Thermolabile methylenetetrahydrofolate reductase, homocysteine, and cardiovascular disease risk: the European Concerted Action Project[1–3]

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ABSTRACT

Background: Homozygotes for the thermolabile mutation (*TT* genotype) of the methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20) enzyme have elevated plasma concentrations of the cardiovascular disease risk factor homocysteine, particularly if folate depleted.

Objective: We examined the relations between thermolabile MTHFR, plasma homocysteine, plasma folate, and vascular disease risk.

Design: This was a case-control comparison in 711 vascular disease cases and 747 controls from 9 European countries.

Results: The *TT* genotype was associated with higher homocysteine and lower plasma folate than the *CC* and *CT* genotypes in both cases and controls and a nonsignificant increase in vascular disease risk (1.26; 95% CI: 0.88, 1.81; $P = 0.20$). The frequency of the *TT* genotype in cases was not significantly different from that in controls (12.8% compared with 10.8%). After adjustment for traditional risk factors, the *TT* genotype was associated with an odds ratio of 1.48 (1.0, 2.20) for risk of vascular disease. This risk was attenuated after further adjustment for homocysteine. In subgroups with homocysteine concentrations ≥ 9 μmol/L, risk tended to be higher in *CC* than in *TT* subjects. However, *CC* subjects were characterized by a higher prevalence of the conventional risk factors associated with both elevated plasma homocysteine and serum creatinine. After adjustment, the risk of vascular disease associated with each genotype was not significantly different.

Conclusions: There was a strong graded association between homocysteine and vascular risk in all genotypes. *MTHFR* genotype is a key determinant of plasma total homocysteine concentrations. The initially nonsignificant risk estimate associated with the *TT* genotype was strengthened after adjustment for conventional cardiovascular disease risk factors but was attenuated after adjustment for plasma folate and total homocysteine. The modest risk increase conferred by the *TT* genotype is mediated mainly by increased total homocysteine and low plasma folate concentrations. *Am J Clin Nutr* 2003;77:63–70.

KEY WORDS Homocysteine, methylenetetrahydrofolate reductase, MTHFR, ischemic heart disease, vascular disease risk, folate, European Concerted Action Project

INTRODUCTION

Plasma concentrations of the cardiovascular disease risk factor total homocysteine (tHcy) (1–3) are modulated by several factors (4), including the activity of methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20). This enzyme catalyzes the formation of 5-methyltetrahydrofolate, the methyl donor in the remethylation of homocysteine to methionine (5). A variant of MTHFR, characterized by reduced activity and thermolability (6) and attributed to a C-to-T nucleotide transition at position 677 (677C→T; 7, 8), has been associated with raised plasma tHcy concentrations and with risk of ischemic heart disease (9). Many further studies have examined thermolabile MTHFR as a risk factor in vascular disease, but with few exceptions (10–14), the results of most of these studies (14–32) do not support such an association.

These inconsistencies in the association between thermolabile MTHFR and cardiovascular disease risk may relate to differences in the folate status of the various study populations (16, 22, 23). The thermolabile homozygous (*TT*) *MTHFR* genotype may be associated with elevated tHcy only in the presence of low serum folate (16, 22, 23, 33–35), and the same genotype could be associated with variable risk depending on folate status. In addition, the effect of genotype on risk may not be similar for all categories of vascular disease, as indicated by a recent meta-analysis (25). Finally, the ethnic origin of the population under study may be important, because hyperhomocysteinemia and the thermolabile *MTHFR* genotype may

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TABLE 1
Methylenetetrahydrofolate reductase genotype distribution and concentrations of total homocysteine (tHcy) and plasma folate in 747 controls and 711 cases

	All subjects	Genotype			*P* for main effect of genotype[1]
		CC	*CT*	*TT*	
No. of subjects [*n* (%)]					
Controls	747 (100)	352 (47.1)	314 (42.1)	81 (10.8)	
Cases	711 (100)	313 (44.0)	307 (43.2)	91 (12.8)	
P[2]	0.36				
tHcy (μmol/L)[3]					
Controls	9.8 ± 0.12	9.3 ± 0.11	9.7 ± 0.12	12.2 ± 0.17	
Cases	11.3 ± 0.16	10.7 ± 0.14	10.9 ± 0.15	14.5 ± 0.23	<0.001
P[4]	<0.001				
Plasma folate (nmol/L)[3]					
Controls	9.7 ± 0.22	9.8 ± 0.21	10.0 ± 0.22	8.6 ± 0.23	0.02
Cases	8.3 ± 0.25	8.4 ± 0.23	8.3 ± 0.26	7.7 ± 0.27	
P[4]	<0.001				

[1] Main effect comparison between genotypes (ANOVA for the interaction term was nonsignificant and omitted).
[2] Comparison of genotype distribution between cases and controls (chi-square).
[3] Geometric $\bar{x}$ ± SD on log 10 scale.
[4] Comparison between cases and controls (ANOVA for the interaction term was nonsignificant and omitted).

interact with other genetic traits that are associated with thrombophilia (14, 36, 37). The present case-control study from the European Concerted Action Project (3) examines the relations between the thermolabile *MTHFR* polymorphism, folate status, tHcy concentrations, and cardiovascular disease risk.

SUBJECTS AND METHODS

Between 1990 and 1992, the European Concerted Action Project "Homocysteine and Vascular Disease" recruited 750 cases with established coronary, cerebral, or peripheral vascular disease and 800 age and sex-matched controls from 19 centers in 9 European countries. The selection criteria for the cases and controls were reported previously (3). Briefly, subjects with coronary artery, cerebrovascular, and peripheral vascular disease were included. More than one-half of the frequency-matched control subjects were selected from free-living communities, one-third came from medical insurance screening programs, and one-sixth were selected from hospital staff members. All subjects were < 60 y old. For inclusion, cases had to have clinical and objective evidence of new onset vascular disease and controls had to be free of overt vascular disease. Subjects with diabetes, thyroid disease, exposure to nitrous oxide within 3 mo of the index vascular event, severe renal impairment, or evidence of nonatherosclerotic vascular disease were excluded. The consent of the local ethics committee was sought and obtained.

All blood samples, collected ≥ 3 mo after the index vascular event, were drawn and placed on ice, protected from light, and stored at −70 °C within 1 h of appropriate sample preparation. Fasting plasma tHcy concentrations were measured by HPLC and fluorescence detection (38). Plasma folate was measured by using a microbiological assay (39).

Genotype analysis

Genotyping was carried out in diluted blood samples (1:20 in 1% ascorbic acid) originally prepared for the determination of red blood cell folate. In ≈70% of the samples, genotyping was successful with the conventional technique developed by Frosst et al (8), in which ≈500 μL diluted blood is used. Samples were also genotyped in 10 μL diluted blood with the use of a multiinjection capillary-electrophoresis technique (40); ≈92% of the samples were assayed by this method. In 86 subjects, insufficient blood was available and genotyping was performed by using DNA remnants in plasma as the template, as previously described (40).

There was good agreement (> 90% concordance) between the results obtained by use of the conventional technique and those obtained by use of capillary electrophoresis and laser-induced fluorescence detection of DNA fragments. Discrepancies were resolved by performing additional analysis with the capillary electrophoresis–laser-induced fluorescence readout, because experience to date suggests that this method is more sensitive.

Statistical analysis

Geometric mean values of fasting tHcy and plasma folate concentrations were derived because of the positively skewed distributions of these variables. Linear regression on the log-transformed variables was also used. Two-way analysis of variance with interaction terms was used to examine the relations between fasting tHcy (as a continuous variable) and concentrations of plasma folate and genotype. Conditional logistic regression analysis was carried out to obtain odds ratios of vascular disease, allowing for stratification by age, sex, and center, and to adjust for other variables. Analyses were performed with SAS (versions 6.12 and 8.2 for WINDOWS; SAS Institute Inc, Cary, NC).

RESULTS

Blood indexes and genotypes in cases and controls

Of a total of 1550 subjects (cases and controls), the *MTHFR* 677C→T polymorphism was determined for 1458. The frequencies of the *CC*, *CT*, and *TT* genotypes among cases (n = 711) and controls (n = 747) are shown in **Table 1**. The frequency of the *TT* genotype was not significantly different between cases (12.8%) and controls (10.8%; P = 0.36).

Fasting tHcy concentrations were significantly higher in cases than in controls, and for both groups combined, fasting tHcy concentrations were significantly higher in *TT* homozygotes than in *CC* homozygotes or *CT* heterozygotes (Table 1). Plasma folate concentrations were significantly lower in cases than in controls

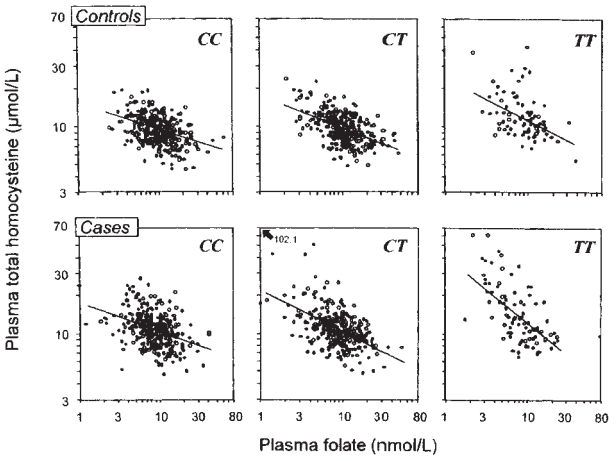

FIGURE 1. Plasma total homocysteine and plasma folate concentrations according to methylenetetrahydrofolate reductase genotype and vascular disease status. *CC*, nonthermolabile homozygotes, $n = 352$ controls, 313 cases; *CT*, thermolabile heterozygotes, $n = 314$ controls, 307 cases; *TT*, thermolabile homozygotes, $n = 81$ controls, 91 cases.

TABLE 2
Odds ratios (95% CIs) for risk of all vascular disease and of ischemic heart disease according to methylenetetrahydrofolate reductase genotype

	All vascular disease ($n = 711$)	Ischemic heart disease ($n = 356$)
Model 1[1]		
CC	1.0	1.0
CT	1.0 (0.77, 1.29)	1.14 (0.83, 1.59)
TT	1.48 (1.0, 2.20)	1.87 (1.13, 3.11)
Model 2[2]		
CC	1.0	1.0
CT	0.98 (0.75, 1.26)	1.13 (0.81, 1.57)
TT	1.14 (0.75, 1.73)	1.49 (0.97, 2.53)
Model 3[3]		
CC	1.0	1.0
CT	0.97 (0.75, 1.26)	1.14 (0.82, 1.59)
TT	1.16 (0.77, 1.76)	1.54 (0.90, 2.63)

[1] Adjusted for age, center, and sex and also adjusted for smoking, hypertension (systolic blood pressure >160 mm Hg, diastolic blood pressure >95 mm Hg, or receiving treatment) and hyperlipidemia (total cholesterol ≥6.5 mmol/L or receiving treatment).

[2] As model 1 but also adjusted for total homocysteine.

[3] As model 2 but also adjusted for plasma folate.

(Table 1), and plasma tHcy increased as a function of decreasing folate concentrations for all genotypes (**Figure 1**). Although not statistically significantly different, this inverse relation tended to be more pronounced in *TT* than in *CT* and *CC* subjects and in cases than in controls.

MTHFR genotype, folate, and tHcy as independent risk factors

Compared with the *CC* genotype, the odds ratio for risk of vascular disease, adjusted for age, sex, and center, associated with the *TT* genotype was statistically nonsignificant: 1.26 (95% CI: 0.88,1.81; $P = 0.20$). However, after adjustment for the conventional risk factors, the association was strengthened (odds ratio: 1.48; 95% CI: 1.00, 2.20; **Table 2**). Further adjustment for tHcy weakened this association, indicating that the relation between MTHFR and risk was mediated largely by an elevated tHcy concentration. Adjustment for plasma folate had no further effect on the relation (Table 2), and additional adjustment for creatinine in any of the 3 models used had no effect on the significance of the relation (data not shown).

The adjusted risk conferred by the *TT* genotype was investigated separately in patients with ischemic heart disease ($n = 356$), peripheral vascular disease ($n = 205$), and cerebrovascular disease ($n = 150$). This analysis showed that the increased risk was confined to patients with ischemic heart disease (odds ratio, adjusted for conventional risk factors: 1.87; 95% CI: 1.13, 3.11; Table 2); the effect was nonsignificant in the other vascular disease groups (data not shown). Once again, adjustment for creatinine had no significant effect on the strength of the relation (data not shown).

Considering the relation between vascular disease risk and plasma folate concentration irrespective of genotype, decreasing plasma folate concentrations significantly increased the risk of vascular disease. With the use of a plasma folate concentration >10.76 nmol/L as a reference (top two-fifths of the controls), the odds ratio for disease associated with a concentration ≤6.6 nmol/L (bottom one-fifth of the controls) was 1.84 (95% CI: 1.32, 2.57) after adjustment for conventional cardiovascular disease risk factors (P for trend across folate quintiles = 0.004). This relation lost significance when additionally adjusted for tHcy (odds ratio: 1.41; 95% CI: 0.98, 2.03; P for trend across folate quintiles = 0.15 after adjustment for tHcy).

The effect of plasma folate on the genotype-risk relation was also examined. Within each stratum of plasma folate, the *TT* genotype was associated with a nonsignificantly increased risk of all vascular disease and particularly ischemic heart disease (data not shown). This effect was more marked, but still nonsignificant, when adjustment was made for conventional risk factors.

MTHFR genotype, tHcy, and risk

The relations between genotype, tHcy, and risk are examined in **Table 3**. Increasing fasting tHcy concentrations were associated with a significantly increased risk of vascular disease in all subjects. The strength of this relation was weakened but still significant after adjustment for the conventional risk factors (Table 3) and plasma folate concentration (data not shown).

Although not an a priori hypothesis, we explored the possibility that the vascular disease risk associated with any given concentration of tHcy might differ according to genotype. We used conditional logistic regression to obtain odds ratios for vascular disease at increasing tHcy concentrations for each genotype. With the use of a tHcy concentration <9 μmol/L in *CC* subjects as a reference, an approximate 5-fold increase in risk at tHcy concentrations >15 μmol/L in *CC* subjects after adjustment for age, sex, and center was noted (1.0 compared with 4.98). In *TT* subjects after adjustment for age, sex, and center, a 3-fold increase in risk was observed only at tHcy concentrations >30 μmol/L (1.82 compared with 6.03). Hence, at intermediate tHcy concentrations (15–30 μmol/L), the risk of disease tended to be higher in *CC* than in *TT* subjects. After adjustment for serum creatinine, smoking, hypercholesterolemia, and hypertension, the odds ratio for vascular disease risk in each genotype was not significantly different: [3.38 (95% CI: 1.49, 7.67) compared with 3.43 (95% CI: 1.62, 7.28)].

TABLE 3
Case and control distributions according to methylenetetrahydrofolate reductase genotype and total homocysteine (tHcy) concentration and odds ratio (OR) for vascular disease[1]

tHcy	No. of subjects (controls/cases)			Risk of vascular disease OR (95% CI)[2]				Risk of vascular disease OR (95% CI) adjusted[3]			
(μmol/L)	*CC*	*CT*	*TT*	All	*CC*	*CT*	*TT*	All	*CC*	*CT*	*TT*
<9	164/92	133/87	16/17	1.0	1.0	1.05 (0.71, 1.55)	1.82 (0.85, 3.88)	1.0	1.0	1.10 (0.72, 1.70)	1.90 (0.84, 4.32)
9–14.99	177/180	164/182	47/39	1.47 (1.14, 1.89)	1.59 (1.17, 2.26)	1.6 (1.12, 2.29)	1.36 (0.81, 2.30)	1.48 (1.12, 1.96)	1.67 (1.13, 2.46)	1.54 (1.03, 2.29)	1.70 (0.95, 3.02)
≥15	11/41	17/38	18/35	3.26 (2.14, 4.98)	4.98 (2.36, 10.51)	2.92 (1.49, 5.73)	2.99 (1.52, 5.89)	2.90 (1.81, 4.65)	3.38 (1.49, 7.67)	2.74 (1.31, 5.73)	3.43 (1.62, 7.28)
15–20	11/29	15/23	9/12	2.42 (1.48, 3.95)	4.96 (2.35, 10.46)	2.24 (1.06, 4.75)	2.13 (0.8, 5.66)	2.02 (1.16, 3.51)	3.37 (1.49, 7.66)	1.87 (0.81, 4.29)	2.99 (1.03, 8.71)
20–30	0/12	2/11	7/14	5.44 (2.47, 11.99)	—	7.15 (1.5, 32.9)	3.19 (1.16, 8.77)	5.60 (2.38, 13.19)	—	8.90 (1.80, 44.12)	3.56 (1.17, 10.88)
>30	0/0	0/4	2/9	7.73 (1.66, 36.04)	—	—	6.03 (1.22, 29.77)	6.49 (1.26, 33.32)	—	—	4.78 (0.81, 28.03)

[1] Risk of vascular disease for all genotypes together is based on a comparison with the reference group with a tHcy concentration <9 μmol/L. Risk of vascular disease for individual genotypes is based on a comparison with the reference *CC* group with a tHcy concentration <9 μmol/L. The last 3 lines of the table refer to the tHcy distribution >15 μmol/L.

[2] In model 1, adjustment was made for age, sex, and center. Risk of vascular disease is considered first in all genotypes together as well as individually.

[3] As model 1, but also adjusted for creatinine, smoking, hypertension (systolic blood pressure >160 mm Hg, diastolic blood pressure >95 mm Hg, or receiving treatment) and hyperlipidemia (total cholesterol ≥6.5 mmol/L or receiving treatment)

TABLE 4
Prevalence of selected characteristics among controls and cases according to plasma total homocysteine (tHcy) concentration and thermolabile methylenetetrahydrofolate reductase genotype

Variable	Total population	tHcy < 9 μmol/L			tHcy 9–15 μmol/L			tHcy > 15 μmol/L		
		CC	*CT*	*TT*	*CC*	*CT*	*TT*	*CC*	*CT*	*TT*
No. of subjects										
Controls	747	164	133	16	177	164	47	11	17	18
Cases	711	92	88	17	180	181	39	41	38	35
Mean age (y)										
Controls	43.9	40.1	41.6	42.8	46.6	45.9	41.4	47.5	48.9	37.8
Cases	47.2	45.8	43.4	46.7	48.0	47.9	49.4	51.2	47.4	45.1
Male (%)										
Controls	72.0	62.8	55.6	75.0	80.2	82.9	72.3	90.9	82.4	72.2
Cases	73.0	55.4	60.2	47.1	84.4	76.8	66.7	80.5	71.1	85.7
Smokers (%)										
Controls	33.2	33.5	33.1	37.5	39.6	25.0	36.2	36.4	35.3	27.8
Cases	55.0	50.0	40.9	52.9	58.3	55.3	51.3	73.2	71.1	51.4
Hypertension (%)[1]										
Controls	11.9	10.4	11.3	0.0	10.7	16.5	8.5	9.1	23.5	11.1
Cases	38.1	35.9	31.8	35.3	38.3	39.8	33.3	61.0	42.1	25.7
Hyperlipidemia (%)[2]										
Controls	36.8	34.2	22.6	25.0	42.9	45.7	27.8	36.4	58.8	33.3
Cases	52.9	45.7	52.3	47.1	55.6	57.5	48.7	61.0	50.0	37.1
Creatinine > 100 μmol/L (%)										
Controls	1.5	0.0	0.8	0.0	1.7	3.1	2.1	9.1	0.0	0.0
Cases	4.2	1.1	0.0	0.0	3.3	4.4	0.0	19.5	13.2	5.7
Plasma folate < 6.6 nmol/L (%)										
Controls	18.8[3]	9.8	2.3	12.5	24.4[4]	20.7	29.8	72.7	70.6	44.4
Cases	30.8[5]	14.3[6]	9.2[6]	0.0	31.8[6]	29.4[6]	28.2	53.7	73.0[6]	71.4
Plasma cobalamin < 120 pmol/L (%)										
Controls	5.2	1.8	1.5	0.0	8.5	6.1	2.1	9.1	11.8	27.8
Cases	6.5	5.4	1.1	0.0	3.3	7.2	7.7	14.6	21.1	14.3

[1] Systolic blood pressure > 160 mm Hg, diastolic blood pressure > 95 mm Hg, or receiving treatment.
[2] Serum total cholesterol ≥ 6.5 mmol/L or receiving treatment.
[3] One control missing: based on 746 controls.
[4] One control missing.
[5] Five cases missing: based on 706 cases.
[6] One case missing.

At higher tHcy concentrations (20–30 μmol/L), *TT* subjects were still at increased risk compared with those with lower tHcy concentrations. However, the CIs widened, reflecting the small numbers of subjects with these higher tHcy concentrations.

Determinants of elevated tHcy and vascular disease according to genotype

In total, 89 of 747 controls and 271 of 711 cases were hypertensive (systolic blood pressure > 160 mm Hg, diastolic blood pressure > 95 mm Hg, or receiving treatment), whereas 275 of 747 controls and 376 of 711 cases were hyperlipidemic (total cholesterol ≥ 6.5 mmol/L or receiving treatment). Because the effect of adjustment for conventional risk factors differed in *CC* and *TT* subjects at higher tHcy concentrations (> 15 μmol/L), we examined the frequencies of determinants of both plasma tHcy concentration and cardiovascular disease risk (4) in each genotype (**Table 4**).

The frequency of the *TT* genotype was significantly higher in those with plasma tHcy concentrations > 15 μmol/L (39% of controls, 31% of cases) than in those with concentrations between 9 and 15 μmol/L (12.1% of controls, 10% of cases) or < 9 μmol/L (5.0% of controls, 8.6% of cases) ($P < 0.01$; Table 4). Low concentrations of plasma folate (< 6.6 nmol/L) and cobalamin (< 120 pmol/L) were found significantly more often among those with tHcy > 15 μmol/L than in those with lower tHcy or in the study group as a whole ($P < 0.01$), but case-control differences in plasma vitamin concentrations were not seen in this group.

Cases with tHcy concentrations > 15 μmol/L had significantly higher frequencies of conventional cardiovascular disease risk factors than did cases with concentrations < 15 μmol/L ($P < 0.01$). As expected, cases overall had a more adverse risk factor profile than did controls, but this relation was more evident in cases with the *CC* and *CT* genotypes than in those with the *TT* genotype. Therefore, among cases with plasma tHcy concentrations > 15 μmol/L, individuals with the *TT* genotype had a lower prevalence and individuals with the *CC* genotype had a higher prevalence of conventional risk factors than did the remainder of the study population.

DISCUSSION

The relation between elevated plasma tHcy and vascular disease risk is independent of traditional risk factors (1, 41). Although the association is stronger in case-control studies (41) and in subjects with established vascular disease (42–44) than in prospective studies (29, 45–48), a recent meta-analysis of prospective studies indicates a robust relation (49). Two further studies

suggest that the plasma tHcy concentration is a strong predictor of cardiovascular events in the early follow-up period and that this risk relation may become attenuated over time (50, 51). Because the *TT MTHFR* genotype is associated with increased plasma tHcy concentrations, this mutation could be associated with increased risk of vascular disease (8). In contrast with 4 studies (10–13), a meta-analysis of 23 studies failed to support such a hypothesis (52). However, Kluijtmans et al (25) suggested that risk due to the *TT* genotype may be confined to those with coronary disease.

The large size of the present study facilitated an examination of the thermolabile *MTHFR* genotype as an independent risk factor for vascular disease and of the relations between genotype, tHcy, plasma folate, and risk. However, the inclusion criterion of age < 60 y at the time of diagnosis may have resulted in the selection of cases with a heavy burden of cardiovascular disease risk factors. Testing the strength of these relations would be best undertaken prospectively. A case-control study can include only surviving cases, and if the *TT* genotype were associated with an adverse prognosis, subjects with this trait would be underrepresented among cases. So far, the evidence seems inconsistent (53–56). Another potential limitation of the present study is the population variation in allele frequencies (10, 25, 34, 37, 57). Because the estimated risk is based on a summary odds ratio, the role of MTHFR within any one group may be masked. However, because the plasma tHcy–MTHFR relation is folate dependent (34), although the *TT* genotype may predict disease in a population with low plasma folate concentrations (58), a multinational study provides an opportunity to examine a wider range of folate concentrations. In addition, although not measured in the present study, recent evidence suggests that the plasma riboflavin concentration should also be considered in any analysis of the relation between *MTHFR* genotype, tHcy concentration, and vascular disease risk (59). Finally, impaired renal function is associated with raised plasma tHcy concentrations, and the glomerular filtration rate surpasses serum creatinine as a predictor of plasma tHcy concentrations (60, 61). Because it was not practicable to estimate the glomerular filtration rate in so large a study, we controlled for serum creatinine. Therefore, it remains possible that the higher tHcy concentrations found in cases was a consequence of vascular disease–mediated impairment of renal function not reflected in circulating creatinine concentrations.

In both cases and controls we found higher tHcy concentrations in *TT* subjects with low folate concentrations, a relation consistently shown by others (14, 22, 28, 34, 35, 52). Plasma tHcy concentrations in *TT* subjects may be more sensitive to folate (Figure 1). Univariate analysis showed a nonsignificant association between *TT* genotype and vascular risk, but after adjustment for risk factors that are associated with elevated plasma tHcy concentrations (4, 41), the *TT* genotype (compared with *CC*) was independently associated with an ≈50% increase in risk. In contrast with earlier work indicating that risk is confined to those with low plasma folate concentrations (22, 33, 35), a nonsignificant increase in risk was found across the spectrum of the plasma folate distribution. The sample size does not allow a definitive conclusion to be drawn but, as in the meta-analysis by Kluijtmans et al (25), when patients with ischemic heart disease were analyzed separately, the increase in risk was higher. In the total case population and in the ischemic heart disease patients, the odds ratio for risk of disease was markedly reduced by further adjustment for tHcy. On this basis but without implying causality, it is reasonable to suggest that the effect of the *TT* genotype on vascular risk may be mediated through high plasma tHcy and low plasma folate concentrations. Others have taken the weakness of the relation between the *TT* genotype and risk as evidence that the relation between elevated tHcy and vascular risk is itself not significant (52, 62), but the present analysis does not support this view.

The increase in risk associated with the *TT* genotype may seem small considering that the tHcy concentration is usually 2–4-μmol/L higher in *TT* than in *CC* subjects (52) (Table 1). Some prospective (63, 64) and case-control (1, 41) studies suggest a 20–40% increase in vascular risk caused by such an increase in tHcy. Therefore, our finding that the *TT* genotype is associated with a 50% increase in risk is slightly higher than expected.

In contrast with earlier studies of the *MTHFR* genotype and despite assuming, perhaps incorrectly (65), that genotype should not affect the distribution of risk factors, we adjusted for those factors that influence plasma tHcy concentrations (4, 41). This adjustment led to different effects on the risk associated with each genotype. In an exploratory analysis, we examined the prevalence of smoking, hypertension, hypercholesterolemia, and elevated creatinine in the separate genotypes at various concentrations of tHcy. Notably, *TT* cases with tHcy concentrations > 15 μmol/L had significantly lower frequencies of these risk factors than did the remainder of the population. By inference, the elevated tHcy concentration was attributed to poor B vitamin status associated with the *TT* genotype itself. In contrast, in hyperhomocysteinemic *CC* and *CT* subjects (especially cases), the frequencies of these traditional risk factors were higher. Because a markedly high mortality rate among *TT* subjects seems unlikely (55), an explanation for this unexpected observation is that the etiology of hyperhomocysteinemia differs between the genotypes. In *CC* subjects, the risk factor profile that "qualifies" them for entry to a case-control study is mediated by conventional risk factors and hyperhomocysteinemia, whereas in *TT* subjects, the risk is largely attributed to isolated hyperhomocysteinemia due to folate deficiency. Interaction effects between tHcy and conventional risk factors (3) may explain why the relation between tHcy and vascular disease risk or mortality seems to be consistently stronger in patients with preexisting disease and in the early follow-up period. Risk relations are stronger in case-control studies (1, 41) and in prospective studies of patients with coronary artery disease (44), renal failure (43), or systemic lupus erythematosus (42) than in prospective studies of subjects who are initially healthy (45, 46, 63, 64).

In conclusion, elevated tHcy is an independent cardiovascular disease risk factor in the 3 *MTHFR* genotypes. In *TT* subjects, the increased risk is associated with elevated tHcy largely attributable to low folate status, whereas in *CC* and *CT* subjects, additional determinants of tHcy and risk such as smoking and hypertension may enhance vascular risk. These interaction effects may explain why *CC* and *CT* subjects tend to have higher risk than do *TT* subjects at a similar tHcy concentration. Multivariate analysis suggests that the *TT* genotype, mostly through its effect on the tHcy concentration, is associated with a modest but significant risk, particularly in patients with ischemic heart disease.

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