Low Folate Levels and Thermolabile Methylenetetrahydrofolate Reductase as Primary Determinant of Mild Hyperhomocystinemia in Normal and Thromboembolic Subjects

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Abstract—Several studies have indicated that mild to moderate hyperhomocystinemia is a common cause of arterial occlusive disease. Whether hyperhomocystinemia per se is an independent risk factor for vein thromboembolism (VTE) is still somewhat controversial. Both genetic and nutritional factors influence plasma homocysteine levels. Therefore, we evaluated plasma total homocysteine (tHcy), folate, and vitamin B₁₂ levels and established, by polymerase chain reaction, the presence of the C677T mutation (A223V) in the methylenetetrahydrofolate reductase (MTHFR) gene in 220 cases with VTE without well-established prothrombotic defects. As a control group, 220 healthy subjects from the same geographic area as the cases were investigated. Hyperhomocystinemia was defined as a plasma tHcy level above the 95th percentile in the controls (18.05 μmol/L). Hyperhomocystinemia was found in 16% of cases (odds ratio = 3.59; P < 0.001); deficiencies of folate (<2.47 ng/mL) or vitamin B₁₂ (<165 pg/mL), defined as values below the 5th percentile in controls, were found in 17.7% (P < 0.001) and 12.3% (P = 0.015) of cases, respectively. The homozygous condition for the MTHFR mutation (VV) was present in 28.2% of cases and 17.7% of controls (odds ratio = 1.82; P = 0.013). Comparing only the idiopathic forms of VTE (n = 80/220; 36.3%) with normal controls, individuals with hyperhomocystinemia, or individuals homozygous for MTHFR mutation increased the odds ratios to 4.03 (P = 0.005) and 2.11 (P = 0.018), respectively. No statistically significant difference was observed in the MTHFR genotype distribution of cases and controls with hyperhomocystinemia (P = 0.386); however, the normal MTHFR genotype (AA) appeared in control subjects only when tHcy levels were below the 80th percentile (10.57 μmol/L) of the distribution, whereas in case patients, it was present at the highest tHcy levels. A strong association between mutated homozygosity (VV), low folate levels, and hyperhomocystinemia was found in both groups. We conclude that in patients with VTE who do not have coexisting prothrombotic defects, hyperhomocystinemia increases the risk of developing idiopathic and venous thrombosis; the homozygous condition for the MTHFR mutation confers a moderate risk but, together with low folate levels, it is the main determinant of mild hyperhomocystinemia in normal and thromboembolic populations. (Arterioscler Thromb Vasc Biol. 1999;19:1761-1767.)

Key Words: hyperhomocystinemia n homocysteine n metabolism n thermolabile MTHFR n C677T mutation n venous thrombosis

Homocysteine lies at the branch point of methionine metabolism, between the remethylation and transsulfuration pathways, and forms methionine and cystathionine, respectively. Several enzymes balance and regulate this pathway under normal conditions.¹,² Methionine formation is tightly tied to a vitamin B₁₂-dependent enzyme, methionine synthase, which uses 5-methyltetrahydrofolate as a carbon donor; this donor is synthesized by the methylenetetrahydrofolate reductase (MTHFR) gene from 5,10-methylenetetrahydrofolate. Reduction in the activity of these enzymes caused by congenital defects and/or deficiencies in folate, vitamin B₁₂, or vitamin B₆ (due to anomalous intake or malabsorption) may affect the normal homocysteine pathway.³–⁵ A correlation between hyperhomocystinemia and arterial vascular disease is well established.⁶–⁸ Several studies have investigated the role of hyperhomocystinemia in recurrent vein thromboembolism (VTE); some attribute it to a causal relation,⁹–¹¹ in particular with juvenile VTE,¹² whereas others demonstrate that hyperhomocystinemia is not a frequent cause of VTE¹³,¹⁴ in subjects with inherited...
thrombophilia\textsuperscript{15} or that hyperhomocystinemia increases the risk of VTE only in subjects not exposed to circumstantial risk situations.\textsuperscript{16} A thermolabile variant of \textit{MTHFR} has been described in patients with coronary and peripheral artery disease\textsuperscript{17,18}; it reduced specific activity caused by the C677T homozygous mutation\textsuperscript{19} in a highly conserved residue of the molecule A223V. Association of VTE with C677T homozgyosity is still controversial\textsuperscript{15,16,20–23}; in fact, the frequency of the mutated allele was quite high, depending on the ethnic group analyzed.\textsuperscript{18,19,24–26} The correlated plasma total homocysteine (tHcy) levels seem strongly dependent on the folate status of each individual; they are high only under a certain folate concentration, even in mutated homozygous subjects.\textsuperscript{27} Folate supplements reduce plasma tHcy levels, and the response to folate supplements is affected by the number of the 677T alleles in the \textit{MTHFR} gene, with the strongest response in 677TT homozygotes.\textsuperscript{28} We studied 220 patients with VTE and 220 healthy control subjects to correlate the prevalence of hyperhomocystinemia with the incidence of the C677T mutation, and we investigated the role of folate and vitamin B\textsubscript{12} in the establishment of hyperhomocystinemia in association with VTE in subjects with thermolabile \textit{MTHFR}.

### Methods

#### Subjects

All subjects gave informed consent before the study began. Patients who had had at least one vein thrombosis or pulmonary embolism episode (confirmed by phlebography or pulmonary scintigraphy) and who had been free of thrombotic episodes for at least 6 months were enrolled from those who came to our Center between October of 1996 and February of 1997. A total of 220 unrelated patients (from the 880 examined) were selected from the files of the anticoagulant unit of our Center and enrolled in the study. The control group (n = 220) consisted of blood donors without a familial history of VTE in the same geographic region of Northern Italy (Emilia Romagna) as the patient group. Deep venous thrombosis or pulmonary embolism not associated with cancer, surgery, recent childbirth, trauma, or contraceptive treatment were considered idiopathic. The exclusion criteria for both groups were as follows: age >75 years; pregnancy or recent childbirth; therapies including methionine, betaine, choline, folate, vitamin B\textsubscript{12}, or vitamin B\textsubscript{12}; presence of any form of cancer; and liver or renal insufficiency. Finally, subjects who fasted for <10 hours were not admitted for blood sampling. In addition, patients with antithrombin, protein C, or protein S deficiencies; factor V Leiden (R506Q mutation); or who were taking lupus anticoagulants were also excluded from the study. Excluding control subjects with similar defects did not change the statistical analysis; therefore, such subjects were included in the study. The study protocol was approved by the Ethics Committee of the University of Ferrara.

#### Blood Collection

All blood samples were collected after an overnight fast (>10 hours) by venipuncture into a Vacutainer containing 0.129 mol/L sodium citrate (Becton Dickinson). Platelet-poor plasma was obtained by double centrifugation at room temperature for 15 minutes at 2000g. The plasma aliquots were immediately frozen at −70°C until use.

#### Coagulation Findings

To screen defects in the activated protein C pathway, we used the global test (ProC Global, Dade Behring) based on the activation of endogenous plasma protein C, as previously described.\textsuperscript{29,30} Specialized assays for protein C, protein S, and activated protein C resistance, including detection of the factor V Leiden mutation, were performed as previously described.\textsuperscript{31} Antithrombin activity (Chromogenix AB) was measured according to the supplier’s instructions. The search for lupus anticoagulants was performed according to the method of Exner et al.\textsuperscript{32}

### Statistical Analysis

The distributions of plasma tHcy and vitamin concentrations were positively skewed; therefore, they were transformed logarithmically to approximate normal distribution, and such data were analyzed statistically. The statistical significance of the differences between cases and controls was performed with Student’s t test and Chi-square test. When appropriate, Yates’ correction or Fisher’s exact test were applied. P ≤ 0.05 was considered statistically significant. Odds ratios (OR) and their 95% confidence intervals (CI) were used to estimate the risk for vein thromboembolism. Adjusted ORs were calculated by logistic-regression models that controlled for age, sex, and menopausal status.

### Results

Table 1 shows the relevant characteristics of the 220 normal controls and the 220 thromboembolic cases investigated.

![Figure 1](http://atvb.ahajournals.org/)

#### Laboratory Determinations

Plasma tHcy concentration was determined by high-performance liquid chromatography with fluorometric detection; hyperhomocystinemia was defined as tHcy levels above the 95th percentile in the control group (18.05 μmol/L). Folate and vitamin B\textsubscript{12} plasma concentrations were determined by standard immunoassays (Sanofi Diagnostics Pasteur). Folate and vitamin B\textsubscript{12} levels below the respective 5th percentile of the distribution of the controls were considered deficiencies (2.47 ng/mL and 165 pg/mL, respectively, for folate and vitamin B\textsubscript{12} levels).

### MTHFR Mutation Detection

C677T \textit{MTHFR} gene mutation was detected by Hinf I restriction analysis of a 198-bp polymerase chain reaction–amplified fragment in the gene for \textit{MTHFR}, according to Frosst et al.\textsuperscript{19}

#### Table 1. Biological Parameters in the Normal Controls and in the Thrombotic Cases

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Controls (n = 220)</th>
<th>Cases (n = 220)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>46.5 ± 13.6</td>
<td>53.5 ± 14.05</td>
</tr>
<tr>
<td>Sex, male/female</td>
<td>135/85</td>
<td>124/96</td>
</tr>
<tr>
<td>Mean tHcy level, μmol/L</td>
<td>9.1 ± 6.6</td>
<td>11.7 ± 10.8*</td>
</tr>
<tr>
<td>Mean folate level, ng/mL</td>
<td>4.95 ± 2.2</td>
<td>4.38 ± 2.9</td>
</tr>
<tr>
<td>Median folate level, ng/mL</td>
<td>4.10</td>
<td>3.70</td>
</tr>
<tr>
<td>Mean vitamin B\textsubscript{12} level, pg/mL</td>
<td>297 ± 92.9</td>
<td>308 ± 149.8</td>
</tr>
<tr>
<td>Median vitamin B\textsubscript{12} level, pg/mL</td>
<td>284</td>
<td>277</td>
</tr>
</tbody>
</table>

\textit{MTHFR} genotype, n (%)

<table>
<thead>
<tr>
<th></th>
<th>AA</th>
<th>AV</th>
<th>WV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>65 (29.5)</td>
<td>116 (52.7)</td>
<td>39 (17.7)</td>
</tr>
<tr>
<td></td>
<td>51 (23.2)</td>
<td>107 (48.6)</td>
<td>62 (28.2)</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SD unless otherwise specified.

*P = 0.029 between cases and controls; †P = 0.013 between cases and controls.
Figure 1 shows that among the 11 hyperhomocysteinemic controls, 9 (82%) had the VV genotype, 2 (18%) were heterozygotes, and none had the AA genotype; among the 35 hyperhomocysteinemic cases, 22 (63%) had the VV genotype, 9 (26%) were heterozygotes, and 4 (11.4%) had the AA genotype. Although no statistically significant difference was observed between the MTHFR genotype distribution of cases and controls with hyperhomocysteinemia ($P=0.386$), no AA control subject was found up to the 80th percentile (10.57 μmol/L) of tHcy distribution. However, the number of mutated homozygotes (62/220 patients [28.2%]) in cases and 39/220 subjects [17.7%] in controls [OR = 1.82; 95% CI, 1.15 to 2.86; $P=0.013$] and the frequency of the mutated allele (52.5% of cases and 44% of controls [$P=0.015$]) were significantly different. The OR for mutated homozygosity slightly increased when only idiopathic VTE was considered (OR = 2.11, 95% CI, 1.17 to 3.78; $P=0.018$); this raise was not statistically significant ($P=0.708$).

The cutoff point to define folate or vitamin $B_{12}$ deficiency was the 5th percentile of the respective vitamin distribution in the controls (2.47 ng/mL and 165 pg/mL, respectively, for folate and vitamin $B_{12}$). The number of individuals with vitamin deficiency was significantly higher in case subjects (39/220 [17.7%] for folate [OR = 4.09; 95% CI, 2.03 to 8.22; $P=0.001$] and 27/220 [12.3%] for vitamin $B_{12}$ [OR = 2.65; 95% CI, 1.28 to 5.5; $P=0.015$]). When the two examined groups were adjusted for age, sex, and menopausal status, the new ORs for folate and vitamin $B_{12}$ were 3.31 ($P=0.005$) and 2.11, 95% CI, 1.17 to 3.78; $P=0.018$), respectively, for any and idiopathic VTE.

Table 2 shows the crude ORs of developing any VTE versus developing idiopathic VTE at different cutoff points of tHcy concentration. A statistically significant association was observed between high tHcy levels and VTE in both groups for the cutoffs considered. Although the ORs in the group of idiopathic VTEs were higher if compared with those of any VTE, the difference was not statistically significant ($P=0.860$ at the 95th percentile).

<table>
<thead>
<tr>
<th>tHcy Percentile (mean, μmol/L)</th>
<th>Any VTE (n=220)</th>
<th>Iidiopathic VTE (n=80)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% CI</td>
</tr>
<tr>
<td>97.5 (26.6)</td>
<td>3.17</td>
<td>1.2–8.1</td>
</tr>
<tr>
<td>95.0 (18.05)*</td>
<td>3.59</td>
<td>1.8–7.3</td>
</tr>
<tr>
<td>90.0 (15.15)</td>
<td>2.58</td>
<td>1.5–4.4</td>
</tr>
<tr>
<td>85.0 (12.05)</td>
<td>2.07</td>
<td>1.3–3.2</td>
</tr>
<tr>
<td>80.0 (10.57)</td>
<td>1.90</td>
<td>1.2–2.9</td>
</tr>
</tbody>
</table>

*After adjusting for age, sex, and menopausal status, the ORs at the 95th percentile were $2.54$ (95% CI, 1.25–5.29; $P=0.018$) and $3.0$ (95% CI, 1.25–7.28; $P=0.020$), respectively, for any and idiopathic VTE.
By performing a multivariate analysis comparing the risk of developing future VTE from an increase in tHcy levels caused by low folate levels, low vitamin B₁₂ levels, or to the 677TT MTHFR genotype, we discovered that risk decreased as follows: low folate levels (P<0.001), 677TT MTHFR genotype (P<0.039), low vitamin B₁₂ level (P<0.094); the risk was not statistically significant only for low vitamin B₁₂ levels.

Table 3 shows tHcy levels in cases and controls stratified by MTHFR genotype. The mean tHcy level increased as the copies of the mutated amino acid V223 increased for both cases and controls. Moreover, the ranges of tHcy distribution were wider in cases than in controls, and in the former, they were similarly expanded, despite the three different MTHFR genotypes. For this reason, we subdivided the tHcy levels of cases and controls by stratifying by MTHFR genotype in folate levels below and above the respective median (4.1 ng/mL for controls and 3.7 ng/mL for cases).

Table 4 shows that hyperhomocystinemic individuals occurred in both the subgroups of cases among all 3 genotypes: AA, AV, or VV; however, they were mainly found in the low folate subgroup. In addition, those with very high tHcy concentrations showed low folate levels (Figure 2). Hyperhomocystinemic controls only occurred in the low folate subgroup mainly with the VV genotype. The data from Tables 3 and 4 suggest that in controls, the association between VV MTHFR genotype and low folate level may be considered the main cause of hyperhomocystinemia and that in cases, defects besides thermolabile MTHFR must be taken into account.

To better correlate plasma tHcy concentrations, folate levels, and MTHFR genotypes, we divided the two groups of subjects investigated (cases and controls) by the three possible MTHFR genotypes and then further stratified them by folate and tHcy concentrations (Figure 2). Figure 2A shows that among the individuals with VV genotype and hyperhomocystinemia, none had folate levels above the median

### TABLE 3. Plasma tHcy Levels in Cases and Controls by MTHFR Genotype

<table>
<thead>
<tr>
<th>MTHFR Genotype</th>
<th>Plasma tHcy Level, μmol/L</th>
<th>Cases (n=220)</th>
<th>Controls (n=220)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA n=51</td>
<td>8.8±10.3 (3.5–74.4)</td>
<td>n=65</td>
<td>6.6±1.9* (3.7–11.44)</td>
</tr>
<tr>
<td>AV n=107</td>
<td>10.2±8.1 (4.3–73.8)</td>
<td>n=116</td>
<td>8.3±3.1* (3.8–19.6)</td>
</tr>
<tr>
<td>VV n=62</td>
<td>16.4±13.3 (4.4–69.2)</td>
<td>n=39</td>
<td>15.6±12.7 (4.3–64.4)</td>
</tr>
</tbody>
</table>

Values shown are mean±SD (range) of tHcy levels for each MTHFR genotype.

*P<0.05 if compared with the respective value in cases.

### TABLE 4. Plasma tHcy Levels in Cases and Controls by MTHFR Genotype and Folate Level

<table>
<thead>
<tr>
<th>MTHFR Genotype</th>
<th>Cases (n=220)</th>
<th>Controls (n=220)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n Folate&gt;Median</td>
<td>n Folate&gt;Median</td>
<td>n Folate&gt;Median</td>
</tr>
<tr>
<td>AA 27 (3.5–18.2)</td>
<td>24 (4.5–74.4)</td>
<td>40 (3.7–10.5)</td>
</tr>
<tr>
<td>AV 60 (4.8–27.1)</td>
<td>47 (4.3–73.8)</td>
<td>59 (4.5–12.7)</td>
</tr>
<tr>
<td>VV 8.6±4.5 (4.4–23.6)</td>
<td>37 (4.3–69.2)</td>
<td>15 (4.3–16.1)</td>
</tr>
</tbody>
</table>

Values shown are mean±SD (range) of tHcy levels for each MTHFR genotype.

Figure 2. Levels of tHcy in plasma stratified by folate concentrations in cases (●) and controls (○) showing the VV (A), AV (B) and AA (C) MTHFR genotypes. Horizontal broken line indicates 95th percentile (18.05 μmol/L) of tHcy distribution in controls, and vertical broken line indicates 5th percentile (2.47 ng/mL) of folate concentration in controls. No subjects with VV MTHFR genotype had tHcy levels below 95th percentile and folate concentrations below 5th percentile (dashed area in A).
subgroup; this is in contrast to Ridker et al., who found significant risk values only for idiopathic VTE. This could be explained in part by the different selection criteria used for the case groups. We excluded any person with an identified prothrombotic defect from cases, which would tend to reduce the found idiopathic events (36% in this study and 50% in that of Ridker et al; $P=0.011$), with a not statistically significant difference between the two subgroups of our cases. These data support the idea that hyperhomocystinemia is also an independent risk factor for venous thromboembolism.

Hyperhomocystinemia is caused by a combination of inherited and environmental factors. Thermolabile MTHFR is the most frequent inherited defect of the homocysteine pathway. Individuals homozygous for the MTHFR mutation have significantly higher tHcy levels than heterozygotes or normal homozygotes, and they can have an increased risk for arterial thrombosis. Because not all studies have reported a direct association between C677T homozygosity and VTE, it is possible that hyperhomocystinemia plays a greater role in developing VTE than C677T homozygosity. Although a high frequency of the mutated allele was found in controls, it was significantly different from cases ($P=0.015$) and the number of mutated homozygotes ($P=0.013$). This led to a slight but statistically significant risk for mutated homozygotes to develop any VTE ($OR=1.82; P=0.013$), and it is not significantly different from that obtained considering only idiopathic VTE ($OR=2.11; P=0.018$). These data support the hypothesis that being homozygous for the MTHFR mutation is also an independent risk factor for venous thromboembolism.

It is unclear whether hyperhomocystinemia from different causes gives the same risk of thrombosis. After performing a multivariate analysis, we found a higher risk associated with hyperhomocystinemia caused by low folate levels than that caused by the MTHFR mutation. This seems to agree with the idea that high tHcy levels are present in the plasma of homozygous subjects with the MTHFR mutation only under a certain folate concentration, whereas low folate levels per se affect the homocysteine pathway. We found a significantly higher number of subjects with vitamin deficiencies in cases than in controls, implying a significant association between VTE and low vitamin levels. When the two groups were completely adjusted for age, sex, and menopausal condition, the new ORs remained significant only for low folate levels, suggesting that some form of decreased vitamin-dependent enzyme activity may be connected to thrombosis. A different response to folate supplementation has been described that depends on the number of 677T alleles in the MTHFR gene. We found that mean plasma tHcy levels increased as the number of the mutated alleles increased in the MTHFR genotype for both groups investigated. The 677T-related tHcy levels and the presence of high tHcy concentrations mainly in the low folate subgroups suggest a modulatory role of MTHFR and folate in tHcy regulation. On the other hand, cases with hyperhomocystinemia who were heterozygous or null for the 677T mutation generally had low folate levels, suggesting that hyperhomocystinemia in these cases might be caused by folate-related defects besides thermolabile MTHFR.
Among the mutated homozygotes, no subject had hyperhomocysteinemia and folate levels above the median, and no subject had normohomocystinemia with folate levels below the 5th percentile. Moreover, among the mutated homozygotes, no clear cutoff point exists to separate individuals with normal tHcy concentrations from those with high tHcy concentrations, but there is a continuous distribution from the lower to the higher values of tHcy that crosses the line of the 95th percentile. This was also observed, in part, among the heterozygotes, although the percentage of subjects with hyperhomocysteinemia was lower and cases had high tHcy values and folate levels in the high-normal range. In these cases, hyperhomocysteinemia could be explained by non-folate-related defects. The distribution of tHcy in the normal homozygous subjects was different, and a clear-cut division was observed between hyperhomocysteinemic and normohomocysteinemic subjects. This was confirmed by considering the mean and standard deviation of the tHcy levels in the normal homozygotes below the 95th percentile; they were significantly lower ($P<0.0001$) than those obtained in the two corresponding subgroups of heterozygous- and homozygous-mutated subjects (data not shown). All this means that the homozygous condition of the MTHFR mutation strongly influences tHcy plasma levels when folates are in the low-normal range and that the heterozygous form also had a slight but statistically significant ability to influence tHcy levels, with values surrounding the cutoff point of the distribution. Therefore, the heterozygous subjects with very high tHcy levels could be the combined result of the mutated MTHFR allele and other inherited or acquired defects. Moreover, it should be noted that a strong reduction in the number of hyperhomocysteinemic controls occurred when the mutated MTHFR alleles disappeared (eg, 4/51 cases versus 0/65 controls with hyperhomocysteinemia and AA MTHFR genotype; $P=0.069$) and that controls with the AA MTHFR genotype did not have tHcy levels above the 80th percentile. Although a significant difference was not completely assessable, we could speculate that thermolabile MTHFR is the only defect of the homocysteine pathway present in healthy people. It is more correct to conclude that this occurred because of the significantly lower number of hyperhomocysteinemic subjects found in the controls; its significance, if any, could be ascertained only in a larger population study.

In conclusion, our data show that in selected patients without coexisting prothrombotic defects, hyperhomocysteinemia can be considered an independent risk factor for VTE and that the homozygous condition for the MTHFR mutation confers a moderate risk; however, the mutation’s strong association with low folate levels should be considered the main determinant of mild hyperhomocysteinemia in normal and thromboembolic populations. Moreover, the presence of a common mutation in the MTHFR gene that affects the tHcy pathway when folate levels are in the low-normal range is an important example of gene-environment interaction, Dr Giampiero Cappelletti for his excellent technical assistance, and Drs Hassett Shawn and Adams Russell for revising the manuscript.

References
21. Aruyma VR, von Zuben PM, Chiappini LC, Annichino-Bizzacchi JM, Costa FF. The mutation Ala677→Val in the methylene tetrahydrofolate


