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Determinants of Fasting and Post-Methionine Homocysteine Levels in Families Predisposed to Hyperhomocysteinemia and Premature Vascular Disease

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Abstract—Elevated plasma total homocysteine (tHcy) levels, either measured in the fasting state or after oral methionine loading, are associated with an increased risk of atherothrombotic disease. Fasting and post-methionine hyperhomocysteinemia (HHC) overlap to a limited extent; both can occur as familial traits. We investigated determinants of fasting, postmethionine and delta (ie, post-methionine minus fasting levels) tHcy levels in 510 subjects of 192 HHC-prone families including 161 patients with clinical vascular disease and 349 without vascular disease. We focused on tHcy levels in relation to levels of vitamin B_{12}, B_6 and folate and the methylenetetrahydrofolate reductase (MTHFR) C677T mutation. Multivariate linear analyses adjusted for the presence of vascular disease showed that fasting tHcy was significantly related to folate and vitamin B_{12}, and the presence of the MTHFR *TT* genotype and the *T* allele, and to age, smoking habits, and serum levels of creatinine. Both post-methionine and delta tHcy levels were related to serum folate levels, and the presence of the MTHFR *TT* genotype and the *T* allele, and to postmenopausal status, and body mass index. An interaction was found between MTHFR *TT* genotype and serum folate levels for both fasting and post-methionine tHcy, ie, for a given decrease in serum folate, homocysteine levels increased more in subjects with the *TT* genotype than in those with the *CC* genotype. Fasting, post-methionine and delta tHcy were higher in patients with vascular disease than in their healthy siblings, but these levels were less dependent on serum folate levels ($P<0.05$), whereas the effect of MTHFR genotype was stronger ($P=0.01$). This study found evidence that post-methionine and delta tHcy levels are not only influenced by factors affecting homocysteine transsulfuration but also by factors that affect remethylation. The explained variances of fasting, post-methionine and delta tHcy were 49%, 62%, and 78%, respectively. We also found evidence, in patients with premature vascular disease but not in their healthy siblings, for a factor that increases tHcy levels but weakens the normal inverse relation between folate and tHcy and amplifies the effect of the MTHFR genotype. **(*Arterioscler Thromb Vasc Biol*. 1999;19:1316-1324.)**

Key Words: homocysteine ■ methionine loading test ■ methylenetetrahydrofolate reductase ■ vitamins ■ familial hyperhomocysteinemia ■ premature vascular disease

Elevated plasma total homocysteine (tHcy) levels, either measured in the fasting state or after oral methionine loading, are associated with an increased risk of atherothrombotic disease.[1–4] Fasting and post-methionine hyperhomocysteinemia (HHC) overlap to a limited extent,[3,5–8] so that it is of interest to gain more insight in the determinants of both fasting and post-methionine tHcy levels.

Important factors governing homocysteine levels are folate, vitamin B_{12} and vitamin B_6 status, and homozygosity for the C677T mutation of the methylenetetrahydrofolate reductase (MTHFR) gene.[3,9–23] Except for vitamin B_6, these variables are thought to be involved in homocysteine remethylation and thus to be associated specifically with the fasting rather than the post-methionine level, the latter being thought to reflect (vitamin B_6-dependent) homocysteine transsulfuration.[24] Recently, fasting tHcy levels have in addition been shown to be positively correlated with male sex, age, smoking, blood pressure and serum levels of cholesterol and creatinine.[25]

Both fasting and post-methionine HHC can occur as familial traits.[6,26–29] It is not known to what extent vitamin and MTHFR mutation status determine fasting and post-methionine tHcy levels in families in which HHC is prevalent. The first aim of this study, therefore, was to investigate this in 510 subjects of 192 HHC-prone families.[6,7] In addition, if fasting and post-methionine tHcy do indeed reflect

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homocysteine remethylation and transsulfuration, respectively, it can be hypothesized that the determinants of post-methionine tHcy differ from those of fasting tHcy, but this has not been extensively studied.[30] The second aim of this study thus was to investigate this by comparing the determinants of fasting with those of post-methionine tHcy. Because the level after methionine loading will, to at least some extent, be influenced by the fasting level, we also studied the delta homocysteine level, ie, the post-methionine minus the fasting level.[5]

Subjects and Methods

Subjects

In April 1993, we started a HHC screening program among consecutive patients with clinically manifest arterial occlusive disease with onset before the age of 55 years. Definitions of vascular disease have been described in detail elsewhere.[6]

From April, 1993, to January, 1995, we screened 737 patients. We then randomly selected 192 patients for family studies, with ≈3:1 oversampling of patients with versus those without HHC after a methionine loading test, as described below. We here report on 510 subjects from 192 different families. Of these 510 subjects, 161 had clinical vascular disease (patients) and 349 did not (healthy siblings). In 31 families the index patient could not be studied. Of the 161 patients, 122 (76%) had post-methionine HHC. Subjects (n=20) using multivitamin supplementation or folic acid, vitamin B_{12} or vitamin B_6 were asked to stop this for at least 5 months before the methionine loading test.

All subjects underwent a methionine loading test and additional investigations, as detailed below. All subjects gave informed consent for this study, which was approved by the local ethics committee.

Methionine Loading Test and Additional Investigations

After an overnight fast, venous blood samples were taken between 9 and 10 AM according to a strict protocol. Subjects were asked to refrain from smoking and from using alcohol from 10 PM on the evening before blood sampling. A second blood sample was obtained 6 hours after an oral methionine load (0.1 g/kg body weight). (One tHcy measurement after 6 hours is commonly used to obtain a reasonable if imperfect description of the area under the tHcy curve after methionine loading.) Plasma was separated immediately after sampling; samples were stored at −30°C until use. Homocysteine was determined within 1 week of blood sampling. Total (free plus protein-bound) homocysteine concentrations were measured by using tri-n-butylphosphine as the reducing agent and ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate as the fluorochromophore, followed by high-pressure liquid chromatography with fluorescence detection.[31] Reference values for fasting and post-methionine homocysteine in our laboratory are <18 and <54 μmol/L, respectively, in men, <15 and <51 μmol/L, respectively, in premenopausal women, and <19 and <69 μmol/L, respectively, in postmenopausal women. (These reference values were obtained from a group of healthy volunteers recruited from the hospital staff who had vitamin B_6, B_{12}, and folic acid concentrations within the reference ranges. The mean ages [SD] for men [n=23] and women [n=41 pre- and n=27 postmenopausal women] were 36.9 [5.7] and 42.3 [9.5], respectively.) Delta homocysteine was defined as the post-methionine minus the fasting homocysteine level.

Just before the methionine loading test, venous blood samples were taken for measurement of serum levels of folate (radioassay, Becton Dickinson; reference, >3.4 nmol/L), vitamin B_{12} (radioassay, Becton Dickinson; reference, >80 pmol/L) and vitamin B_6 (measured as pyridoxal-5-phospate by high-pressure liquid chromatography with fluorescence detection after precolumn derivatization with semicarbazide; reference, >17 nmol/L).[32] We also measured serum creatinine levels (modified Jaffé reaction) and serum lipids (total and high density lipoprotein [HDL] cholesterol and triglycerides [enzymatically]). Low density lipoprotein [LDL] cholesterol was calculated by Friedewald's formula.[33]

For the C677T MTHFR mutation analysis, DNA was obtained from the buffy coat of EDTA blood. The mutation creates a *Hin*fI restriction site, which was used for analysis. The polymerase chain reaction (PCR) conditions and the sequence of the primers used in the amplification of the part of the gene containing the mutation were taken from Frosst et al.[15] *Hin*fI restriction enzyme analysis of the PCR products and subsequent electrophoresis in a 3% agarose gel were used to determine the mutation status of the subject, with *TT*, *CT*, and *CC* indicating subjects homozygous and heterozygous for the mutation and homozygous for the wild type, respectively.

At the time of blood sampling we recorded age, body weight and height, menopausal status (post-menopause was defined as absence of menstrual bleeds for >1 year), presence of hypertension (WHO criteria), and current and past smoking habits. Body mass index was calculated as weight/height.[2]

Statistical Analysis

Data were analyzed with the statistical package SPSS for Windows 6.1. Descriptive data are given as mean (SD) or, when skewed, as median (range). The distributions of homocysteine levels were skewed with a long tail toward high values. Log-transformed homocysteine values were therefore used in the linear regression analyses and the results were transformed back to the orginal scale.

Our main interest was the relation between tHcy levels on the one hand and serum levels of folate, vitamin B_{12} and vitamin B_6, and MTHFR genotype, on the other. We analyzed this in 3 steps. We first did preliminary analyses adjusted only for age and sex, and, because of the selection procedure (see above), for the presence of vascular disease. We next investigated possible confounding variables: menopausal status, body mass index, smoking habits, pack years (calculated by multiplying the number of cigarette packages smoked per day by the number of years the subject smoked), the presence of hypertension, serum lipids and serum creatinine. Third, to examine whether relationships between various determinants of plasma homocysteine concentrations were independent of each other, we entered all variables that had a P value of <0.2 in the preliminary analyses into an extended multivariate linear model, except for the presence of vascular disease and serum vitamin levels, which were entered into all extended multivariate analyses. Relative changes in homocysteine levels per the indicated change in the independent variables (ie, back-transformed regression coefficients) are given with their 95% confidence intervals (CIs). The MTHFR genotype was entered into the regression models in 2 different ways (one at a time): taking *CC* as the reference category, we studied either the contrast between *TT* and *CC* or that between *CT* and *CC*. Furthermore, we investigated the presence of possible interactions among vitamin levels, MTHFR genotype and presence of vascular disease by adding the appropriate interaction terms to the extended multivariate models.

All testing was 2-tailed with 0.05 as the level of significance.

Results

Table 1 shows characteristics of the study population. The distribution of the C677T polymorphism of the MTHFR gene in our study population followed the Hardy-Weinberg equilibrium (data not shown). As expected, the oversampling of patients with versus those without post-methionine HHC resulted in higher fasting, post-methionine and delta tHcy in the patients than in the siblings (all P values <0.0001). They also had a higher prevalence of the MTHFR *T* allele (72.7% versus 62.8%; P=0.03), but not of the *TT* genotype (19.4% versus 16.7%; P=0.45), and were older and were more often smokers, had a higher number of pack years, higher serum levels of creatinine, cholesterol and triglycerides, and lower serum levels of HDL cholesterol, folate, vitamin B_{12} and vitamin B_6 (data not shown).

Fasting Homocysteine Level

The extended multivariate analyses showed that fasting tHcy levels were significantly related to folate and vitamin B_{12}, the

TABLE 1. Laboratory and Clinical Characteristics of 510 Subjects From 192 Families With an Index Patient With Premature Arterial Occlusive Disease

n (%), M/F	249 (48.8)/261 (51.2)
Postmenopausal state, n (% women)	82 (31.4)*
Age, y	44.5±9.7
Preexisting vascular disease, n (%)	161 (31.6)
Plasma homocysteine in the entire population, (μmol/L)†	
Fasting	11.4 (7.2, 8.9, 15.3, 21.8)
Post-methionine	51.6 (29.8, 36.7, 68.0, 88.3)
Delta	36.7 (20.7, 26.2, 49.8, 70.8)
Plasma homocysteine in patients, (μmol/L)‡	
Fasting	14.3 (3.3–98.6)
Post-methionine	61.3 (15.5–214.9)
Delta	44.7 (9.6–147.4)
Plasma homocysteine in siblings, (μmol/L)‡	
Fasting	10.8 (4.8–174.1)
Post-methionine	42.9 (12.7–196.3)
Delta	31.6 (0.4–133.0)
Current smokers, n (%)	219 (42.9)
Current or past smokers, n (%)	328 (64.3)
Pack years‡§	17.5 (1.0–98.0)
Body mass index (kg/m^2)	25.1±4.0
Hypertension, n (%)	115 (22.5)
Creatinine (μmol/L)	85.2±13.6
Total cholesterol (mmol/L)	6.0±1.2
HDL cholesterol (mmol/L)	1.3±0.6
LDL cholesterol (mmol/L)	3.9±1.1
Triglyceride (mmol/L)	1.7±1.0
Vitamin B_6 (nmol/L)‡	27.0 (4.0–272.0)
Vitamin B_{12} (pmol/L)‡	260.0 (53.0–727.0)
Folate (nmol/L)‡	11.4 (0.6–93.0)
Methylenetetrahydrofolate reductase, n (%)‖	
Homozygous normal (*CC*)	167 (34.1)
Heterozygous mutant (*CT*)	237 (48.4)
Homozygous mutant (*TT*)	86 (17.6)
T allele carrier	323 (65.9)

Data are given as number (n) with percentages in parentheses or as mean with SD. Delta homocysteine levels indicate post-methionine levels minus fasting levels.

*Four postmenopausal women used hormone replacement therapy; †Median and 10th, 25th, 75th, and 90th percentile; ‡Skewed data are given as median (range).

§Pack years were calculated by multiplying the number of cigarette packages smoked per day by the numbers of years the patient smoked.

‖In 20 subjects, MTHFR genotype was not available.

presence of the MTHFR *TT* genotype and the *T* allele, patient status, and to age, current smoking, pack years, and serum levels of creatinine (Table 2). When we repeated this analysis with exclusion of folate and vitamin B_{12}, fasting homocysteine levels were not related to vitamin B_6 (relative change per 30 nmol/L increase of vitamin B_6=0.999; CI, 0.998 to 1.0004, P=0.19).

Post-Methionine Homocysteine Level

Post-methionine and delta tHcy were related to serum folate levels, the presence of the MTHFR *T* allele and the MTHFR *TT* genotype, patient status, and to postmenopausal status, and body mass index. When we repeated the extended multivariate analysis with exclusion of folate, post-methionine tHcy was related to vitamin B_6 (relative change per 30 nmol/L increase of vitamin B_6=0.96; CI, 0.92 to 0.99, P=0.03). Delta tHcy was related to vitamin B_6 after exclusion of both vitamin B_{12} and folate (relative change=0.95; CI, 0.90 to 0.99, P=0.05). We repeated the extended multivariate analyses for post-methionine tHcy by adding fasting tHcy to the model, which gives a more precise adjustment for the influence of fasting on post-methionine tHcy than studying delta tHcy. The results were similar, except that the relations with the presence of the MTHFR *T* allele and *TT* genotype were no longer significant (relative change=1.06; CI, 0.98 to 1.15, P=0.17 for the *T* allele and =1.11; CI, 0.97 to 1.26, P=0.13 for the *TT* genotype).

We repeated all analyses after exclusion of subjects with tHcy >60 μmol/L fasting (n=8), >120 μmol/L post-methionine (n=14), and/or >105 μmol/L delta (n=10). The results were similar (data not shown).

Interaction Analyses

We next studied the possibility of an interaction between MTHFR genotype and folate status on tHcy levels.[12] For both fasting and post-methionine tHcy levels, the interactions between serum folate levels and MTHFR *TT* were significant. For each 10-nmol/L decrease in folate levels, fasting and post-methionine tHcy increased with factors of 1.16 and 1.11 in subjects with the *CC* and with factors of 1.74 and 1.31 in those with the *TT* genotype (P=0.0001 and =0.003, Table 3, Figure 1A and 1B). For delta tHcy, the interaction between *TT* genotype and folate status was not significant, although the point estimates were similar to those of post-methionine tHcy (Table 3). Interactions between *CT* genotype and serum folate levels were not significant for any tHcy level (Table 3).

We next found, for fasting, post-methionine and delta tHcy, an interaction between the presence of vascular disease and serum folate. In healthy siblings, each 10-nmol/L decrease of folate levels raised fasting, post-methionine and delta tHcy with, respectively, a factor of 1.36 (CI, 1.24 to 1.46), 1.29 (CI, 1.19 to 1.39), and 1.22 (CI, 1.11 to 1.34), as compared with a factor of 1.06 (CI, 1.01 to 1.14), 1.06 (CI, 1.001 to 1.12), and 1.004 (CI, 1.001 to 1.006) in patients (P<0.05 for all interactions; Figure 2A and 2B).

For post-methionine tHcy, we also found an interaction between serum vitamin B_{12} levels and presence of vascular disease. For each 30-pmol/L decrease in vitamin B_{12}, post-methionine tHcy increased with a factor of 1.008 (CI, 1.001 to 1.02) in siblings versus a factor of 1.04 (CI, 1.01 to 1.06) in patients (P<0.05; Figure 3). For fasting and delta tHcy, the interactions between serum vitamin B_{12} levels and presence of vascular disease were not significant: for each 30-nmol/L decrease in vitamin B_{12}, fasting and delta tHcy increased with factors of 1.008 (CI, 1.007 to 1.011) and 0.99 (CI, 0.98 to 1.01) in siblings versus factors of 1.02 (CI, 1.006 to 1.04) and 1.03 (CI, 0.996 to 1.06) in patients (P for interaction =0.37 and =0.10, respectively).

Finally, we found an interaction between the presence of the *TT* genotype and the presence of vascular disease with respect to tHcy. Among those with the *TT* genotype, fasting tHcy was a factor of 1.55 higher in patients compared with

TABLE 2. Results of Preliminary and Extended Multivariate Linear Analyses of Determinants of Homocysteine Levels Among 510 Subjects

	Fasting		Post-methionine		Delta	
	Relative Change	*P*	Relative Change	*P*	Relative Change	*P*
			Preliminary multivariate			
Folate (per 10 nmol/L)	0.85 (0.81–0.90)	<.0001	0.986 (0.982–0.99)	<.0001	0.90 (0.85–0.96)	.001
Vitamin B_6 (per 30 nmol/L)	0.97 (0.93–1.01)	.18	0.94 (0.91–0.98)	.003	0.94 (0.90–0.98)	.01
Vitamin B_{12} (per 30 pmol/L)	0.98 (0.97–0.99)	.0002	0.987 (0.975–0.998)	.02	0.995 (0.98–1.01)	.50
Methylenetetrahydrofolate reductase§						
Homozygous normal (CC)	0.80 (0.73–0.88)	<.0001	0.88 (0.82–0.95)	.001	0.90 (0.82–0.99)	.04
Heterozygous mutant (CT)	1.16 (1.06–1.25)	.0008	1.10 (1.01–1.19)	.02	1.09 (0.98–1.20)	.03
Homozygous mutant (TT)	1.56 (1.39–1.74)	<.0001	1.26 (1.13–1.40)	.0001	1.16 (1.003–1.34)	.05
T allele carrier	1.24 (1.14–1.36)	<.0001	1.13 (1.05–1.22)	.001	1.11 (1.01–1.21)	.04
Male sex	1.18 (1.09–1.30)	.0001	...	...	0.92 (0.84–1.0005)	.05
Postmenopausal state	...	...	1.12 (0.995–1.30)	.06	1.21 (1.04–1.41)	.01
Age (per 1 year)	...	...	1.003 (0.999–1.007)	.14	1.004 (0.999–1.009)	.009
Current smokers (yes/no)	1.10 (1.02–1.20)	.02	...	...	...	...
Pack years* (per 1)	1.002 (0.999–1.004)	.19	...	...	...	...
Body mass index (per 1 kg/m^2)	...	...	1.01 (1.0006–1.02)	.04	1.02 (1.01–1.03)	.004
Creatinine (per 10 μmol/L)	1.05 (1.01–1.08)	.01	...	...	...	...
Total cholesterol (per 1 mmol/L)	0.97 (0.93–1.01)	.13	...	...	1.03 (0.99–1.08)	.17
HDL cholesterol (per 0.1 mmol/L)	...	...	0.992 (0.985–0.995)	.04	0.992 (0.983–1.0003)	.06
LDL cholesterol (per 1 mmol/L)	...	...	1.04 (0.995–1.08)	.08	1.05 (0.99–1.11)	.08
Triglyceride (per 1 mmol/L)	...	...	1.03 (0.99–1.08)	.14	...	...
			Extended multivariate†			
Folate (per 10 nmol/L)	0.82 (0.78–0.88)	<.0001	0.85 (0.80–0.90)	<.0001	0.86 (0.79–0.90)	.0004
Vitamin B_{12} (per 30 pmol/L)	0.97 (0.94–0.99)	.002	...	...	...	...
Methylenetetrahydrofolate reductase‡						
Homozygous normal (*CC*)	0.87 (0.80–0.95)	.001	0.89 (0.81–0.97)	.009	0.87 (0.78–0.98)	.02
Heterozygous mutant (*CT*)	1.09 (0.99–1.19)	.07	1.08 (0.99–1.19)	.10	1.11 (0.97–1.27)	.11
Homozygous mutant (*TT*)	1.36 (1.22–1.52)	<.0001	1.21 (1.06–1.38)	.004	1.28 (1.05–1.56)	.02
T allele carrier	1.16 (1.06–1.26)	.0011	1.12 (1.02–1.23)	.01	1.14 (1.01–1.29)	.03
Vascular patient (yes/no)	1.19 (1.07–1.31)	.0008	1.24 (1.12–1.38)	<.0001	1.24 (1.09–1.41)	.001
Postmenopausal state	...	...	1.19 (1.03–1.37)	.02	1.25 (1.04–1.50)	.02
Age (per 1 year)	1.005 (1.0002–1.01)	.04	...	...	...	...
Current smokers (yes/no)	1.11 (1.02–1.21)	.01	...	...	...	...
Pack years* (per 1)	1.003 (1.0006–1.06)	.02	...	...	...	...
Body mass index (per 1 kg/m^2)	...	...	1.01 (1.002–1.02)	.02	1.02 (1.003–1.03)	.02
Creatinine (per 10 μmol/L)	1.05 (1.02–1.1)	.005	...	...	...	...
Explained variance of the model (%)	49	.0001	62	.05	78	.03

For preliminary analyses, all $P<0.2$ are shown adjusted for age, sex, and preexisting vascular disease. For the extended analyses, all $P<0.05$ are shown. Relative changes in homocysteine level per the indicated change in the independent variable are shown with 95% confidence intervals. (The relative change corresponds to the antilog of the regression coefficient; see Methods). Delta homocysteine levels indicate post-methionine levels minus fasting homocysteine levels.

*Pack years were calculated by multiplying the number of cigarette packages smoked per day by the numbers of years the patient smoked.

†Adjusted for vitamin B_6, B_{12}, and folate and for all variables with $P<0.2$ in preliminary analyses as shown in the upper part of the table, without interaction terms.

‡In 20 subjects, MTHFR genotype was not available.

healthy siblings; for the *CC* genotype, this was 1.19 ($P=0.01$ versus *TT*; Table 3). For post-methionine and delta tHcy, the interaction between genotype and patient status was not significant (Table 3).

To check whether the interactions of (1) MTHFR genotype with folate, and (2) folate and vitamin B_{12} with patient status (see above) were related to the differences in tHcy level distribution between (1) subjects with the *TT* and those with *CC* genotype, and (2) patients and siblings, we added the appropriate squared terms of folate and vitamin B_{12} to the interaction analyses. The interactions between MTHFR genotype and folate remained significant, as did that between folate and patient status for fasting tHcy. For post-methionine and delta tHcy, the interaction between patient status and folate lost significance, as did that with vitamin B_{12} for post-methionine tHcy. However, the squared term was sig-

TABLE 3. Interactions for Fasting, Post-Methionine, and Delta (ie, Post-Methionine Minus Fasting Homocysteine Levels) Homocysteine Levels Between MTHFR Genotypes and Serum Folate Levels and Patient Status, Respectively

	MTHFR Genotype				
	CC	*CT*	*TT*	*P*†	*P*‡
		Folate*			
Homocysteine levels (μmol/L)					
Fasting	1.16 (1.04–1.29)	1.10 (1.03–1.16)	1.74 (1.43–1.96)	.0001	.18
Post-methionine	1.11 (1.01–1.22)	1.10 (1.01–1.16)	1.31 (1.25–1.47)	.003	.58
Delta	1.11 (0.96–1.27)	1.12 (1.07–1.19)	1.34 (1.09–1.64)	.13	.90
		Patient status§			
Homocysteine levels (μmol/L)					
Fasting	1.19 (1.02–1.38)	1.07 (1.03–1.40)	1.39 (1.24–2.10)	.01	.40
Post-methionine	1.22 (1.01–1.48)	1.15 (1.04–1.40)	1.29 (1.12–1.67)	.30	.10
Delta	1.22 (1.06–1.42)	1.14 (0.98–1.44)	1.03 (0.91–1.23)	.83	.24

Relative change (95% confidence intervals) in homocysteine level per 10 nmol/L decrease of serum folate level* (upper part) and for patients vs sibling status§ (lower part). (The relative change corresponds to the antilog of the regression coefficient; see Methods.)

*Per 10 nmol/l decrease of serum folate levels.

†*P* value for interaction indicating subjects with *TT* versus subjects with *CC* genotype.

‡*P* value for interaction indicating subjects with *CT* versus subjects with *CC* genotype.

§Interaction comparing those with (patients) with those without (siblings) vascular disease.

nificant only for the folate-patient interaction with regard to post-methionine tHcy (data not shown).

When we repeated the interaction analyses for post-methionine tHcy by adding fasting homocysteine levels to the models, the results were similar and did not reveal any new interactions (data not shown).

Discussion

We studied determinants of fasting, post-methionine and delta tHcy levels in 510 subjects from 192 families in which both HHC and premature vascular disease are prevalent.[6,7] The main findings were twofold. First, fasting, post-methionine and delta tHcy were all influenced by serum folate concentrations, by the MTHFR genotype and by the interaction between these 2 variables (Tables 2 and 3 and Figure 1). Second, there were intriguing interactions between, on the one hand, patient status, and, on the other hand, folate and vitamin B_{12} levels and MTHFR genotype: the effect of folate on fasting, post-methionine and delta tHcy was less strong in the patients than in their healthy siblings, whereas, in contrast, the effects of vitamin B_{12} levels and that of the MTHFR genotype on post-methionine tHcy were stronger (Figures 2 and 3).

We made these observations in a group of subjects with, by design, a high prevalence of high tHcy levels and thus of the *TT* genotype.[9,17–19,21,34–37] For example, 44%, 73%, and 73% had fasting, post-methionine and delta tHcy levels >12, 38, and 27 μmol/L, respectively (ie, the upper quintiles in a recent large study).[3] Our study design thus was very efficient with respect to the investigation of determinants of high tHcy, but it must be emphasized that the prevalences of hyperhomocysteinemia and its determinants that we observed cannot be extrapolated to the general population. On the other hand, there is no a priori reason to believe that this selection procedure should bias the interrelationships between tHcy level and its determinants. Thus it is likely that our findings can in this respect be extrapolated to the general population and thus are broadly relevant to the study of the determinants of cardiovascular risk.

Fasting Homocysteine Level

Our findings on fasting tHcy, especially its relation with serum levels of folate and vitamin B_{12}, the MTHFR genotype, and the interaction between folate and *TT* genotype, are in accordance with previous studies.[11,12,14,21,25,34,38] In addition, fasting tHcy was slightly higher among subjects with the *CT* than among those with the *CC* genotype, which together with other data[34] raises the possibility that heterozygosity for the C677T mutation of the MTHFR gene may have some effect on fasting tHcy. Fasting tHcy was not related to postmenopausal status, which is in agreement with Andersson et al[38] but not with others.[40,41] Fasting tHcy was also not related to vitamin B_6 levels, which contrasts with previous findings.[3,39,42,43] These studies,[3,39,42] however, except one,[43] did not adjust the effect of vitamin B_6 for that of vitamin B_{12} and folate.

Post-Methionine Homocysteine Level

Post-methionine tHcy was related to folate levels, the MTHFR genotype and the postmenopausal state, in agreement with previous data,[1,14,15,34,35,41] but the present study is the first to show that the effect of the *TT* genotype increased with decreasing folate levels, ie, that there was an interaction similar to that for fasting tHcy (Figure 1). Moreover, these relations were of similar magnitude as those with fasting tHcy (Table 2). Post-methionine tHcy, in addition, was inversely related to vitamin B_6 levels, but this disappeared after further adjustment for folate (Table 2). This finding contrasts with previous studies[38] but supports others,[39,44] but it must be noted that the analyses in these studies[38,39] were not adjusted for folate and vitamin B_{12}.

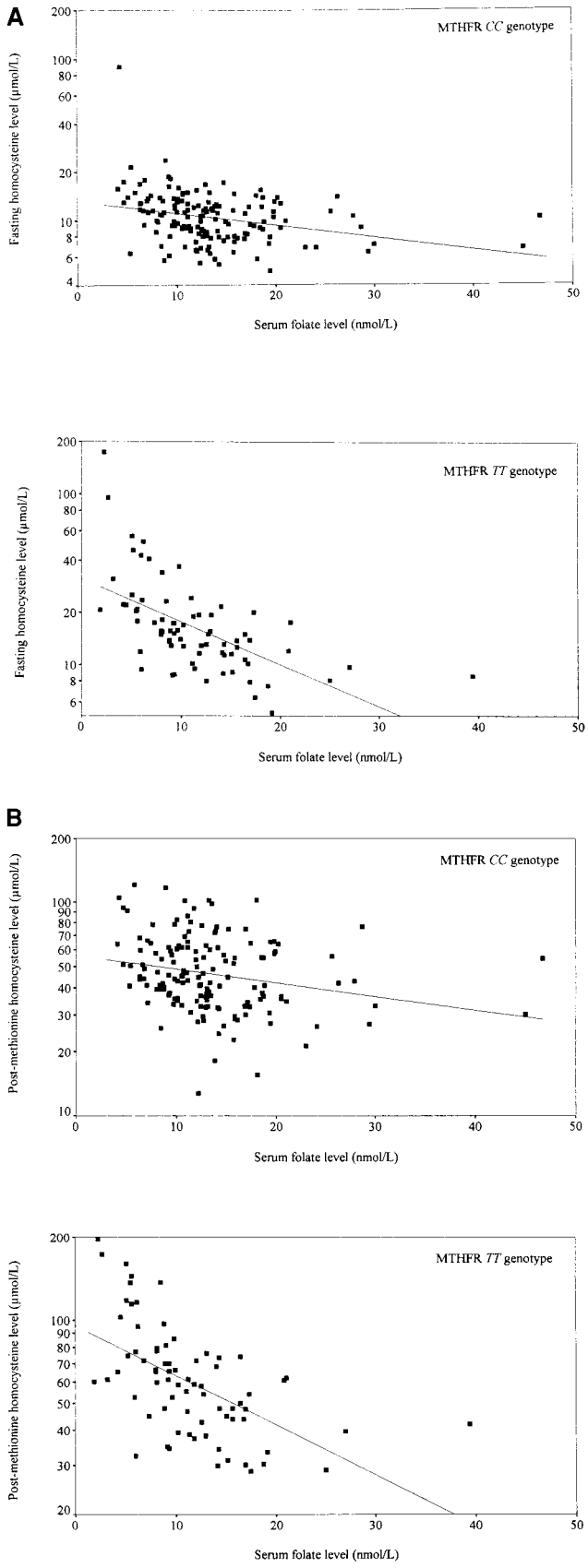

Figure 1. A, Relation between fasting homocysteine levels (log scale) and folate levels for subjects with the MTHFR *CC* genotype (top) and for those with the *TT* genotype (bottom). B, Relation between post-methionine homocysteine levels (log scale) and folate levels for subjects with the MTHFR *CC* genotype (top) and for those with the *TT* genotype (bottom).

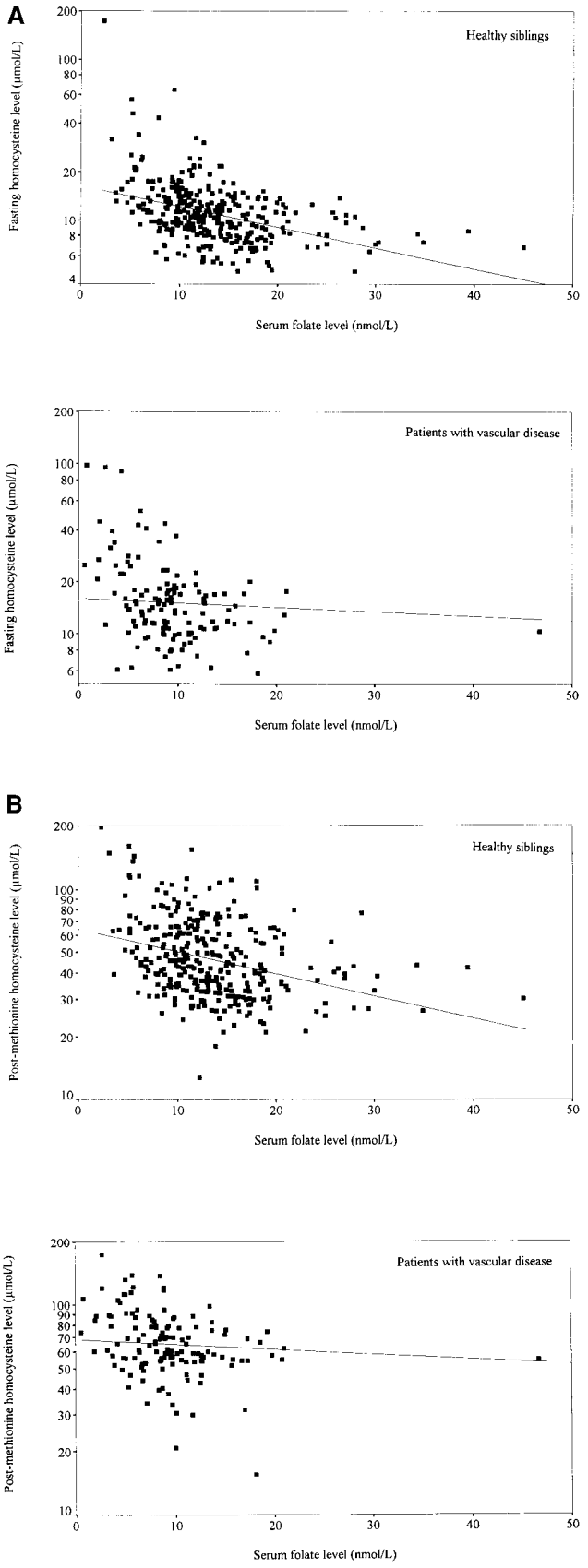

Figure 2. A, Relation between fasting homocysteine levels (log scale) and folate levels for subjects without (top) and for those with vascular disease (bottom). B, Relation between post-methionine homocysteine levels (log scale) and folate levels for subjects without (top) and for those with vascular disease (bottom).

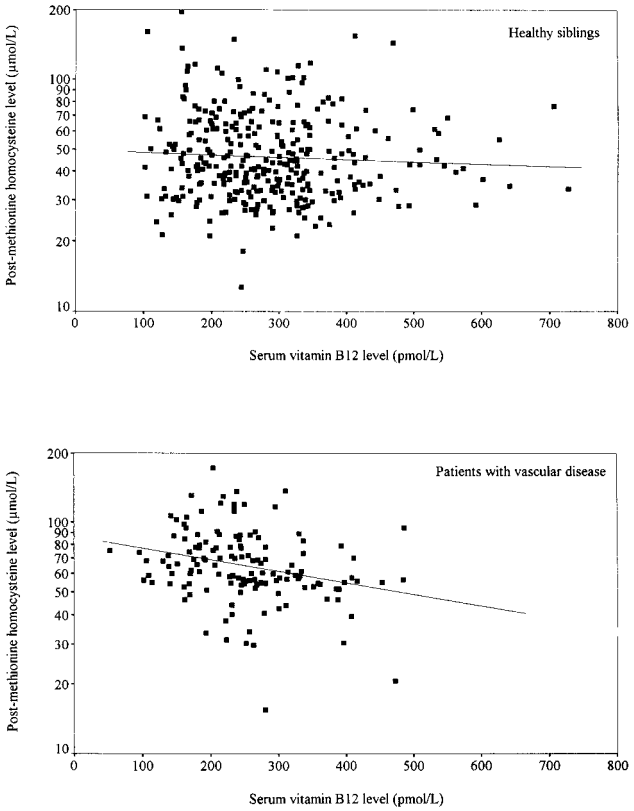

Figure 3. Relation between post-methionine homocysteine levels (log scale) and serum vitamin B_{12} levels for subjects without (top) and for those with vascular disease (bottom).

The investigation of the determinants of post-methionine tHcy is complicated by the fact that post-methionine tHcy, to some extent, depends on fasting levels. We dealt with this in 2 ways: by studying delta tHcy and by adjusting the multivariate analyses of post-methionine tHcy for fasting levels. The results of these analyses largely confirmed the initial analyses with post-methionine tHcy as dependent variable (Table 2).

Fasting and post-methionine (or delta) tHcy levels are thought to reflect homocysteine remethylation and transsulfuration, respectively.[24,45,46] Our data on folate, vitamin B_{12} and MTHFR genotype, all of which affect remethylation, and fasting tHcy are in accordance with this view, but those on post-methionine (or delta) tHcy apparently are not. On the other hand, previous work has convincingly demonstrated that both vitamin B_6 and cystathionine-β-synthase deficiency, which are primary factors affecting transsulfuration, do lead to increased post-methionine tHcy.[46,47] Taken together, these[46,47] and the present findings thus strongly suggest that post-methionine and delta tHcy are influenced not only by factors that affect transsulfuration, but also by factors that affect remethylation. This hypothesis needs further investigation, eg, with kinetic modeling of methionine metabolism by infusion of labeled methionine.[48]

The Effect of Patient Status

A final intriguing finding was that the effect on tHcy of folate, vitamin B_{12}, and the *TT* genotype differed between patients and healthy siblings. We cannot fully exclude that the higher tHcy levels in the patients play a role. Nevertheless, taken together, these data raise the possibility of the presence, in the patients but not in their healthy siblings, of a factor that increases tHcy, weakens the normal inverse relation between folate and tHcy, and, to some extent, amplifies the effects of vitamin B_{12} and of the *TT* genotype on post-methionine tHcy. The most parsimonious hypothesis is that this reflects a factor affecting homocysteine remethylation, such as an additional mutation in the MTHFR gene or a mutation in the methionine synthase gene. The clinical implication of this finding is that any such factor may also be linked to vascular disease. These hypotheses require further investigation.

Study Limitations

The explained variances of fasting, post-methionine and delta tHcy were 49%, 62%, and 78% respectively (Table 2). Other determinants thus must play a role,[49] which we did not assess. In addition, we used serum creatinine, an imprecise estimate of renal function. Nevertheless, we adjusted for many other variables (Table 2), so important residual confounding appears unlikely. Moreover, we found that exclusion of subjects with very high tHcy levels did not materially affect our results. As noted above, it is plausible that our findings on the determinants of tHcy can be extrapolated to the general population, but we cannot fully exclude bias. In any case, our results are relevant for patients with premature atherothrombotic disease and their families. Finally, we assumed that low folate levels cause an increase in tHcy, but our study was cross-sectional, so we cannot exclude that, to some degree, a high tHcy level is a marker of processes that decrease serum folate.[50,51]

Conclusions

Fasting, post-methionine and delta tHcy were all influenced by serum folate concentrations, by the MTHFR genotype and by the interaction between these 2 variables, suggesting that post-methionine and delta tHcy are influenced not only by factors that affect transsulfuration but also by factors affecting homocysteine remethylation. Furthermore, we found some evidence, in the patients with premature vascular disease but not in their healthy siblings, for a factor that increases homocysteine levels but weakens the normal inverse relation between folate and homocysteine and amplifies the effect of the MTHFR genotype. Whether these latter findings are related to the development of vascular disease needs further investigation.

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