Hyperhomocysteinemia and Other Inherited Prothrombotic Conditions in Young Adults With a History of Ischemic Stroke

Pasquale Madonna, MD; Valentino de Stefano, MD, PhD; Antonio Coppola, MD; Ferdinando Cirillo, PhD; Anna Maria Cerbone, MD; Giuseppe Orefice, MD; Giovanni Di Minno, MD

Background and Purpose—The mechanisms of ischemic stroke in young adults are poorly understood. During the last years, several studies suggested a role for genetic factors predisposing to thrombophilia and for moderate hyperhomocysteinemia in this setting.

Methods—We evaluated in 132 consecutive patients (66 males, 66 females; mean ± SD age, 38.4 ± 11.7 years; mean ± SD age at first event, 34.8 ± 10.9 years; range, 6 months to 50 years) referred to our center between January 1997 and December 1999 for a history of young adult ischemic stroke (age at first event, <51 years) the prevalence of factor V (FV) Leiden, prothrombin (FII) G20210A, and C677T and 5,10-methylene tetrahydrofolate reductase (MTHFR) gene mutations and fasting serum total homocysteine levels. Two hundred sixty-two apparently healthy subjects (117 males, 145 females; mean ± SD age, 36 ± 13.2 years) served as controls.

Results—Total homocysteine levels differed significantly (P = 0.004, t test) between patients and controls: 13.03 ± 18.61 versus 10.75 ± 6.24 μmol/L (mean ± SD), respectively. In contrast, homozygosity for the TT mutation of the MTHFR gene was 30 of 132 (22.7%) in patients and 45 of 262 (17.2%) in controls; this difference was not statistically significant (P > 0.05, χ² test). However, when we stratified the whole population according to genotype, fasting serum homocysteine levels were significantly higher in TT patients than in TT controls (25.3 ± 36.8 versus 15 ± 11.6 μmol/L; P = 0.02, t test). Mutations of FV Leiden and of FII G20210A gene are currently reported to be associated with a tendency toward ischemic stroke. Their frequencies were not statistically significantly different between patients and controls in this setting: 7 of 132 (5.3%) versus 17 of 262 (6.5%) for FV Leiden and 10 of 132 (7.6%) versus 16 of 262 (6.1%) for FII G20210A, respectively (all P > 0.05, χ² test).

Conclusions—In the present cohort of patients, moderate hyperhomocysteinemia is the only variable that helps to identify young adults with a history of ischemic stroke. (Stroke. 2002;33:51-56.)

Key Words: genetics ■ homocysteine ■ stroke, ischemic ■ young adults

Ischemic stroke is a leading cause of death in developed countries; its prevalence among young adults (aged 15 to 45 years) ranges from 3% to 5%.1,2 Over the last years, several studies have been performed to elucidate the mechanisms of this ischemic event. Factor V (FV) Leiden and G20210A variant of the prothrombin (FII) gene are clotting factor mutations that are associated with an increased tendency toward venous thrombosis.3,4 The evidence of a role for these gene variants in the risk of ischemic stroke is controversial.5–11 Case-control12,13 and prospective14,15 studies have suggested an association between moderate hyperhomocysteinemia and risk of ischemic stroke, with a meta-analysis of the data indicating that hyperhomocysteinemia confers at least a 2.5-fold increased risk of stroke.16 Homozygosity for the C to T substitution at nucleotide 677 of the gene of 5,10-methylene tetrahydrofolate reductase (MTHFR) is associated with a 50% reduction of the activity of this enzyme17 and is the most common inherited cause of moderate hyperhomocysteinemia. Several studies18–21 have shown a significant association of homozygous C677T MTHFR variation and coronary artery disease, peripheral artery disease, and venous thrombosis. However, these data have been disputed,22–26 with the combination of the homozygous mutant genotype with low plasma folate levels thought to predispose subjects to vascular disease.27,28 In regard to the association of homozygous C677T MTHFR gene mutation and the risk of cerebrovascular disease, the results are conflicting as well.29–32 In young adults with a history of ischemic arterial strokes, we evaluated the role of these inherited prothrombotic factors.
Subjects and Methods

Study Population

Patients and controls gave informed consent before entering the study, which was approved by the ethics committee of our institution. The study included 132 consecutive unrelated patients (66 males, 66 females; mean±SD age, 38.4±11.7 years) referred, between January 1997 and December 1999, to the Thrombosis Unit of University Hospital “Federico II” of Naples, Italy. The employees of the University Hospital were evaluated within the framework of a large survey on the distribution of risk factors for arterial/vein thrombosis in apparently healthy individuals. This survey was performed almost in parallel with the present study. Two hundred sixty-two of these apparently healthy individuals (117 males, 145 females; mean±SD age, 36±13.2 years), belonging to the same ethnic background as stroke subjects, served as controls. Patients were enrolled in the study if they had suffered from 1 or more previous ischemic stroke(s) that had occurred at least 6 months before the study. The patients’ mean age at first event was 34.8±10.9 years (range, 6 months to 50 years). The clinical diagnosis of stroke was defined as an acute focal neurological deficit lasting ≥24 hours. In each case, the ischemic event was confirmed by CT and/or MRI scan within 72 hours from the onset of the symptoms. A complete clinical summary, with emphasis on predisposing factors to ischemic stroke, alcohol consumption, and use of drugs, was obtained from all subjects by a well-trained staff. Habitual alcohol consumption was considered present if there was a history of alcohol intake for ≥5 dwk. Information concerning major risk factors for arterial disease was also collected. The risk factors were defined as follows: hypertension (diastolic blood pressure >90 mm Hg on repeated measurements); diabetes mellitus (repeated fasting serum glucose levels >7.0 mmol/L), hyperlipidemia (total cholesterol >6.18 mmol/L and/or triglycerides >2.39 mmol/L), and current use or a history (>1 year from cessation) of cigarette smoking. Women’s history of oral contraceptive use and pregnancy was also assessed. Patients and controls receiving vitamin supplementation or substances affecting homocysteine metabolism as well as those with transient ischemic attacks and migraine, defined according to the revised International Headache Classification,33 were excluded. Patients were also excluded from the study if they had overt cancer or abnormal serum creatinine and/or liver function tests.

Materials

KCl, dNTP, MgCl₂, gelatin, and mineral oil were from Perkin Elmer-Cetus; proteinase K was from USB Corp; sucrose, Triton X-100, HEPES, Tris-HCl, EDTA, ethidium bromide, and SDS were from Sigma Chemical Co. Restriction enzymes X-100, HEPES, Tris-HCl, EDTA, ethidium bromide, and SDS were from Perkin Elmer-Cetus; proteinase K was from USB Corp; sucrose, Triton X-100, HEPES, Tris-HCl, EDTA, ethidium bromide, and SDS were from Sigma Chemical Co. Homocysteine microplate enzyme immunoassay was from Bio-Rad Laboratories Diagnostic Group.

Blood Collection

From each subject, after 12 to 15 hours of overnight fasting, 18 mL of blood was drawn between 9 and 9:30 AM from an antecubital vein, via a 21-gauge scalp vein needle, into sterile Vacutainer tubes (Beckton Dickinson), 2 of which contained 0.129 mol/L trisodium citrate (9:1), and centrifuged within 60 minutes at 3000 rpm for 10 minutes. All tubes were kept at 4°C during all procedures, until snap-freezing of serum and storage at −80°C.

Isolation of DNA and Genotype Analysis

DNA was extracted according to Miller et al.34 The primers and the experimental conditions used to detect the homozygous MTHFR mutation, the FV Leiden, and the G20210A FII variant by polymerase chain reaction technique have been previously described.35 The amplification products were electrophoretically resolved in 3% agarose gels by a 40 mmol/L Tris-acetate buffer (pH 7.7) containing 1 mmol/L EDTA, stained with 0.5 μg/mL of ethidium bromide, and visualized under UV light.

Measurements

Serum total homocysteine, ie, the sum of free homocysteine, cysteine-homocysteine mixed disulfide, and protein-bound forms, was evaluated by an ELISA method and spectrophotometrically measured (normal levels, <15 μmol/L).

Statistical Analysis

The Statistical Package for Social Sciences for personal computer was used for statistical analysis. The results are expressed as mean±SD. Continuous variables were analyzed by Student’s t test and the Scheffé post hoc test. Categorical variables were analyzed by χ² statistics; when appropriate, odds ratios (ORs) and their 95% CIs are reported. Multiple regression analysis (dependent variable: homocysteine) was performed with a stepwise method. In each case, values of P<0.05 were considered statistically significant.

Results

Demographic and Clinical Characteristics of the Study Sample

Of the 132 patients, 66 were males (50%), while 44.6% of controls were males (117/262). Mean age of the patients was 38.4±11.7 years, and mean age at first event was 34.8±10.9 years. Mean age of controls was statistically comparable (36±13.2 years; P>0.05, t test). Cases were more often current smokers (66.7% versus 32.4%; OR=4.2; 95% CI, 2.5 to 6.8; P<10⁻⁶, χ² test), hypertensives (38.8% versus 9.3%; OR=6.2; 95% CI, 3.3 to 11.6; P<10⁻⁶, χ² test), diabetics (7.3% versus 5.1%; OR=1.5; 95% CI, 0.5 to 4; P>0.05, χ² test), and hyperlipidemics (41.5% versus 33.3%; OR=1.6; 95% CI, 0.8 to 2.3; P>0.05, χ² test), although in the last 2 cases the difference compared with controls did not reach statistical significance. According to the dietary questionnaire, 2 patients and 3 controls met the requirements of habitual alcohol consumption (ie, a history of documented alcohol intake for ≥5 days a week). Because of the limited number of subjects with habitual alcohol consumption, alcohol was not taken into consideration in the analysis. We found low plasma levels of antithrombin III in 1 case, low plasma levels of protein C in 1 case, and functional protein S deficiency in 2 cases, while no case or control had abnormally low fibrinogen plasma levels. Antiphospholipid syndrome was detected in 3 patients (Table 1). In regard to the type of events, CT and/or MRI scans showed that 59 individuals had a lacunar stroke (thrombosis of intracranial arteries). In the remaining cases, transsphincteric and/or transesophageal echocardiography as well as Doppler ultrasonography of extracranial carotid arteries confirmed that 4 individuals had a cardioembolic stroke and 22 had had an atherothrombotic event (large-vessel disease, ie, at least 1 internal carotid and/or vertebral artery with ≥50% stenosis). Because of the small sample size, among these 85 patients, no significant association was detected between genetic predisposing factors, homocysteine levels, and type of stroke. Since echocardiography and/or Doppler ultrasonography could not be obtained in the other 51 cases, differentiation between atherothrombotic and cardioembolic events could not be established.

Prevalence of Genetic Polymorphisms Among Patients and Controls

Among cases, the frequency of heterozygosity for FV Leiden and for FII G20210A gene mutation were 7 of 132 (5.3%) and
There was no difference in the prevalence of FV and of FII mutations when the study population was stratified according to sex; this was true in the whole population and when patients and controls were analyzed separately (7.1% and 5.2% in males and females, respectively, for FII; all 5.2% in males and females, respectively, for FV Leiden; 8.7% and 4.7% in males and females, respectively, for FII). Only 1 case subject (0.8%) carried both mutations as well (0.4%; P<0.05, Fisher’s exact test). Homozygosity for C677T mutation of the MTHFR gene was detected in 30 of 132 patients (22.7%) and in 45 of 262 controls (17.2%); the difference was not statistically significant (P>0.05, χ² test). The same lack of significance was obtained for MTHFR TT genotype by dividing the study population according to sex (20.8% and 17.5% for males and females, respectively; all P>0.05, χ² test). Among patients, homozygosity for TT genotype of MTHFR was found to be associated with heterozygosity for FII variant in 3 cases (2.3%), while no case of association between FV Leiden and MTHFR TT genotype was found. The prevalence of the associations of FV Leiden and FII variant with TT genotype of MTHFR was not statistically different in controls, at 1 of 262 (0.4%) for FV Leiden and 4 of 262 (1.5%) for FII variant (all P>0.05, χ² test) (Table 1).

**Determinants of Serum Homocysteine Levels**

To identify the main determinants of homocysteine levels, a multiple regression analysis with a stepwise method was performed. In the equation, homocysteine was the dependent variable, and sex, age, and MTHFR genotype were the independent variables.

### Table 1: Patients’ Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>Controls</th>
<th>OR (95% CI)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients (F/M)</td>
<td>132 (66/66)</td>
<td>262 (145/117)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean age, y</td>
<td>38.4±11.7</td>
<td>36±13.2</td>
<td></td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Age at first event, y</td>
<td>34.8±10.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acquired factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cigarette smoking</td>
<td>66.7%</td>
<td>32.4%</td>
<td>4.2 (2.5–6.8)</td>
<td>&lt;10⁻⁶</td>
</tr>
<tr>
<td>Hypertension</td>
<td>38.8%</td>
<td>9.3%</td>
<td>6.2 (3.3–11.6)</td>
<td>&lt;10⁻⁶</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>41.5%</td>
<td>33.3%</td>
<td>1.6 (0.8–2.3)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>7.3%</td>
<td>5.1%</td>
<td>1.5 (0.5–4)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Genetic factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FV Leiden</td>
<td>7 (5.3%)</td>
<td>17 (6.5%)</td>
<td>0.8 (0.3–2.1)</td>
<td>0.8</td>
</tr>
<tr>
<td>FII G20210A</td>
<td>10 (7.6%)</td>
<td>16 (6.1%)</td>
<td>1.2 (0.5–3)</td>
<td>0.73</td>
</tr>
<tr>
<td>MTHFR C677T</td>
<td>30 (22.7%)</td>
<td>45 (17.2%)</td>
<td>1.4 (0.8–2.5)</td>
<td>0.23</td>
</tr>
<tr>
<td>FV+Fil</td>
<td>1 (0.8%)</td>
<td>1 (0.4%)</td>
<td>&gt;0.05†</td>
<td></td>
</tr>
<tr>
<td>MTHFR+Fil</td>
<td>0 (0%)</td>
<td>1 (0.4%)</td>
<td>&gt;0.05†</td>
<td></td>
</tr>
<tr>
<td>MTHFR+Fil</td>
<td>3 (2.3%)</td>
<td>4 (1.5%)</td>
<td>&gt;0.05†</td>
<td></td>
</tr>
</tbody>
</table>

*Patients vs controls, χ² test.
†Patients vs controls, Fisher’s exact test.

### Table 2: Serum Homocysteine Levels According to Sex and Genotype

<table>
<thead>
<tr>
<th></th>
<th>Patients (n=125)</th>
<th>Controls (n=252)</th>
<th>P (t Test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>13±18.6</td>
<td>10.8±6.2</td>
<td>0.004</td>
</tr>
<tr>
<td>Males</td>
<td>13.6±11.1</td>
<td>13.3±8.1*</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Females</td>
<td>12.5±23.9</td>
<td>8.7±2.9</td>
<td>0.000</td>
</tr>
<tr>
<td>TT</td>
<td>25.3±36.8†</td>
<td>15±11.6*</td>
<td>0.02</td>
</tr>
<tr>
<td>CT</td>
<td>9.4±3.2</td>
<td>9.9±3.8</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>CC</td>
<td>9.6±2.7</td>
<td>9.7±3.9</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

Values are mean±SD, expressed in micromoles per liter.
*Males vs females, P=0.003, t test.
†CC vs CT vs TT, P<0.05, Scheffé post hoc test.
covariates. Multiple $R$ of the regression was 0.24. The first variable that was entered in the equation was MTHFR genotype ($\beta=0.24$; $t=4.90$; $P=0.000$), and the second was sex ($\beta=0.15$, $t=3.07$; $P=0.002$); age was not in the equation as a variable and therefore was not associated with total homocysteine levels ($\beta=0.08$; $t=1.52$; $P=0.128$) (Table 3).

**Discussion**

In our setting of patients with young adult ischemic stroke, we investigated the prevalence of the most common genetic polymorphisms associated with an increased risk of venous thrombosis and their correlation with the event. The prevalence of FV Leiden and the 20210A FII variant was not significantly higher among patients than in the control population. These data are comparable with several reports showing that the presence of FV Leiden5,7,11 or the FII variant6–8,10,11 is not associated with an increased risk of arterial thrombosis. However, they are at variance with those by Margaglione et al10, who found an OR of 2.56 for FV Leiden, and those by De Stefano et al,9 who found an OR of 5 for the FII variant. Because it is a reference center, our institution admits unselected patients with arterial thrombotic events. In the study population by Margaglione et al,10 a history of venous thrombosis was significantly more frequent in patients than in controls. In addition to the high selection (young patients with no atherogenic risk factors), the group of patients evaluated by De Stefano et al9 was small ($n=72$). Moreover, FV Leiden and FII 2010A play a major role in venous thrombosis, including that taking place in the veins of the brain.36–39

Previous reports have shown that hyperhomocysteinemia is closely associated with the occurrence of stroke. In a cohort of middle-aged British men, Perry et al15 identified hyperhomocysteinemia as a strong, graded, independent risk factor for stroke. Coull et al12 reported that moderate hyperhomocysteinemia is an independent risk factor for stroke of any type. Our data confirm and extend the concept that total homocysteine serum levels are significantly higher among patients with ischemic stroke than in controls; this statement was still true when patients with major thrombophilic factors, such as antithrombin III deficiency ($n=1$), protein C deficiency ($n=1$), protein S deficiency ($n=2$), or antiphospholipid syndrome ($n=3$), were excluded from the analysis.

The frequency of homozygosity for C677T mutation of the MTHFR gene, the main genetic determinant of moderate hyperhomocysteinemia, was investigated in the present setting as well. The TT mutation was more frequent in patients than in controls (22.7% versus 17.2%), but the difference was not statistically significant ($P=0.05$, $\chi^2$ test). Thus, our data support the widely held opinion that homozygosity for MTHFR TT variant is not a risk factor for arterial ischemic stroke.11,30–32 This concept has been disputed in only 1 case.29 However, when patients and controls were divided according to genotypes, a significant difference in total homocysteine levels was found only when TT homozygotes were compared. Furthermore, as reported by several authors,29,32 we observed higher serum homocysteine levels in subjects with TT genotype than in those with CC or CT genotype, while no differences were observed for total homocysteine levels among CC and CT genotypes in patients as well as in controls. Finally, when we performed a multiple regression analysis to identify the main determinants of moderate hyperhomocysteinemia, MTHFR genotype was the first (strongest) variable that entered the equation. Despite the fact that the prevalence of the TT mutation among patients was not statistically higher than in controls, these findings are consistent with a dominant role for the TT genotype in moderate hyperhomocysteinemia.

When patients and controls were divided according to sex, males exhibited raised homocysteine levels only among controls. The interaction with other genetic risk factors40,41 and the intake of folic acid42,43,28,42–44 or vitamin B12,44,45 which ameliorates the effect of thermolabile MTHFR on homocysteine levels, may well account for the lack of difference that we have observed when the whole population was stratified according to sex. Accordingly, multiple $R$ of our regression analysis was only 0.24, perhaps because of the absence in our equation of other important variables such as vitamin levels.

Although the main thrust of the present report is devoted to homocysteine and other inherited prothrombotic conditions in young adults with ischemic stroke, over the last 10 years, vascular thrombosis (eg, ischemic stroke) among young patients has been shown to be a multifactorial disease in which inherited predisposing factors play an increasingly relevant role. More recently, the interaction of inherited factors with environmental variables has further clarified our understanding of the mechanisms leading to ischemic stroke. Hyperhomocysteinemia is a “new” predisposing condition in which inherited and acquired factors interact cumulatively. It is increasingly recognized that single genetic abnormalities are seldom the sole cause of stroke. As clarified in the present report (Table 1), subjects with a history of stroke were more often hypertensives and heavy smokers than controls matched for sex and age. This is in agreement with previous reports on this topic and supports the concept that gene/environment interactions (eg, those leading to hyperhomocysteinemia) may be important determinants of a propensity to develop stroke events.

Recently, the homocysteine-lowering effects of folate and vitamins B12 and B6 have been extensively described,46,47 and the possibility of vitamin supplementation as a therapeutic strategy for vascular diseases has received attention. At this time, the beneficial effects of lowering plasma homocysteine by vitamins, in relation to the risk of vascular disease, have not yet been established. In view of this, prospective studies using vitamin B12 and/or folate supplementation are needed to carefully quantify the risk reduction in primary and in secondary prevention studies. This information is of special

**TABLE 3. Main Determinants of Serum Homocysteine Levels ($R=0.24^*$)**

<table>
<thead>
<tr>
<th></th>
<th>$\beta$</th>
<th>$t$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR</td>
<td>0.24</td>
<td>4.90</td>
<td>0.000</td>
</tr>
<tr>
<td>Male sex</td>
<td>0.15</td>
<td>3.07</td>
<td>0.002</td>
</tr>
<tr>
<td>Age</td>
<td>0.08</td>
<td>1.52</td>
<td>0.128</td>
</tr>
</tbody>
</table>

$^*$Multiple regression analysis (dependent variable: homocysteine).
interest in patients with a history of young adult ischemic stroke.

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References


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