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Rate of T alleles and TT genotype at MTHFR 677C->T locus or C alleles and CC genotype at MTHFR 1298A->C locus among healthy subjects in Turkey: Impact on homocysteine and folic acid status and reference intervals

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Methylenetetrahydrofolate reductase (MTHFR) is important for folate and homocysteine (Hcy) metabolism. MTHFR 677C->T and 1298A->C MTHFR are two most common mutations which can affect folate and total homocysteine (tHcy) status. This study was designed to determine the rate of MTHFR 677C->T and 1298A->C mutations, and their influence on serum folate, Hcy and vitamin B12 status and the reference intervals in 402 healthy Turkish adults. The rate of MTHFR 677C->T or 1298A->C mutations was 50.7% or 54.7%, respectively. The MTHFR 677C->T mutation-specific reference intervals for serum folate and tHcy were characterized by marked shifts in their upper limits. In homozygote subjects for MTHFR 677C->T serum folate concentration was lower and serum tHcy concentration was higher than those in the wild genotype; all subjects had lower serum folate and 54% of the subjects had higher tHcy concentrations than the cutoff values of $\leq$10 nmol/L and $\geq$12 μmol/L, respectively. Serum vitamin B12 status was similar in all genotypes. Serum tHcy concentrations were inversely correlated with serum folate and vitamin B12 concentrations in all genotypes. These data show that the rate of MTHFR 677C->T and 1298A->C mutations is very high in Turks and serum folate and tHcy status are impaired by these mutations.

KEY WORDS — MTHFR; mutations; homocysteine; folate; vitamin B12; reference intervals; Turkey

INTRODUCTION

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in folate and homocysteine metabolism.[1–3] MTHFR (EC.1.5.1.20) catalyzes the biologically irreversible reduction of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which provides the methyl group for the remethylation of homocysteine to methionine.[1–3] Methionine is in turn converted to S-adenosylmethionine, the common methyl donor for the methylation processes of DNA, proteins, phospholipids and neurotransmitters.[1–3]

The human MTHFR gene has been mapped to the chromosomal region 1p36.3 and comprises 11 exons, encoding a protein of 656 amino acids.[4,5] Several single nucleotide polymorphisms in the MTHFR gene have been identified including the two most important single nucleotide polymorphisms 677C->T and 1298A->C 6-10]. The 677C->T mutation is the most common mutation in MTHFR gene which involves a cytosine (C) to a thymine (T) substitution at position 677 of the MTHFR gene, and causes alanine to replace valine in the enzyme. The 677C->T mutation increases thermolability of MTHFR and reduces its catalytic activity by about 70% in homozygotes (677 T->T) or by 40% in heterozygotes (677 C->T).[6,7] The 1298A->C mutation in MTHFR gene is a point mutation at position 1298 which converts an adenine (A) into a cytosine (C), and is also associated with decreased enzyme activity, although to a lesser extent than the 677C->T polymorphism.[8,9]

Accumulating evidence indicates that these MTHFR polymorphisms can affect folate and total homocysteine (tHcy) status.[1,2,9,12] Individuals who are homozygous for MTHFR 677C->T or double heterozygous for both MTHFR 677C->T and MTHFR 1298A->C mutations, have lower blood folate and higher plasma tHcy concentrations than those with non-mutated gene.[6–11] MTHFR 677C->T mutation results in decreased plasma folate concentrations accompanied by increased plasma tHcy concentrations in parallel with the amount of folate in consumed diet.[12] Impaired folate status and elevated plasma Hyc in MTHFR

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gene polymorphisms are postulated to increase the risk of cardiovascular disease,[6,13–18] cancer,[19] neuropsychiatric diseases[20,21] and the risk of pregnancy complications, including neural tube defects.[8,22,23] Therefore, gathering detailed information on the rate of these MTHFR mutations and genoytpes and their influence on Hcy and folate status and reference intervals might confer significant benefit in determining the risk beared by the population for certain diseases. The reference interval is the most widely used medical decision-making tool. It is central to the assessment as to whether or not an individual is healthy and/or under risk for disease.[24,25] Determination of reference intervals is a delicate task which is required following the recommendations of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC).[26–31] To the best of our knowledge, the reference intervals in healthy adults for tHcy and folate among MTHFR 677C->T and 1298A->C genotypes have not yet been investigated systematically.

Considering the described negative effects of MTHFR mutations on folate and tHcy status, this study was aimed to determine: 1) the rate of MTHFR 677C->T and 1298A->C and their concomitance, 2) the mutation-specific reference values and intervals for plasma folate and tHcy concentrations, 3) impact of MTHFR 677C->T and 1298A->C genotypes on the serum folate and tHcy status, and 4) the relations between serum folate and tHcy concentrations in different genotypes of adult Turkish population. In addition, we also determined vitamin B12 status and reference values, because it is known that vitamin B12 acts as a co-factor in remethylation of Hcy to methionine.[3]

MATERIALS AND METHODS

Subjects

Four hundred and two healthy unrelated individuals of whom 179 were males and 223 were females at 18–45 years of age were included in our study in Bursa, Turkey, to determine genotype distributions of MTHFR polymorphisms and serum folate, Hcy and vitamin B12 status and the reference intervals. The sample size was determined based on the statistical recommendations of International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) [30]. The study group was mainly formed by random selection of individuals from the reference group for Bursa population based on the recommendations of IFCC[27] and the Clinical Laboratory Standards Institute (CLSI) C-28-A2 document.[32] A health status questionnaire form was filled by each subject and physical examinations were performed. Participants with a history of chronic diseases, thrombosis, stroke, coronary heart disease, hepato-renal diseases or individuals with abnormal laboratory tests results for hepato-renal functions were excluded. Participants taking therapeutic drugs and multivitamins containing folic acid and B12 complex (i.e., B12, B6) were also excluded.

The study was approved by the Ethical Committee of Uludag University Medical School. All subjects were given a written informed consent to participate in the study.

Blood Collection and preparation

The preanalytical phase was standardized based on the recommendations in the IFCC document.[28] Blood samples were collected from ante cubital vein into plain tubes and tubes containing EDTA (Vacutainer, Becton Dickinson, U.K.) from each participant after overnight fasting between 08:00 and 10:00 hrs. Blood samples were centrifuged within 30 min. Serum was isolated by centrifugation at 2000x *g* for 10 minutes and then frozen at $-80\ ^{\circ}C$ until the samples were analyzed.

Genotyping

Venous blood samples containing EDTA were used for mutation analysis. Mutation analyses which can detect MTHFR 677C->T and1298A->C gene mutations at the same time were performed using CVD StripAssay (ViennaLab Labordiagnostika GmbH, Austria), a system based on the reverse hybridization principle for mutations analysis included three successive steps: DNA extraction, Multiplex PCR and Reverse Hybridization, according to manufacturer's recommendations.

Chemical Analysis

Serum folate and vitamin B12 concentrations were determined using the Immulite 2000® Chemiluminescent Immunoassay System (Diagnostic Products Corporation, Los Angeles, CA, USA). Serum tHcy concentration was determined by fluorescence polarization immunoassay method using the AXSYM® System (Abbott, Wiesbaden, Germany. Analytical aspects were in accordance with the IFCC recommendations.[29] Samples were measured in batches of 10 per day. Control materials were measured in duplicates at the beginning and at the end of the assays.

Statistics

Statistical analysis was performed using SigmaStat V2.03 (SPSS Science Software GmbH, Erkrath, Germany) for Windows. The allelic and genotyping frequencies of MTHFR mutations were estimated by counting alleles and genotypes and calculating sample proportions; the statistical significance of differences of frequencies between groups was compared by a Chi-square (χ^2) test. The distribution of MTHFR mutations were tested for Hardy-Weinberg equilibrium using χ^2 goodness-of-fit test. The Kolmogorov-Smirnov test was used for normality. Mean, median, quartiles, percentiles and minimum-maximum values of folate, tHcy and vitamin B12 were determined by the descriptive statistical methods. The central 95% reference limits for folate, tHcy and vitamin B12 were calculated by use of a non-parametric statistical method in accordance with the IFCC recommendations.[30] The 90% confidence intervals of reference limits were estimated according to the IFCC document,[30] as described previously.[33,34] Difference in serum folate, tHcy and vitamin B12 values in different genotypes, and male and female

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subjects was determined by the Kruskal-Wallis one way analysis of variance (ANOVA) by ranks followed by Dunn's pairwise multiple comparison method. The statistical significance of the ratio was determined by the z-test. The relationship between two variables was determined by Spearman Rank Order correlations and multiple regression analysis. Data are given as mean ± standard error of the mean (SEM) or as median with 5% and 95% percentiles. A p-value < 0.05 was considered statistically significant in all test.

RESULTS

Rate of MTHFR 677C->T and MTHFR 1298A->C mutations

The rate of MTHFR 677C->T or MTHFR 1298A->C mutations was 50.7% or 54.7%, respectively (Table 1). The frequency of the mutated T allele for MTHFR 677C->T and the mutated C allele for MTHFR 1298A->C were 0.277 and 0.323, 0.302 and 0.335, or 0.341 and 0.338 in 179 male, 223 female or in total of 402 healthy subjects, respectively. The observed allelic frequencies for MTHFR 677C->T or MTHFR 1298A->C were in accordance with the Hardy-Weinberg law of equilibrium.

Table 2 shows the distribution of nine possible genotypes regarding the two common MTHFR polymorphisms. In 54 out of 402 subjects either MTHFR 677C->T or MTHFR 1298A->C mutation, or both was present. The rate of mutated subjects was 86.6% (348/402). Six different genotypes were determined based on the presence and/or absence of MTHFR 677C->T and/or 1298A->C mutations (Table 2). Both MTHFR 677C->T and MTHFR 1298A->C heterozygote mutations were determined in 76 subjects (Table 2), and the co-existence rate was 19.0% (76/402).

Reference intervals for folate, vitamin B12 and tHcy in MTHFR 677C->T and 1298A->C mutated subjects

Table 3 shows the reference intervals for serum folate, tHcy and vitamin B12 in our study group. Upper limit for tHcy in MTHFR 677C->T mutated individuals was (26.6 μmol/L) much higher than those observed (21.2 μmol/L) in all subjects (Table 3). The upper and lower limits of folate were slightly low in MTHFR 677C->T-mutated-individuals (Table 3). Reference intervals for serum tHcy were narrowed moderately in the MTHFR 1298A->C- mutated subjects compared with those in 677C->T mutated subjects (Table 3). In male subjects the upper limit for folat was much lower, while the upper limit for serum tHcy was much higher than those in observed in female subjects (Table 3).

Table 2. Rate of genotypes based on MTHFR 677C->T and 1298A->C mutations in MTHFR gene

MTHFR 677C->T Genotype	MTHFR 1298A->C Genotype	N	Rate (%)
677C->C	1298A->A	54	13.4
677C->C	1298A->C	92	22.9
677C->C	1298C->C	52	12.9
677C->T	1298A->A	89	22.1
677C->T	1298A->C	76	19.0
677C->T	1298C->C	0	0
677T->T	1298A->A	39	9.7
677T->T	1298A->C	0	0
677T->T	1298C->C	0	0

Serum folate, vitamin B12 and tHcy concentrations in MTHFR 677C->T and 1298A->C mutated subjects

Mean values of serum folate, vitamin B12 and tHcy concentrations were 7.5 ± 0.2, 6.9 ± 0.1 or 7.1 ± 0.2 nmol/L, 287 ± 5.1, 284 ± 7.0 or 287 ± 7.1 pmol/L and 11.1 ± 0.2, 12.0 ± 0.4 or 10.5 ± 0.2 μmol/L in all of 402 subjects, 204 of MTHFR C677- mutated subjects or 220 of MTHFR A1298C mutated subjects, respectively.

When analyzed in six different genotypes based on MTHFR 677C->T and MTHFR 1298A->C mutations, serum tHcy and folate concentrations showed considerable variations in their characteristics. As seen in Table 4, serum median tHcy concentrations were highest in male and

Table 1. Rate of MTHFR 677 C ->T and MTHFR 1298A->C mutations in 402 healthy individuals

	Male (N = 179)		Female (N = 223)		Total (Male + Female) (N = 402)	
Mutations/Genotypes	N	Rate (%)*	N	Rate (%)*	N	Rate (%)*
MTHFR 677C->T						
CC (normal-wild)	91	50.8 (52.3)	107	48.0 (45.9)	198	49.3 (48.7)
TT (homozygote)	11	6.2 (7.7)	28	12.6 (10.4)	39	9.7 (9.1)
CT (heterozygote)	77	43.0 (40.0)	88	39.4 (43.7)	165	41.0 (42.2)
MTHFR 1298A->C						
AA (normal-wild)	79	44.1 (44.2)	103	46.2 (43.5)	182	45.3 (43.8)
CC (homozygote)	20	11.2 (11.2)	32	14.3 (11.6)	52	12.9 (11.5)
AC (heterozygote)	80	44.7 (44.6)	88	39.5 (44.9)	168	41.8 (44.7)

*Numbers in parenthesis indicate the expected frequencies (as percentage). Statistical analysis, by χ^2 test, showed no difference between the observed and expected frequencies of MTHFR 677 C ->T and MTHFR 1298A->C mutations (677C->T male: $\chi^2 = 1.01$, 677C->T female: $\chi^2 = 2.12$, 677C->T total: $\chi^2 = 0.29$, 1298A->C male: $\chi^2 = 0.0$, 1298A->C female: $\chi^2 = 3.3$, 1298A->C 1298 total: $\chi^2 = 1.78$; df = 2, $p > 0.05$).

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Table 3. Reference intervals and their 90% confidence intervals (90% CI) for serum thcy, folate and vitamin B12 in all, and MTHFR 677C->T or MTHFR 1298A->C mutated subjects

Subjects	tHcy (μmol/L) Reference intervals (lower-upper 90% CI)	Folate (nmol/L) Reference intervals (lower-upper 90% CI)	Vitamin B12 (pmol/L) Reference intervals (lower-upper 90% CI)
All subjects (n = 402)	5.9–21.2	3.3–15.2	141–582
	(5.4–6.3)–(18.9–22.3)	(2.5–3.6)–(14.5–17.1)	(133–147)–(564–592)
Male (n = 179)	7.3–23.9	3.3–14.8	144–584
	(6.2–7.7)–(19.2–27.5)	(3.0–3.6)–(14.3–15.5)	(137–158)–(560–601)
Female (n = 223)	5.8–19.5	3.0–17.1	138–580
	(5.7–6.8)–(23.9–28.1)	(2.8–3.4)–(14.3–18.7)	(131–149)–(555–598)
677C->T mutated subjects (n = 204)	6.0–26.6	2.8–12.5	136–574
	(5.7–6.8)–(23.9–28.1)	(2.5–3.1)–(11.9–15.0)	(130–143)–(552–590)
1298A->C mutated subjects (n = 220)	6.1–18.6	3.6–15.1	139–583
	(5.0–6.6)–(16.9–21.8)	(3.1–4.1)–(14.0–17.1)	(135–142)–(553–594)

female homozygote subjects with MTHFR 677C->T mutation. In this genotype, 82% of male and 43% of female subjects had higher serum tHcy concentration than the cutoff value of ≥12 μmol/L (Table 4). Serum tHcy levels in male subjects were significantly higher than those observed in female subjects in all of the six genotypes (Table 4). Female subjects in the 677C->C/1298C->C, 677C->T/1298A->A or 677C->T/1298A->C genotype had significantly higher serum tHcy concentrations than those in 677C->C/1298A->A genotype (Table 4). Although serum median tHcy concentration was not significantly different, about 65% of male subjects with 677C->C/C1298C->C genotypes had higher serum tHcy concentrations than the cutoff value of ≥12 μmol/L (Table 4).

Serum folate concentrations were lowest in both male and female homozygote subjects for MTHFR 677C->T mutation (Table 4). In this genotype, about all of subjects had lower serum folate concentration than the cutoff value of ≤10 nmol/L (Table 4). Serum folate levels were also slightly, but not significantly, lower in the all genotypes possessing any of the mutated alleles. However, about 80%, 83% and 84% of subjects with 677C->C/1298A->C, 677C->T/1298A->A and 677C->T71298A->C had lower folate levels than the cutoff value. In male subjects with 677C->C/

Table 4. Serum thcy and folate concentrations in male and female subjects in different genotypes and number of subjects who have higher serum thcy or lower serum folate concentration than the cutoff values

Genotype/Subjects	N	tHcy (μmol/L) Median (5%, 95%)	Subjects with tHcy ≥12 μmol/L N (%)	Folate (nmol/L) Median (5%, 95%)	Subjects with folate ≤10 nmol/L N (%)
677C->C/1298A->A					
Male + Female	54	9.5 (6.0, 16.8)	10 (19%)	8.1 (4.9, 17.0)	34 (63%)
Male	27	10.3 (7.8, 17.5)	7 (26%)	8.0 (5.1, 13.3)	17 (63%)
Female	27	7.7 (5.8, 15.5)[#]	3 (11%)	8.1 (4.6, 21.1)	17 (63%)
677C->C/1298A->C					
Male + Female	92	9.7 (6.3, 14,2)	14 (15%)	6.8 (4.3, 14.2)	74 (80%)[♣]
Male	44	10.4 (7.2, 17.3)	12 (27%)	6.8 (3.9, 15.2)	36 (82%)
Female	48	8.8 (5.8, 12.2)[#]	2 (4%)	6.9 (4.5, 13.8)	38 (79%)
677C->C/1298C->C					
Male + Female	52	10.6 (6.6, 17.9)	20 (38%)[♣]	7.3 (3.6, 14.9)	42 (81%)
Male	20	11.2 (8.4, 17.6)	13 (65%)[♣]	6.1 (3.5, 10.8)[*]	19 (95%)[♣]
Female	32	9.9 (6.3, 17.8)[*#]	7 (22%)	8.1 (4.2, 15.0)	23 (71%)
677C->T/1298A->A					
Male + Female	89	10.4 (6.2, 19.8)	27 (30%)	6.8 (3.9, 12.0)	74 (83%)[♣]
Male	41	11.3 (8.2, 21.1)	17 (41%)	6.7 (3.5, 11.5)	37 (90%)[♣]
Female	48	9.5 (6.0, 19.5)[*#]	10 (21%)	6.9 (4.1, 13.2)	37 (77%)
667T->T/1298A->A					
Male + Female	39	12.2 (6.9, 42.2)[*]	21 (54%)[♣]	5.2 (2.7, 8.9)[*]	39 (100%)[♣]
Male	11	15.2 (8.6, 49.9)[*]	9 (82%)[♣]	5.2 (2.1, 7.7)[*]	11 (100%)[♣]
Female	28	11.2 (6.7, 33.9)[*#]	12 (43%)[♣]	5.2 (3.0, 9.6)[*]	28 (100%)[♣]
677C->T/1298A->C					
Male + Female	76	10.4 (5.4, 17.9)	19 (25%)	6.6 (4.2, 11.6)	64 (84%)[♣]
Male	36	11.0 (7.6, 19.1)	13 (36%)	7.2 (4.2, 11.4)	30 (84%)
Female	40	9.6 (6.8, 13.9)[*#]	6 (15%)	6.5 (4.2, 12.3)	34 (85%)

[*]Different ($p < 0.05$–0.001) when compared with the respective values for subjects in the C677C/A1298A (non-mutated subjects for both mutation).
[♣]Significant ($p < 0.05$–0.001) when compared (by z-test) with the respective ratio for subjects in the C677C/A1298A (non-mutated subjects for both mutation).

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1298C->C genotype (homozygote for MTHFR 1298A->C) serum folate concentration was, however, lower ($p < 0.01$) than the value observed in male non-mutated subjects (677C->C/1298A->A genotype) (Table 4); about 95% of males had lower folate levels than the cutoff value.

Serum vitamin B12 concentrations were similar in all six genotypes (data not shown).

Relations between serum folate, vitamin B12 and tHcy concentrations in MTHFR 677C->T and 1298A->C mutated subjects

To examine the distribution of serum tHcy concentrations and their relations with serum folate and vitamin B12 concentrations, we plotted serum tHcy values against serum folate and serum vitamin B12 values from all subjects within the six genotypes. As seen in Figures 1 and 2, serum tHcy concentrations showed inverse relations with both serum folate and vitamin B12 concentrations in all six genotypes. The relations were strongest in the homozygote individuals for MTHFR 677C->T mutation (Figures 1 and 2). These correlations remained significant after adjustment for confounders (i.e., age, folic acid and B12 levels) (data not shown).

Serum folate and vitamin B12 concentrations were positively correlated in all subjects ($r = 0.334$, $p < 0.001$, $n = 402$), non-mutated subjects ($r = 0.403$, $p < 0.01$, $n = 54$), MTHFR 677C->T homozygote ($r = 0.473$, $p < 0.01$, $n = 39$), MTHFR 677C->T heterozygote ($r = 327$, $p < 0.01$, $n = 89$), MTHFR 1298A->C homozygote ($r = 0.465$, $p < 0.001$, $n = 52$), MTHFR 1298A->C heterozygote ($r = 0.278$, $p < 0.01$, $n = 92$) and MTHFR 677C->T/1298A->C compound heterozygote ($r = 0.282$, $p < 0.05$, $n = 76$) subjects.

Discussion

These data show that about 86% of our study population possess either one of MTHFR 677C->T and 1298A->C mutations or both. The rate of MTHFR 677C->T or 1298A->C mutation was 50.7% or 54.7%, respectively. The reference intervals and their upper and lower limits for serum tHcy and folate varied considerably with presence or absence of MTHFR 677C->T and 1298A->C mutations. In the MTHFR 677C->T mutated subjects the reference intervals for serum tHcy and folate were characterized by marked shifts in the upper limits. In the T677T/A1298A genotype (homozygote for MTHFR 677C->T) serum tHcy concentration was higher while serum folate concentration was lower than the values in the wild genotype. In this genotype all subjects had lower serum folate and 54% of subjects had higher tHcy concentrations than the cutoff values of ≤ 10 nmol/L and ≥ 12 μmol/L, respectively. Serum tHcy concentrations were inversely correlated with serum folate and vitamin B12 concentrations in all of the six genotypes.

Our data complement previous studies on MTHFR gene polymorphism in Turks[35,36] and different populations around the world.[37–45] It is clear from previous studies that the rate of MTHFR 677C->T and 1298A->C mutations are common and they show ethnic and regional variations.[42–45] The rate of MTHFR 677C->T among Caucasians varies between 47–70% with homozygosity ranges from 4–26.4% in Europe and America.[42–44] The rate of MTHFR 677C->T homozygosity is found 7.9% in Chinese[45] and 32.2% in Mexicans.[42,43] The rate of MTHFR 1298A->C polymorphism is not as widely studied as that of MTHFR 677C->T, but the homozygous state for this polymorphism has been observed in 8–12% French,[7] Dutch,[8] German,[15] Canadian,[9] white Americans[16] and the Ashkenazi Jewish.[37] In the present study, we observed that the rate of heterozygosity and homozygosity for MTHFR 677C->T or 1298A->C polymorphism was 41.0% and 9.7% or 41.8% and 12.9%, respectively. These values are in good accordance with the values reported recently by Sazci *et al.*,[35] for Turkish population, and comparable with the values given above for most Caucasians in Europe, Canada and America.

With respect to these two common polymorphisms (667C->T and 1298A->C) in the MTHFR gene, we found that six of the nine combined genotypes were present in our population; 19% of these were compound heterozygotes. This was comparable with the reported ranges of MTHFR 677C->T/1298A->C compound heterozygosity as 19% in the United States,[16] 20% in the Netherlands,[8] 23% in Germany,[15] 23.5% in France[7] and 21.6% in Turkey.[35] Comparable with reports by other investigators,[8,9,15,35,37,38] we found no individuals who were homozygous for both polymorphisms (677T->T/1298C->C genotype). We also detected no individuals who were homozygous for one polymorphism and heterozygous for the other (Table 1). Other studies have shown that the rate of the 677T->T/1298A->C or 677C->T/1298C->C genotype is very low (i.e., 0.2% in white Americans[21] and 1% in Turkish[35]).

The present study is the first to provide the MTHFR 677C->T/1298A->C mutations-specific reference intervals for serum tHcy, folate and vitamin B12 concentrations in apparently healthy adults. The reference intervals for serum folate and tHcy in our whole study group were comparable with the reported values in Turkish adult healthy populations.[32] We found that the reference intervals for serum tHcy and folate, but not vitamin B12, concentrations vary considerably in MTHFR 677C->T-mutated individuals. In the MTHFR 677C->T-mutated subjects the reference intervals for serum folat and tHcy were characterized by marked shifts in the upper limits (Table 3). These marked shifts in the upper limits may have resulted from the impairment in both serum folate and tHcy status in subjects with MTHFR 677C->T mutation. This view was supported directly by the genotype-specific data on serum folate and tHcy concentrations from the present study (see below and Table 4). It may also be necessary to note that the upper limit for tHcy in the present study was comparable with the upper limit for tHcy in males reported by our laboratory which derived from the patient data.[33]

In accordance with data on the mutation-specific reference intervals described above, serum folate and tHcy

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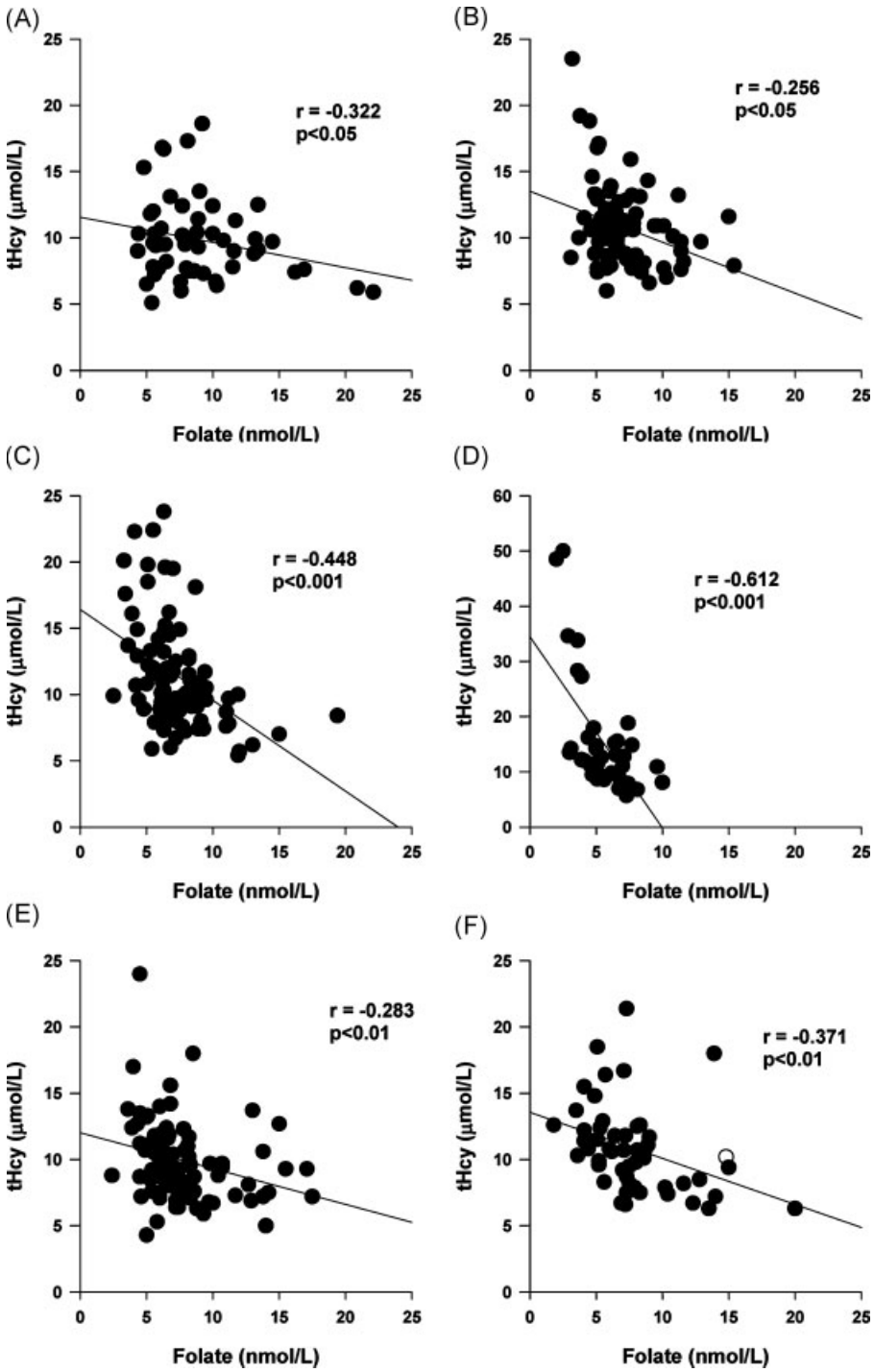

Figure 1. Relations between serum tHcy and folate concentrations in subjects with six different combined MTHFR 677C->T/1298A->C genotypes. Serum tHyc concentrations were plotted against serum folate concentrations in blood samples collected from individuals with six different combined MTHFR 677C->T/1298A->C genotypes. A = 677C->C/1298A->A (wild/wild, normal); B = 677C->T/1298A->C (compound hetorozygotes); C = 677C->T/1298A->A (heterozygote/wild); D = 677T->T/1298A->A (homozygote/wild); E = 677C->C/1298A->C (wild/heterozygote); F = 677C->C/1298C->C (wild/homozygote)

status varied considerably in the six different genotypes regarding with MTHFR 677C->T and 1298A->C mutations. We found that the individuals who are homozygous for MTHFR 677C->T have higher serum tHcy and lower folate concentrations than those with non-mutated individuals, as shown previously.[6–11] In some,[6–10] but not all,[11,16,46] previous studies it has also been shown that the individuals who are double heterozygous for both MTHFR

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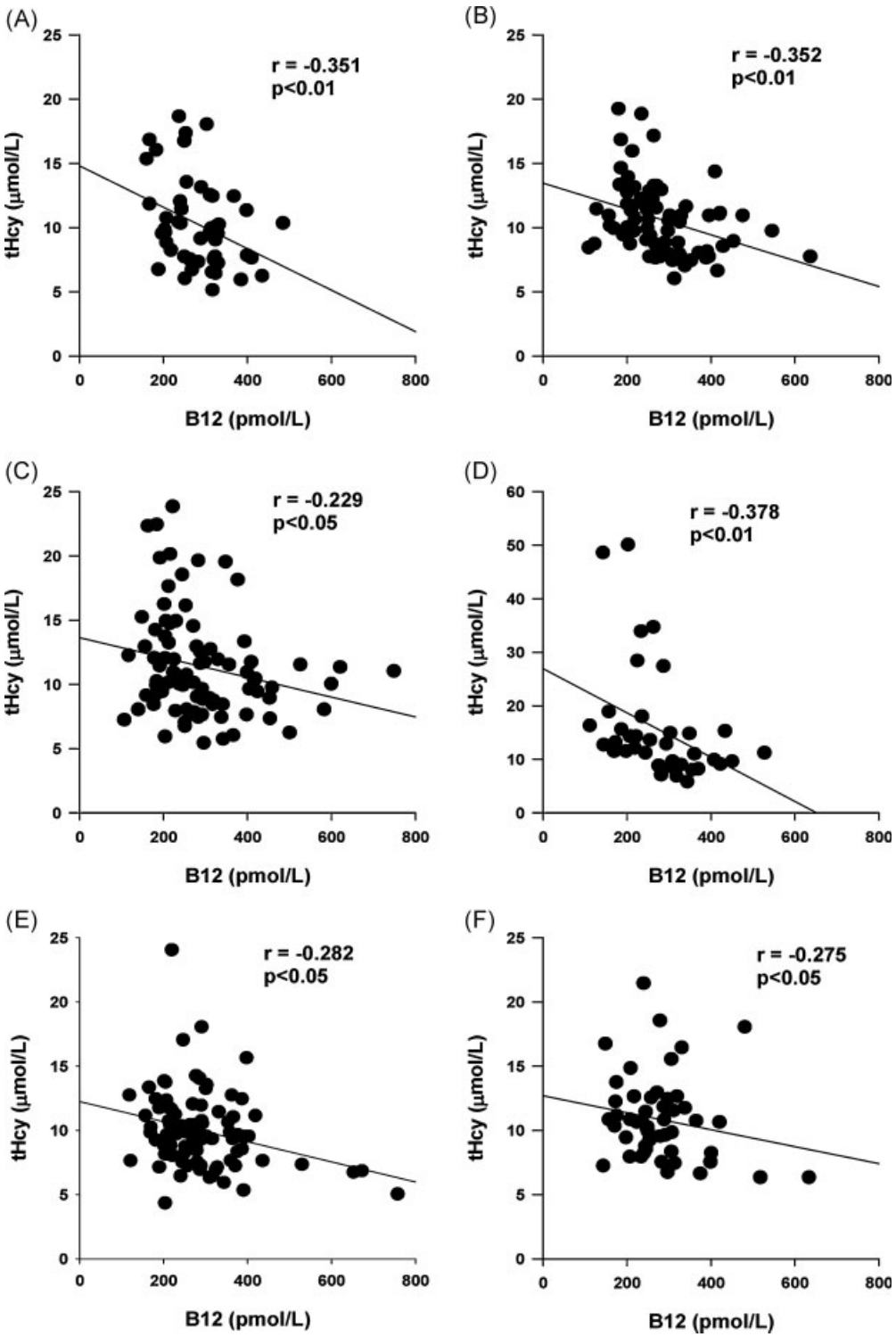

Figure 2. Relations between serum tHcy and vitamin B12 concentrations in subjects with six different combined MTHFR 677C->T/1298A->C genotypes. Serum tHyc concentrations were plotted against serum vitamin B12 concentrations in blood samples collected from individuals with six different combined MTHFR 677C->T/1298A->C genotypes. A = 677C->C/1298A->A (wild/wild, normal); B = 677C->T/1298A->C (compound hetorozygotes); C = 677C->T/1298A->A (heterozygote/wild); D = 677T->T/1298A->A (homozygote/wild); E = 677C->C/1298A->C (wild/heterozygote); F = 677C->C/1298C->C (wild/homozygote)

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677C->T and MTHFR 1298A->C mutations, have lower blood folate and higher plasma tHcy concentrations than those with non-mutated individuals.[6–10] Although we failed to confirm these observations for males, we found that female subjects with the double heterozygosity had significantly higher tHcy concentrations than the wild type females (Table 4). Furthermore, female subjects heterozygous only for MTHFR 677C->T or homozygous for MTHFR 1298A->C also had higher serum tHcy concentrations than those in wild type females. Despite, male subjects in all of the six genotypes had significantly higher serum tHcy concentrations than the female subjects. The higher serum tHcy concentrations in males have been attributed primarily to differences in muscle mass and/or concentrations of sex hormones.[37,38,46,47]

On the basis of the gradually increasing cardiovascular risk in a multicenter case-control study, the European Concerted Action Project (ECAP) defined the cutoff value of 12.1 μmol/L for fasting serum tHcy.[48] Recently, Castanon *et al*.,[49] proposed a hyperhomocysteinemia cutoff value of 12 μmol/L for venous thrombosis. In the present study we found that 36–82% of males and 4–43% females in 677C->C/1298C->C, 677C->T/1298A->A, 677C->T/1298A->C and 677T->T/1298A->A had serum tHcy concentrations higher than the cutoff value. It is well established that Turks have one the highest prevalence for coronary disease in Europe.[50,51] Lipids and lipoproteins are in focus,[50,51] but impaired tHcy status due to the high rate of MTHFR 677C->T/1298A->C mutations could be involved in part, in the high incidence of cardiovascular disease. Indeed, association with MTHFR 677C->T and/or 1298A->C mutations in Turks with cardiovascular diseases have been shown by some case-control studies.[52–56] Serum tHcy concentrations showed inverse relation with serum folate and B12 concentrations in all six genotypes, with the strongest in individuals homozygote for MTHFR 677C->T (Figures 1 and 2), as shown previously.[57,58] Taken together, these data suggest that MTHFR 677C->T and 1298A->C mutations are associated with a mild hyperhomocysteinemia and folic acid and/or vitamin B12 supplementation may be helpful to reduce tHcy concentrations to below its cutoff value.

Based on the relationship between plasma Hcy and folate, it has been suggested that plasma folic acid cutoff value on functional ground is 10 nmol/L.[59] In the present study we found that about 65% of the non-mutated individuals and 77–95% of the mutated subjects with 677C->T/1298A->C, 677C->C/1298C->C, 677C->T/1298A->A and 677C->T/1298A->C genotypes had lower serum folate concentration than its cutoff values of $\leq$10 nmol/L. Furthermore, we observed that all of subjects with 677T->T/1298A->A genotype have lower folate concentration than its cutoff values of $\leq$10 nmol/L. The observed folate concentrations in the present study are in good accord with data from Turkish people[60] and populations of various European countries such as Norway, the Netherlands, Sweden and Greece.[61] Although folate intake in Turkish people which is estimated to be 271 ± 139 μg[61] is among European ranges (200–300 μg) for daily recommendation for daily folate intake[61] our data show that daily intake of folate is apparently insufficent to maintain normal folate status in most of the subjects included in this study. Our present findings also imply that, to maintain normal folate status in MTHFR mutated subjects, extra folate supplementation is required particularly in people with 677T->T genotype since low folate status both by accompanying impaired tHcy metabolism and, itself, is an independent risk factor for coronary heart disease[60] and neural tube defects.[8,22,23,62]

In conclusion, these data show that MTHFR 677C->T and 1298A->C mutations, particularly homozygosity for MTHFR 677C->T, have negative influence on serum folate and tHcy status and alter reference intervals and values for these two analytes. Homozygote individuals for MTHFR 677C->T were characterized with a severely impaired serum folate and tHcy status and about a half of males with MTHFR C667T and 1298A->C mutations associated with higher serum tHcy concentrations than the cutoff values for the risk of cardiovascular diseases.[48,49] Further clinical studies are required to determine real health impacts of MTHFR 677C->T and/or 1298A->C polymorphisms and impaired folate and/or tHcy status in Turkish population. Data from the present study could facilitate these studies by providing basic information on the rate of these polymorphisms, the mutation-specific reference intervals and the genotype-specific serum folate and tHcy status. The data obtained in the present study may also be applicable to European population since folate status and rate of MTHFR gene polymorphism are similar with those of Turkish people.

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