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ORIGINAL ARTICLE

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A second common variant in the methylenetetrahydrofolate reductase (MTHFR) gene and its relationship to MTHFR enzyme activity, homocysteine, and cardiovascular disease risk

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Abstract Molecular defects in genes encoding enzymes involved in homocysteine metabolism may account for mild hyperhomocysteinemia, an independent and graded risk factor for cardiovascular disease (CVD). We examined the relationship of two polymorphisms in the methylenetetrahydrofolate reductase (MTHFR) gene, the 677C→T and 1298A→C variants, to MTHFR activity, homocysteine concentrations, and risk of CVD in a population of 190 vascular disease patients and 601 apparently healthy controls. The mean specific and residual MTHFR activities were significantly lower in 677CT and 677TT individuals (both $P<0.001$). The 1298A→C mutation alone showed no effect on MTHFR activities. However, when the 677C→T genotype was taken into account, the 1298A→C mutation also caused a significant decrease in MTHFR activities, which was observed in both the homozygous 1298CC ($P<0.001$) and the heterozygous 1298AC states ($P=0.005$). Both the 677TT as the 677CT genotypes were associated with significantly higher fasting and postload homocysteine levels than 677CC ($P<0.001$ and $P=0.003$, respectively). The 1298A→C mutation had no effect on fasting or postload homocysteine levels. Since homocysteine itself is considered to be positively associated with the risk of CVD, these findings indicate that the 1298A→C mutation cannot be considered a major risk factor for CVD.

Keywords Methylenetetrahydrofolate reductase · Homocysteine · Hyperhomocysteinemia · Cardiovascular disease

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Abbreviations *CVD*: Cardiovascular disease · *MTHFR*: 5,10-Methylenetetrahydrofolate reductase · *PCR*: Polymerase chain reaction

Introduction

Homocysteine is a sulfur amino acid that is degraded in the transsulfuration pathway to cystathionine, a step that is catalyzed by cystathionine β-synthase. Alternatively, it can be remethylated to methionine via a pathway regulated by 5,10-methylenetetrahydrofolate reductase (MTHFR). MTHFR converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the primary circulatory form of folate. In both pathways major genetic defects that cause enzyme deficiencies are associated with very high plasma homocysteine levels and excretion of homocystine into the urine. These forms of homocystinuria are characterized by neurological abnormalities and by the development of early arteriosclerotic and thrombotic vascular disease [1, 2].

Wilcken et al. [3] were the first to report an association between mildly elevated homocysteine levels and cardiovascular disease (CVD). Later, numerous investigators have reported the occurrence of elevated plasma homocysteine levels in patients with cerebral, peripheral or coronary artery disease or venous thrombosis [4, 5, 6]. Today a mildly elevated plasma homocysteine concentration is generally [7, 8], although not universally [9, 10] accepted as an independent and graded risk factor for both arterial occlusive disease and venous thrombosis.

Plasma homocysteine levels are influenced by various factors: genetic, dietary, and life-style factors can interfere with transsulfuration and remethylation. Homocysteine blood concentrations have been found to be correlated in monozygotic and dizygotic healthy twins [11, 12] and to be strongly correlated among family members of patients with coronary vascular disease [13, 14] or with various forms of arterial occlusive disease [15].

In 1988 Kang et al. [16] described a thermolabile variant of MTHFR that was associated with decreased enzyme activity and increased heat lability of the enzyme. Frosst et al. [17] reported the 677C→T mutation to be the genetic cause of thermolabile MTHFR. This 677C→T mutation leads to the exchange of an alanine with a valine amino acid and is associated with elevated homocysteine concentrations, especially in those individuals with low folate status [18]. Thermolabile MTHFR, however, accounts for mild hyperhomocysteinemia in approximately 25% of vascular disease patients [19], which indicates that additional mutations in the MTHFR gene or other genes may also affect homocysteine levels. In 1998 we [20] described an A→C change at basepair 1298 that results in the substitution of glutamate by an alanine residue. Subsequently its effect on homocysteine and folate metabolism and its potential role as a risk factor for neural-tube defects was investigated. That study found compound heterozygosity for the 677C→T and 1298A→C variants to be associated with reduced MTHFR specific activity, elevated homocysteine, and decreased plasma folate levels [20].

In the present study we examined this 1298A→C mutation and its potential interaction with the 677C→T mutation as a possible cause of elevated homocysteine concentration and as a possible genetic risk factor for CVD. We assess the effect of both variants on plasma homocysteine levels, MTHFR activity, and residual MTHFR activity in 190 vascular disease patients and 601 apparently healthy subjects.

Materials and methods

Patients and controls

We studied 190 patients with coronary, peripheral, or cerebral vascular disease. Of these, 130 were recruited from persons who underwent coronary angiography in the Zuiderziekenhuis Hospital in Rotterdam, The Netherlands [21], and 60 had documented premature CVD (10 myocardial infarction, 32 cerebral arterial occlusive disease, and 18 peripheral arterial occlusive disease) [22]. The control group consisted of 601 controls, of whom 101 were included from the general population in Rotterdam [21] and 500 were recruited from a general practice in The Hague, The Netherlands [23].

The proportion of men was higher among patients (75.3%) than controls (47.1%; $P<0.001$). The mean age of patients was 49.0±10.2 years, and that of controls was 50.6±12.7 years (n.s.). Table 1 presents the major clinical characteristics of patients and controls. The patient group showed a significantly higher fasting homocysteine level ($P=0.005$), while the postload homocysteine level tended to be higher in patients ($P=0.10$). Specific and residual MTHFR activity did not differ between patients and controls, although the residual MTHFR activity expressed as a percentage of the specific activity tended to be lower in patients ($P=0.06$).

Mutation detection

The 677C→T variant creates a *Hin*fI site, and was analyzed by restriction fragment length polymorphism polymerase chain reaction (PCR) as described elsewhere [17]. The 1298A→C variant changes a glutamate to an alanine residue and abolishes a *Mbo*II restriction site. Mutation analysis was performed by PCR and subse-

Table 1 Clinical characteristics of study population. Age and MTHFR activities are expressed as mean ±SD; homocysteine concentrations as geometric means (95% CI); MTHFR activities as nanomoles of formaldehyde formed per milligram of protein per hour ($n=129$ for controls and $n=42$ for patients)

	Patients ($n=190$)	Controls ($n=601$)	P[a]
Fasting homocysteine (μmol/l)	14.4 (13.7–15.0)	13.1 (12.7–15.1)	0.005
Postload homocysteine (μmol/l)	41.1 (39.7–43.2)	40.1 (39.1–41.1)	0.10
Specific MTHFR activity	17.5±7.1	18.8±6.8	0.74
Residual MTHFR activity	10.1±5.9	11.9±5.6	0.25
Residual MTHFR activity (%)	53.3±15.9	59.4±13.3	0.06

[a] P values are age- and sex-adjusted

quent restriction enzyme analysis with *Mbo*II. The PCR was carried out in a total volume of 50 µl containing 50 ng of the forward primer 5′-ATGTGGGGGGAGGAGCTGAC-3′ and 50 ng of the reverse primer 5′-CCCTCTTCCCATTCAACCCTCTG-3′, 200 µM each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 mM $MgCl_2$ and 1 unit *Taq* polymerase (Life Technologies). PCR conditions were: an initial denaturation step of 3 min at 92°C, followed by 35 cycles of 92°C/60 s (denaturation), 55°C/60 s (annealing), and 72°C/60 s (extension), and a final extension of 7 min at 72°C. The amplified PCR fragment of 240 bp was digested with the restriction enzyme *Mbo*II, followed by gel electrophoresis analysis on a 2% agarose gel. After restriction enzyme analysis the 1298AA genotype results in two fragments of 25 and 215 bp. The 1298CC genotype shows only one fragment of 240 bp [24].

MTHFR enzyme activity assay

Specific and residual MTHFR activities in isolated lymphocytes were determined radiochemically as described by Engbersen et al. [19]. Activities are expressed as nanomoles of formaldehyde formed per milligram of protein per hour. The residual MTHFR activity is the enzyme activity after heat incubation for 5 min at 46°C and is also expressed as a percentage of the specific MTHFR activity before incubation.

Homocysteine

All CVD patients and controls were subjected to a standardized oral methionine loading test (0.1 g L-methionine/kg body weight) [4]. Total homocysteine was measured in plasma using a high-performance liquid chromatography procedure, with reverse phase separation and fluorescence detection, as described by Te Poele-Pothoff et al. [25]. All homocysteine measurements were conducted in our laboratory at the University Medical Center Nijmegen, The Netherlands.

Statistics

Homocysteine concentrations were logarithmically transformed prior to all statistical analyses. Differences between patient and control groups were assessed by Student's *t* test for continuous variables and Pearson's χ^2 test for frequencies. *P* values were age- and sex-adjusted by linear regression analysis. Age- and sex-adjusted odds ratios and 95% confidence interval were calculated by logistic regression analysis to estimate the relative risk of CVD for the various genotype combinations. One-way analysis of variance was used to assess the differences in continuous variables between the different genotypes, followed by Bonferroni-corrected *t* tests. *P* values are two-tailed, and $P<0.05$ was considered statistically significant.

Results

MTHFR genotype distribution and CVD risk

Both 677C→T and 1298A→C genotype distributions were in Hardy-Weinberg equilibrium. We observed an allele frequency of the 1298C allele of 0.30 in patients and of 0.33 in controls ($\chi^2=1.45$, $P=0.48$). The allele frequency of the 677T allele in controls was 0.29, compared with 0.33 in patients ($\chi^2=1.57$, $P=0.46$); thus the frequency of the mutated alleles was similar in patients with CVD and in controls.

Table 2 combines the 677C→T and 1298A→C genotypes to generate composite genotypes and presents calculated odds ratios for risk of CVD for each genotype relative to the 677CC/1298AA genotype. No increased risk for CVD was observed for any of the composite genotypes. The distribution of composite MTHFR genotypes is consistent with the hypothesis that the two variants originated on different alleles.

Table 2 Prevalence and odds ratios (*OR*; adjusted for age and gender) and 95% confidence intervals (*95% CI*) for risk of CVD of the MTHFR polymorphisms in patients and controls

Genotype		Number of		OR[a]	95% CI
677	1298	Patients	Controls		
CC	AA	23	79	1[a]	
CC	AC	48	146	1.22	0.68–2.21
CC	CC	13	57	0.79	0.36–1.76
CT	AA	42	119	1.43	0.77–2.65
CT	AC	32	112	0.99	0.53–1.85
TT	AA	20	52	1.42	0.69–2.93

[a] Reference category

Table 3 Relationship between MTHFR genotypes and MTHFR enzyme activity. Activities are expressed as nanomoles of formaldehyde formed per milligrams of protein per hour; *P* values calculated by analysis of variance

Genotype	*n*	MTHFR activity		
		Specific	Residual	Residual (%)
677[a]				
CC	96	21.8±6.3	14.5±5.1	65.3±8.2
CT	60	15.9±4.4	8.7±3.2	53.1±10.0
TT	15	7.9±2.2	2.4±1.7	27.6±13.4
P		<0.001	<0.001	<0.001
1298				
AA	74	18.8±8.0	11.4±6.6	54.2±16.6
AC	83	18.6±6.2	11.7±5.3	60.3±12.1[b]
CC	13	15.8±3.2	10.1±2.5	62.8±5.4
P		n.s.	n.s.	0.011

[a] $P<0.001$ 677TT vs. 677CC, 677CT vs. 677CC, 677TT vs. 677CT (Bonferroni *t*-test)
[b] $P<0.05$ 1298AC vs. 1298AA (Bonferroni *t*-test)

MTHFR activities

MTHFR activity was measured in isolated lymphocytes of 42 patients and 129 controls. Part of the control group has been used in our previous study [20]. Because the relationship between the MTHFR genotypes and MTHFR enzyme activity was similar in patients and controls (data not shown), we combined the groups to increase statistical power in subsequent analyses.

Table 3 shows the separate effects of the 677C→T and 1298A→C mutations on the MTHFR activity. The mean specific and residual MTHFR activities were significantly lower in individuals with the homozygous 677TT genotype and the heterozygous 677CT genotype

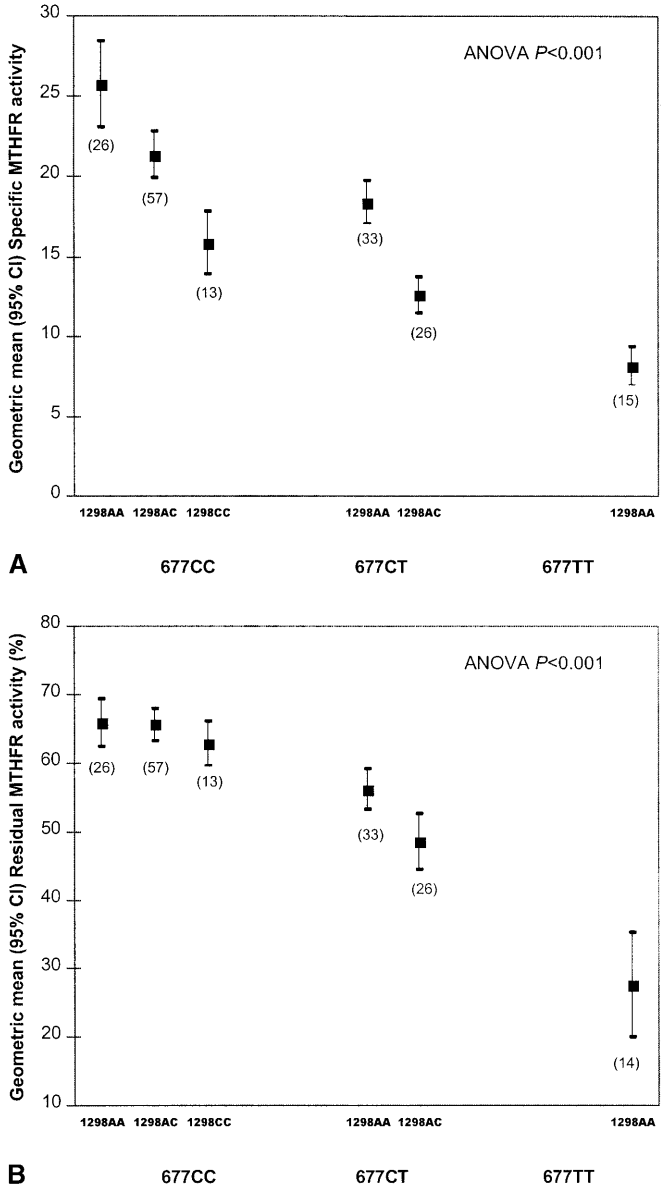


Fig. 1 Relationship between composite MTHFR genotypes and specific (**A**) and residual (**B**) MTHFR activity. *Figures in parentheses* Number of individuals in each genotype group. MTHFR activities are expressed as nanomoles of formaldehyde formed per milligram of protein per hour. Residual MTHFR activity is expressed as a percentage of the MTHFR activity before heat incubation

than in those observed in 677CC homozygotes (P<0.001, analysis of variance). The 1298A→C mutation by itself had no effect on specific and residual MTHFR enzyme activity, but the 1298AC and 1298CC showed higher residual (%) activities than with the 1298AA genotype (P=0.011, analysis of variance; Table 3). Figure 1 shows the effects of the 1298A→C genotypes and MTHFR activity within each of the three 677C→T genotype groups. Among 677CC subjects those with the 1298CC or 1298AC genotype had lower specific MTHFR activity than their 1298AA peers (both P<0.005; Fig. 1A). Among 677CT heterozygotes those with the1298AC genotype also showed significantly lower activities than individuals with the 677CT/1298AA genotype (P<0.001). The mean residual (%) MTHFR activities of the composite genotypes show that within none of the MTHFR 677C→T genotype groups a significant effect of the 1298A→C mutation on residual (%) MTHFR activity (Fig. 1B) could be observed.

Table 4 Relationship between MTHFR genotypes and fasting and postload homocysteine concentrations, expressed as geometric means (with 95% confidence intervals); P values calculated by analysis of variance

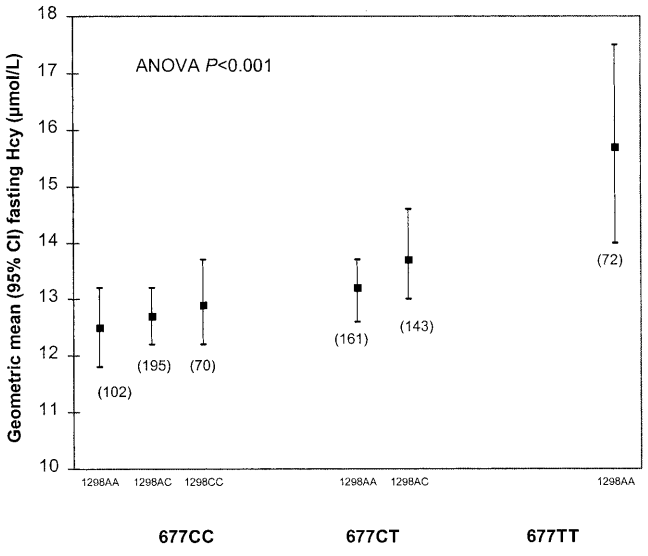
Genotype	n	Homocysteine concentration (μmol/l)	
		Fasting	Postload
677			
CC	378	12.7 (12.4–13.1)	38.9 (37.8–40.1)
CT	317	13.5 (13.1–14.0)*	41.2 (39.8–42.7)*
TT	74	15.6 (14.0–17.4)**,***	46.0 (42.1–50.4)**,4**
P		<0.001	<0.001
1298			
AA	337	13.5 (13.0–14.0)	40.6 (39.3–42.1)
AC	340	13.1 (12.7–13.6)	40.5 (39.0–41.9)
CC	70	12.9 (12.2–13.7)	38.7 (36.6–41.1)
P		n.s.	n.s.

*P<0.05 677CT vs. 677CC, **P<0.001 677TT vs. 77CC, ***P<0.005 677TT vs. 677CT, 4*P<0.05 677TT vs. 677CT (Bonferroni t-test)

Homocysteine levels

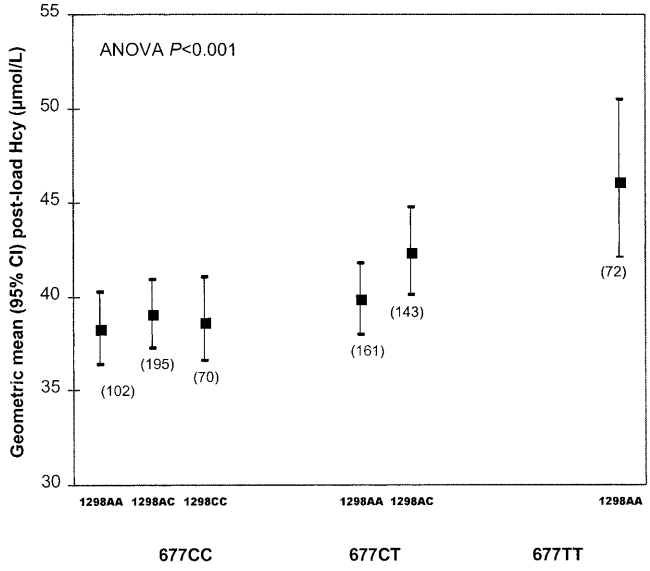
Because separate analysis of the relationship between MTHFR genotype and homocysteine in patients and controls showed the same results (data not shown), we combined the groups to increase statistical power. Table 4 shows the individual effects of the 677C→T and the 1298A→C genotypes on the homocysteine levels. Individuals with the 677TT or 677CT genotype had significantly higher fasting and postload homocysteine levels than 677CC homozygotes (P<0.001 and P=0.003, respectively). The 1298A→C mutation alone showed no effect on fasting or postload homocysteine levels.

Figure 2 shows the combined effects of the 677C→T and the 1298A→C genotypes on both fasting and postload homocysteine levels. Individuals with the composite 677CT/1298AC genotype had slightly higher geometric mean fasting (13.7 μmol/l, 95% CI 13.0–14.6) and postload (42.4 μmol/l, 95% CI 40.1–44.8) homocysteine levels than those with the 677CT/1298AA genotype (13.2 μmol/l, 95% CI 12.6–13.7; and 39.9 μmol/l, 95% CI 38.0–41.8, respectively), but these differences were not statistically significant (P=0.18 and P=0.08, respectively).



P<0.001 for 677TT/1298AA vs 677CC/1298AA;
677TT/1298AA vs 677CC/1298AC;
677TT/1298AA vs 677CC/1298CC
P<0.005 for 677TT/1298AA vs 677CT/1298AA

A



P<0.005 for 677TT/1298AA vs 677CC/1298AA
P<0.05 for 677TT/1298AA vs677CT/1298AA;
677TT/1298AA vs 677CC/1298CC

B

Fig. 2 Relationship between composite MTHFR genotypes and fasting (**A**) and postload (**B**) homocysteine concentrations. *Figures in parentheses* Number of individuals in each genotype group

Discussion

As reported previously by us [17, 22, 26], the MTHFR 677C→T mutation has a significant effect on MTHFR activity. Both the heterozygous 677CT and the homozygous 677TT genotypes are associated with significantly lower MTHFR enzyme activities than the homozygous wild-type genotype. The 677TT genotype is the genetic basis of the so-called thermolabile MTHFR [17]. Some mutations may in particular affect the stability of the enzyme, which becomes manifest at studying thermolability [17, 19].

In this study the individuals with the 677CT or 677TT genotype had significantly lower MTHFR activity and higher homocysteine levels than 677CC individuals. When the MTHFR enzyme activities of the two polymorphisms were analyzed independently, the 1298AC genotype was seen to be associated with a significant increase in residual MTHFR activity (expressed as a percentage of the specific activity), which suggests that the 1298A→C polymorphism affects the thermostability of the protein. After the 677C→T genotype is taken into account, the 1298A→C variant appears to have a significant effect on the specific MTHFR enzyme activity. However, a significant difference in the percentage of residual activity after heat incubation between the 1298A→C genotypes is not observed, suggesting that this variant does not affect the thermostability of the enzyme. This shows that any conclusions about effect of a single polymorphism must be handled carefully, and that the effect must be confirmed in a bacterial expression system before any firm conclusions can be drawn concerning the effect of the putative polymorphism.

Although the 1298A→C polymorphism does have a significant effect on the MTHFR activity (Fig. 1A), neither the 1298AC genotype nor 1298CC genotype results in elevated homocysteine levels. Earlier studies [27, 28] have shown similar findings of the effect of the 1298A→C polymorphism on MTHFR activity; the 677CC/1298AC and 677CC/1298CC genotypes show MTHFR activities of 60–92% and 52–66% compared with the 677CC/1298AA genotypes. The combined heterozygotes (677CT/1298AC) show MTHFR activities between 36 and 62% of the activity associated with the 677CC/1298AA genotype. In these two studies no effect of the 1298A→C polymorphism on homocysteine concentrations was observed. Friedman et al. [29] however, did find a lower homocysteine concentration of the 677CC/1298CC genotype than with the 677CC/1298AA genotype, suggesting that the 1298A→C variant affects homocysteine concentrations. Furthermore, in our earlier study [20] compound heterozygotes had a higher homocysteine concentration than 677CT/1298AA individuals. Jacques et al. [30] reported that subjects who were homozygous for the 677C→T polymorphism had elevated homocysteine concentrations only in the presence of low folate status (<15.4 nmol/l). Girelli et al. [31] showed that in individuals with folate levels below the median (<11.5 nmol/l) fasting homocysteine was significantly higher not only in 677TT but also in 677CT individuals than in 677CC homozygotes. Therefore folate status may explain the discrepancy in the results of the above studies. Other environmental factors, such as nutritional intake, ethnic differences, and body mass index, have also

been reported to affect homocysteine concentrations [32]. A meta-analysis by Jee et al. [33] revealed an association of the 677C→T polymorphism with increased risk of CVD in Japan but not in other populations.

Another plausible explanation is that the decreased MTHFR enzyme activity must reach a certain threshold level before it results in increased plasma homocysteine concentrations. If this threshold level is between 12.61 and 7.89 nmol formaldehyde formed per milligram of protein per hour, i.e., between the level of the combined heterozygotes and the level of the 677TT/1298AA genotype (Fig. 1A), this could explain the different effects of these two genotypes on homocysteine concentrations in this study.

The genotype distributions were similar in patients and controls, indicating that no particular MTHFR genotype is associated with increased CVD risk. The effect of the 1298A→C polymorphism in the MTHFR gene is reflected in the enzyme activity, but a significant effect on homocysteine levels was not found. Since homocysteine itself is considered to be positively associated with the risk of CVD, the results of this study indicate that the 1298A→C mutation cannot be considered a major risk factor for CVD.

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