of $N_5$-methyltetrahydrofolate (5-MTHF) and cobalamin (Cbl, vitamin B12) (1–6) (Fig. 1). The function of the MTHFR enzyme is of great importance for the regulation of available 5-MTHF which is the predominant and biologically active form of circulating folate (1, 7). Genetic defects in the enzymes or dietary deficiency of B-vitamin cofactors and their substrates could be responsible for elevations in the serum total Hcy (tHcy) levels (3-6). Between the two well-known polymorphisms of the MTHFR gene, the C677T (substitution of C to T at the residue 677) and the A1298C (transversion of A to C at nucleotide position 1298), which affect the MTHFR enzymatic activity, only the first appears to be associated with increased serum tHcy levels (5). The difference in the functional properties of these variants is due to their respective positions in the protein, such as the C677T being located in the catalytic domain whereas the A1298C is located in the regulatory domain. Individuals with 677TT or 677CT genotype have, respectively, approximately 30% and 65% the MTHFR enzyme activity of those with the 677CC genotype (5).

It is known that a prolonged exposure to elevated serum tHcy levels, referred to as hyperhomocysteinemia (HHcy) (typically defined as serum tHcy levels ≥15 μmol/L)(4), is associated with a wide range of health problems and conditions including cardiovascular disease, deep vein thrombosis or pulmonary embolism, neurocognitive disorders, pregnancy complications (preeclampsia, placental abruption, pregnancy loss), birth defects, osteoporotic fractures, etc. (4–6, 8–11). The aim of this study was to investigate the associations of serum tHcy levels with various demographic, genetic and clinical characteristics in healthy Greek adults.

## SUBJECTS AND METHODS

Our study population included 383 healthy Greek individuals, residents of Chania, Crete, who had visited the Outpatient Clinic of Internal Medicine of the Naval Hospital of Crete or a private medical office of Internal Medicine between January 2016 and December 2018 in the framework of their periodic medical examination (military personnel) or check-up (non-military personnel). The subjects met the following five criteria: (1) age ≥18 years old; (2) normal renal and thyroid function; (3) absence of a

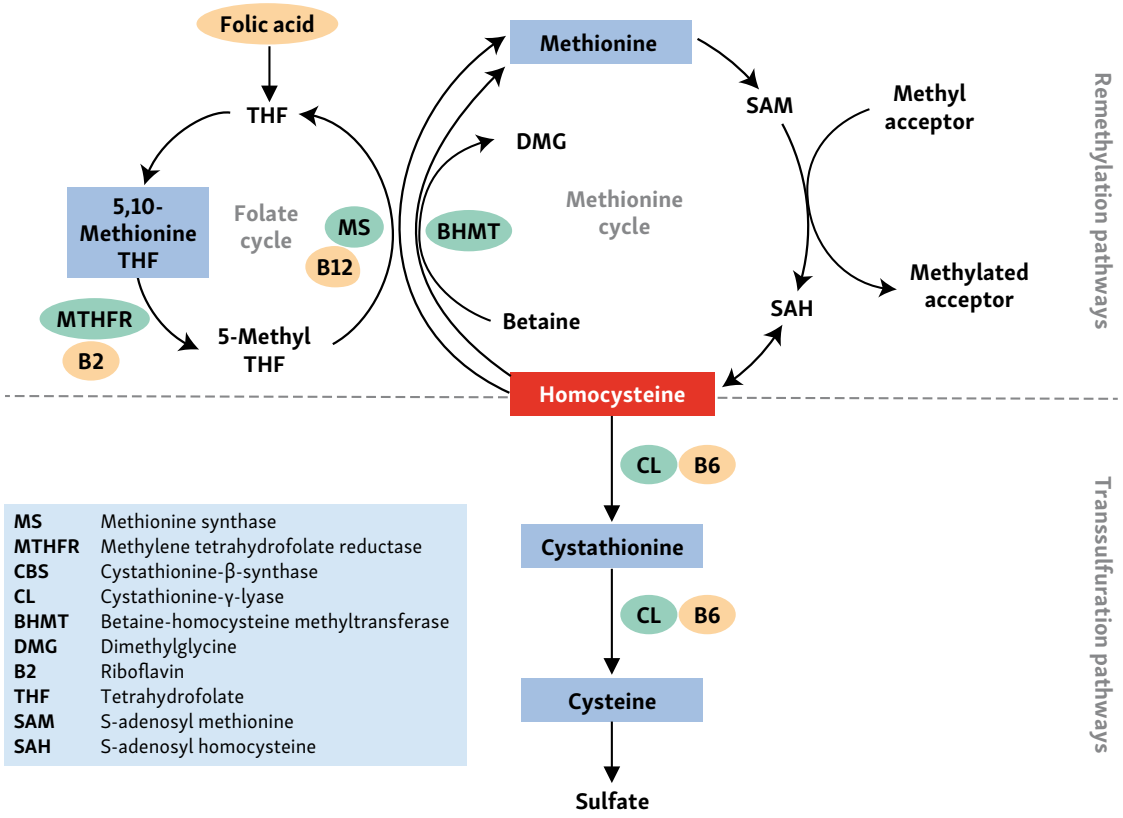


**Fig. 1** Pathways of homocysteine (Hcy) metabolism (2). Hcy is metabolized by one of two divergent pathways: transsulfuration; and remethylation. The transsulfuration of Hcy to cysteine is catalysed by cystathionine-b-synthase (CBS), a process that requires pyridoxal phosphate (vitamin B6) as a cofactor. Remethylation of Hcy produces methionine. This reaction is catalysed either by methionine synthase or by betaine-homocysteine methyltranferase. Vitamin B12 (Cbl) is the precursor of methylcobalamin, which is the cofactor for methionine synthase.

known gastrointestinal disorder (pernicious anemia, gastrectomy/bariatric surgery, inflammatory bowel disease, gastritis, autoimmune metaplastic atrophic gastritis, malabsorption syndrome, Helicobacter pylori infection), diabetes mellitus, coronary heart disease, stroke, malignancy or alcoholism; (4) no consumption of vitamin supplements or drugs affecting folate or Cbl metabolism (i.e. methotrexate, sulphasalazine, antiepileptic drugs, statins, metformin, proton-pump inhibitors) during the last semester, and regarding females (5) no pregnancy or lactation. The subjects were interviewed using a structured form, which included sociodemographic data, lifestyle and dietary habits, such as medical history. Anthropometric parameters of all subjects consisting of height, body weight (BW) and body mass index (BMI), as well as arterial systolic and diastolic blood pressure (SBP, DBP), had been measured and recorded. Records were made of sitting SBP and DBP (two measurements averaged) with a mercury-free sphygmomanometer (*A&D* Medical, model UM-102) by the auscultatory method, standing body height (measured without shoes to the nearest 0.5 cm) with a rigid height meter and BW (without shoes and tunic) with a calibrated balance scale (Fazzini, model S7350HR). BMI was calculated as the BW (kg) divided by the height (m) squared (kg/m$^2$) (12). Blood samples were collected after overnight fasting. A complete blood count and various biochemical parameters including renal and liver function tests, serum glucose, total cholesterol (T-C), high density lipoprotein-cholesterol (HDL-C), triglycerides (TG), folate, Cbl and tHcy (the sum of free and protein-bound homocysteine, homocysteine and homocysteine-cysteine mixed disulfide) were determined by the following standard laboratory procedures. Low density lipoprotein-cholesterol (LDL-C) and atherogenic index (AI; TC/HDL-C ratio) were calculated using the Friedewald (13) and Lauer (14) equations, respectively. Serum folate and Cbl levels were measured with a chemiluminescent microparticle immunoassay method (CMIA) (Architect i1000 System®, Abbott Laboratories, USA). Serum tHcy levels was measured with a fluorescence polarization immunoassay (FPIA) using the commercial kit in the AXSYM® System (Abbott Laboratories, USA). Individuals with serum tHcy levels ≥15 μmol/L were considered to have HHcy.

Total genomic DNA was extracted using standard phenol-chloroform procedures. Screening of the MTHFR C677T and A1298C gene polymorphisms was performed by polymerase chain reaction (PCR) and reverse hybridization (CVD StripAssay® Testing Strip, Vienna Lab, CE IVD kit). Subjects with one copy of the mutant allele on the MTHFR gene were called "heterozygous", but subjects with two copies of the same mutant allele were called "homozygous". Thus, our subjects for C677T and/or A1298C polymorphisms in the MTHFR gene were divided to three genotypes (normal or wild-type (677CC; 1298AA), heterozygous (677CT; 1298AC) and homozygous (677TT; 1298CC).

## STATISTICAL ANALYSIS

Statistical analyses were performed using SPSS software package (version 20, Inc., USA). Data are presented as number (%) for categorical variables and mean ± standard deviation (SD) for continuous variables. Categorical data were analyzed with Fisher's exact test. Continuous variables were compared using Student's t-test for normally distributed variables. One-way analysis of variance (ANOVA) with Bonferroni post hoc test for the significant differences was performed to examine the influence of serum folate levels, serum Cbl levels and the MTHFR C677T and A1298C genotypes on the serum tHcy levels. Correlation between serum Hcy levels and demographic or clinical characteristics was assessed by Pearson's correlation (r). A multivariate linear logistic regression model was developed in order to evaluate the independent effect of the above parameters on serum tHcy levels. All tests were two-tailed and p-values <0.05 were considered to be significant.

**Tab. 1** Demographic, clinical and genetic characteristics of the study population by sex. Values are expressed as mean ± SD and/or No (%).

| Characteristic | Men (n = 199) | Women (n = 184) | p |
|---|---|---|---|
| Age, years | 41.9 ± 14.8 | 43.1 ± 14.6 | 0.448 |
| *Smoking status* | | | |
| Yes | 47 (23.7%) | 42 (22.8%) | 0.904 |
| *Anthropometric values* | | | |
| BW (kg) | 85.0 ± 12.9 | 72.4 ± 12.6 | <0.001 |
| Height (cm) | 177.0 ± 6.0 | 168.0 ± 6.0 | <0.001 |
| BMI (kg/m$^2$) | 27.3 ± 3.9 | 25.5 ± 4.1 | <0.001 |
| *Blood pressure* | | | |
| SBP (mmHg) | 121.1 ± 7.1 | 120.7 ± 7.5 | 0.634 |
| DBP (mmHg) | 69.7 ± 10.3 | 70.0 ± 8.5 | 0.819 |
| *Fasting plasma values* | | | |
| HCT | 43.9 ± 2.8 | 40.6 ± 3.4 | <0.001 |
| HGB | 14.7 ± 1.1 | 13.4 ± 1.3 | <0.001 |
| TC (mg/dl) | 195.5 ± 39.3 | 193.9 ± 34.5 | 0.683 |
| TG (mg/dl) | 124.7 ± 75.2 | 102.8 ± 47.0 | 0.001 |
| HDL-C (mg/dl) | 48.4 ± 11.9 | 55.0 ± 15.3 | <0.001 |
| LDL-C (mg/dl) | 122.2 ± 33.9 | 118.1 ± 30.9 | 0.215 |
| AI | 4.2 ± 1.3 | 3.8 ± 1.2 | <0.001 |
| Folate (ng/mL) | 3.15 ± 1.1 | 3.21 ± 1.1 | 0.560 |
| Cbl (pg/mL) | 275.4 ± 102.8 | 292.0 ± 114.3 | 0.133 |
| tHcy (μmol/L) | 15.07 ± 9.1 | 13.6 ± 6.9 | 0.087 |
| *MTHFR C667T genotypes* | | | |
| Normal (CC) | 41 (20.6%) | 32 (17.4%) | 0.645 |
| Heterozygous (CT) | 105 (52.8%) | 97 (52.7%) | |
| Homozygous (TT) | 53 (26.6%) | 55 (29.9%) | |
| *MTHFR A1298C genotypes* | | | |
| Normal (AA) | 138 (69.3%) | 125 (67.9%) | 0.936 |
| Heterozygous (AC) | 53 (26.6%) | 52 (28.3%) | |
| Homozygous (CC) | 8 (4.0%) | 7 (3.8%) | |

BW: body weight; BMI: body mass index; HCT: hematocrit; HGB: hemoglobin; TC: total cholesterol; TG: triglycerides; HDL-C: high density lipoprotein-cholesterol; LDL-C: low density lipoprotein-cholesterol; AI: atherogenic index (TC/HDL-C); Cbl: cobalamin; tHcy: total homocysteine; SBP: systolic blood pressure; DBP: diastolic blood pressure; MTHFR: $N_5$,$N_{10}$-methylene tetrahydrofolate reductase

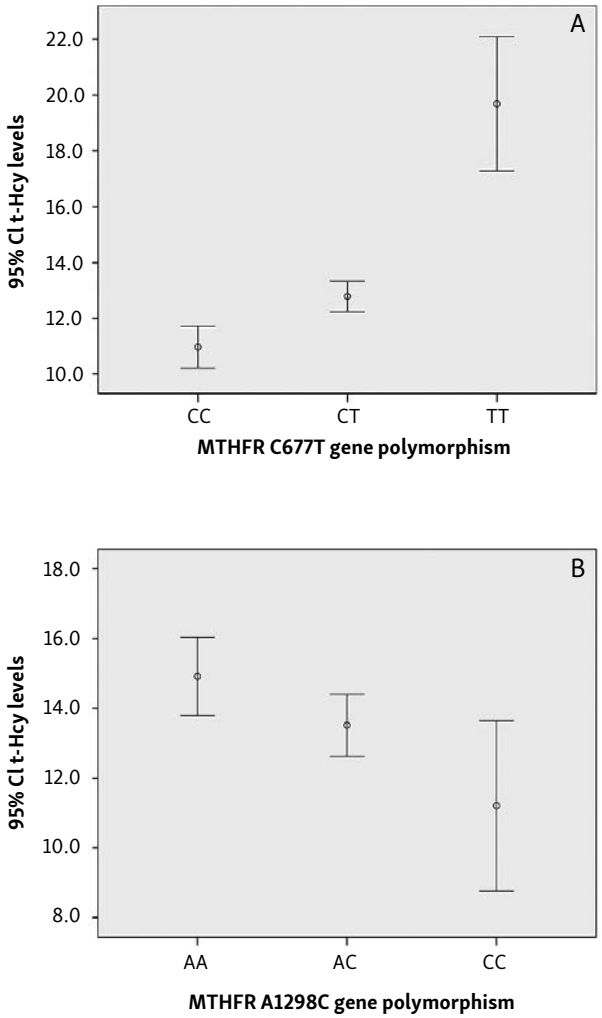


**Fig. 2** Serum tHcy levels according to MTHFR C677T (A) and A1298C (B) gene polymorphisms.

## RESULTS

The demographic, clinical and genetic characteristics of 383 individuals (199 men, 52%) with mean age ± SD: 37.3 ± 8.2 years who participated in our study, divided in 2 groups by sex, are presented in table 1. The serum tHcy levels according to MTHFR C677T and A1298C gene polymorphisms are showed in Fig. 2. The overall C and T allele frequency for the MTHFR C677T gene polymorphism was 45.4% and 54.6%, respectively, and the overall A and C allele frequency for the MTHFR A1298C gene polymorphism was 82.3% and 17.6%, respectively.

Serum tHcy levels had a statistically significant reverse correlation with serum folate and serum Cbl levels, as well as a positive correlation with age ($p < 0.001$, $p < 0.001$ and $p = 0.039$, respectively) (Table 2). Additionally, both MTHFR C677T and A1298C gene polymorphisms had a statistically significant impact on serum tHcy levels. Multivariate linear regression analysis (Table 3) revealed that among them only MTHFR C677T gene polymorphism, serum folate and serum Cbl levels, and age were correlated with tHcy levels independently. As showed in table 4, Bonferroni post hoc test revealed that serum tHcy levels were statistically significantly higher among the subjects with the MTHFR 677TT genotype than the subjects with MTHFR 677CC or 677CT genotypes. Additionally, serum tHcy levels

**Tab. 2** Correlation between the serum tHcy levels and other demographic and clinical characteristics.

| | $r_s$ | p |
|---|---|---|
| Age | 0.105 | 0.039 |
| Sex | 0.088 | 0.087 |
| Smoking status | 0.010 | 0.852 |
| SBP | -0.007 | 0.899 |
| DBP | 0.008 | 0.883 |
| BMI | 0.057 | 0.263 |
| Folate levels | -0.249 | <0.001 |
| Cbl levels | -0.198 | <0.001 |
| TC | 0.043 | 0.399 |
| TG | 0.005 | 0.923 |
| HDL-C | -0.035 | 0.497 |
| LDL-C | 0.069 | 0.178 |
| AI | 0.059 | 0.251 |
| Ht | 0.056 | 0.277 |
| Hb | 0.074 | 0.151 |
| MTHFR C677T gene polymorphisms | 0.388 | <0.001 |
| MTHFR A1298C gene polymorphisms | -0.109 | 0.039 |

$r_s$: Spearman's correlation; SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure; other abbreviations: see table 1

**Tab. 3** Multivariate linear regression analysis.

| Model | | Coefficients[a] | | | t | Sig. |
|---|---|---|---|---|---|---|
| | | Unstandardized | | Standardized | | |
| | | B | Std. Error | Beta | | |
| 1 | (Constant) | 12.156 | 2.300 | | 5.285 | <0.001 |
| | MTHFR C677T gene polymorphisms | 2.314 | 0.297 | 0.389 | 7.784 | <0.001 |
| | Folate | -1.381 | 0.333 | -0.189 | -4.142 | <0.001 |
| | Cbl | -0.013 | 0.003 | -0.173 | -3.809 | <0.001 |
| | Age | 0.052 | 0.025 | 0.094 | 2.072 | 0.039 |
| | MTHFR A1298C gene polymorphisms | 0.421 | 0.363 | 0.058 | 1.160 | 0.247 |

a. Dependent Variable: t-Hcy. For abbreviations: see table 1.

**Tab. 4** Serum tHcy levels distributed by MTHFR genotypes and by serum tHcy levels groups ≥15 or <15). Values are expressed as mean ± SD or No (%).

| MTHFR C677T genotypes | tHcy | tHcy | | p-value |
|---|---|---|---|---|
| | | <15 (n = 258) | ≥15 (n = 125) | |
| CC (n = 73) | 10.96 ± 3.21a | 67 (26.0%) | 6 (4.8%) | <0.001* |
| CT (n = 202) | 12.79 ± 3.91b | 152 (58.9%) | 50 (40.0%) | |
| TT (n = 108) | 19.68 ± 12.59c | 39 (15.1%) | 69 (55.2%) | |
| *Bonferroni post hoc test* | | | | |
| CC vs TT | p < 0.001 | | | |
| CC vs CT | p < 0.001 | | | |
| CT vs TT | p < 0.001 | | | |
| **MTHFR A1298C genotypes** | **tHcy** | **tHcy** | | **p-value** |
| | | <15 (n = 258) | ≥15 (n = 125) | |
| AA (n = 263) | 14.92 ± 9.25 | 177 (68.6%) | 86 (68.8%) | 0.074* |
| AC (n = 105) | 13.5 ± 4.6 | 67 (26.0%) | 38 (30.4%) | |
| CC (n = 15) | 11.2 ± 4.4 | 14 (5.4%) | 1 (0.8%) | |

One-way analysis of variance (ANOVA) with Bonferroni post hoc test test; P < 0.001 by column (a *vs* b *vs* c). * Fisher exact test. For abbreviations: see table 1.

were statistically higher among the subjects with MTHFR 677CT genotype than those with 677CC genotype (Table 4). Regarding the MTHFR C677T gene polymorphism, the existence of the T allele was associated with statistically significant lower serum folate and higher serum tHcy levels than C allele.

Interestingly, even though MTHFR A1298C gene polymorphism had no significant impact on serum tHcy levels (p = 0.09), only one subject out of 15 (6.7%) with MTHFR 1298 CC genotype had serum tHcy levels ≥15 μmol/L. Furthermore, regarding the MTHFR A1298C gene polymorphism, the existence of the C allele was associated with statistically significant lower serum tHcy levels than A allele (p = 0.02).

As showed in table 2 there was no significant correlation between the serum tHcy levels and other demographic or clinical characteristics (sex, BMI, smoking status, SBP, DBP, HGB, HCT, TC, TG, HDL-C, LDL-C, AI).

## DISCUSSION

In the present study of 383 Greek healthy adults, only the MTHFR C677T polymorphism, serum folate and serum Cbl levels were significantly associated independently with serum tHcy levels. Among these three independent factors, MTHFR C677T polymorphism had the greatest influence on those. To our knowledge, this is the first study which was conducted to investigate independent factors affecting the serum tHcy levels in healthy Greek adults.

In our study, the individuals with 677TT genotype had significantly higher serum tHcy levels than individuals with the 677CC or 677CT genotypes, as other studies have also revealed (15–17). This finding could be attributed to thermolability induced in the enzyme MTHFR which results in its lower activity (approximately 70% for 677TT genotype) and therefore inability to efficiently convert 5,10-methylene-THF to 5-MTHF, a conversion necessary for the remethylation of Hcy to methionine (4–6, 16). The significant contribution of the T allele on elevated serum tHcy levels and the statistically significant lower frequency of MTHFR 677CC genotype among individuals with serum tHcy levels ≥15 μmol/L than individuals with serum tHcy levels <15 μmol/L can justify the further investigation of HHcy individuals for a possible existence of MTHFR C677T polymorphism. The significant negative correlation between the serum levels of tHcy and folate or Cbl in our study population was expected considering the participation of folate and Cbl as cofactors in Hcy metabolism (Fig. 1), and justifies on the one hand why two-thirds of cases of elevated serum tHcy levels are due to low serum folate and/or Cbl levels, and on the other hand the administration of folate and Cbl supplements among patients with elevated serum tHcy levels (18). The weak contribution of serum Cbl levels on serum tHcy levels is probably due to the fact that in our study population the majority of individuals (51.7%) had serum Cbl levels between 200 and 300 pg/mL (indicative levels of possible Cbl deficiency) and only 17% had serum Cbl levels <200 pg/mL (indicative levels of Cbl deficiency) (2).

The results of our research did not reveal a significant influence of MTHFR A1298C gene polymorphism on serum tHcy levels. This finding could be explained by the fact that this mutation, despite affecting MTHFR activity, does not result in the synthesis of a thermolabile protein (5, 19, 20). Thus, the investigation of MTHFR A1298C gene polymorphism among individuals with elevated serum tHcy levels is rather unnecessary.

Clinically, the *MTHFR C677T* genotyping mainly among the individuals with HHcy is not only important, it is critical for determining the appropriate folate supplement (folic acid or folinic acid or 5-MTHF) in order to successfully decrease or normalize the elevated serum tHcy concentrations without increasing the levels of unmetabolized folic

acid (UMFA) in the peripheral circulation (18, 21, 22). It is recommended that patients with MTHFR 677TT genotype who have HHcy be treated with 5-MTHF (400-800 μcg daily) and not with folic or folinic acid (22). Moreover, it is well-known that high levels of UMFA may promote the growth of existing cancers and have been associated with decreased natural killer cell activity and increased cancer risk (23–25). Except the monitoring of serum folate levels, the monitoring of serum Cbl levels among HHcy individuals is also necessary considering that low Cbl levels increase the serum tHcy levels because of the impaired activity of Cbl-dependent enzyme MS which regenerates methionine from Hcy (3). Also, a Cbl deficiency can lead to a specific reaction, called methylfolate trap. This trap results from the fact that 5-MTHF can neither be metabolized via the MS pathway, nor reconverted to its precursor 5,10-methylene-THF (Fig. 1), which leads to the 5-MTHF becoming metabolically trapped and unable to be employed anymore (26).

In contrast to other studies (4, 16, 27–33), the serum tHcy levels in our population were not associated with sex, BMI, BP, smoking status, and lipids. A possible explanation for these controversial results may be the differences in studies populations, sample size and the concomitant drugs or diseases of participants. The significant effect of age on serum tHcy concentration in our population is in accordance with other studies (32, 33).

The present study has some limitations. First of all, the sample size was relatively small. Second, other genetic or environmental (e.g. vitamin B6 deficiency) factors involving in the development of HHcy (6) were not evaluated.

## CONCLUSIONS

Serum tHcy levels are influenced by the existence of MTHFR C677T gene polymorphism (mainly 677TT genotype), serum folate and serum Cbl levels. We suggest that the individuals with HHcy should be further investigated for the existence of MTHFR C677T gene polymorphism, with aim to determine the suitable treatment.

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## DISCLOSURE STATEMENT

This study was conducted independently; no company or institution supported it financially.The authors declare that they have no conflicts of interest.

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