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Short communication

Hyperhomocysteinemia increases the risk of venous thrombosis independent of the C677T mutation of the methylenetetrahydrofolate reductase gene in selected Brazilian patients

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Fasting total homocysteine (tHcy) and the methylenetetrahydrofolate reductase (MTHFR) C677T mutation were evaluated in 91 patients with venous thromboembolism and without acquired thrombophilia, and in 91 age-matched and sex-matched controls. Hyperhomocysteinemia was detected in 11 patients (12.1%) and in two controls (2.2%), yielding an odds ratio (OR) for venous thrombosis of 6.1 [95% confidence interval (CI), 1.3–28.4]. After excluding 21 patients and four controls with other known genetic risk factors for venous thrombosis, the OR was not substantially changed (7.0; 95% CI, 1.5–33.1). The prevalence of the MTHFR 677TT genotype was not significantly different in patients (9.9%) and in controls (5.5%), with an OR for venous thrombosis of 1.8 (95% CI, 0.6–5.8). Subjects with the MTHFR 677TT genotype showed higher levels of tHcy compared with the 677CC genotype in patients ($P = 0.010$) and in controls ($P = 0.030$). In conclusion, we found that fasting hyperhomocysteinemia is a risk factor for venous thrombosis in patients without known acquired thrombophilia and other genetic risk factors for venous thrombosis. Although tHcy levels are significantly higher in those homozygous for the MTHFR C677T mutation, this genotype does not increase the thrombotic risk in our study population. *Blood Coagul Fibrinolysis* **13**:271–275 © 2002 Lippincott Williams & Wilkins.

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Introduction

Moderate hyperhomocysteinemia has been associated with an increased risk of arterial disease [1]. In 1994, Falcon *et al*. [2] reported moderate hyperhomocysteinemia as a risk factor for developing venous thrombosis at a young age. Other case–control studies have shown that mild and moderate hyperhomocysteinemia are independent risk factors for venous thrombosis [1].

A thermolabile variant of methylenetetrahydrofolate reductase (MTHFR) is associated with decreased enzyme activity and mild hyperhomocysteinemia [3]. In 1995, Frosst *et al*. elucidated the

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molecular basis of this thermolability, which is due to a C → T substitution at base pair 677 in the MTHFR gene [4]. The homozygosity for the MTHFR C677T polymorphism was associated with an increased risk of venous thrombosis by some authors [5,6], whereas others failed to demonstrate this association [7,8]. Therefore, the role of the MTHFR 677TT genotype as a risk factor for venous thrombosis is still unclear.

The aim of the present study was to determine total homocysteine (tHcy) levels and the MTHFR C677T polymorphism in patients with confirmed venous thrombosis, and in age-matched and sex-matched healthy controls.

Materials and methods

Patients and controls

We studied 91 unrelated patients (34 males and 57 females), with at least one documented episode of venous thromboembolism (VTE), admitted to a Tertiary Care University Hospital in São Paulo between June 1996 and January 2000. Patients were enrolled in the study at least 3 months after the episode of VTE. VTE was confirmed by Doppler ultrasonography and/or contrast phlebography, perfusion and ventilation scans, magnetic resonance imaging or angiography. Thirty-five (38.5%) patients had experienced recurrent episodes of VTE. A preliminary evaluation ruled out patients with acquired disorders that could predispose them to VTE or elevated homocysteine concentration: malignancy, autoimmune diseases, antiphospholipid syndrome, congestive heart failure, hemiplegia, renal failure and use of antifolate drugs.

Patients were asked to look for their own healthy controls according to the following criteria: same sex and age (± 5 years), same geographical origin and no biologic relationship. None of the 91 controls (34 males and 57 females) had a history of thrombosis or known disorders that could predispose them to VTE or elevated homocysteine concentration. Among the patients and controls, approximately 70% were Mestizos (Caucasian and Black descent), 20% were Caucasians and 10% were Blacks.

Informed consent was obtained from all participants and the study was approved by the local Ethics Committee on human research.

Methods

After an overnight fasting of 12 h, venous blood was collected in ethylenediamine tetraacetic acid for tHcy determination and DNA analysis, and in 0.129 mol/l sodium citrate for coagulation assays. Platelet poor plasma aliquots were prepared within 30 min, frozen at −70°C and stored at −20°C until use.

Antithrombin III (ATIII) and protein C were tested by functional methods (Stachrom ATIII, Stachrom Protein C; Diagnostica Stago, Asnières, France) and free protein S antigen was measured by enzyme-linked immunosorbent assay (AsserachromFree Protein S; Diagnostica Stago). Genomic DNA was extracted from leukocytes, and detection of MTHFR C677T mutation, factor V G1691A and prothrombin G20210A were performed as previously reported. DNA analysis was not available for one control.

Plasma tHcy concentration was measured by a Shimadzu high-performance liquid chromatographer (Kyoto, Japan) with fluorimetric detection and isocratic elution [9]. All reagents were obtained from Sigma Chemical Corporation (St. Louis, Missouri, USA). Hyperhomocysteinemia was defined by fasting tHcy exceeding the 95th percentile of the sex-specific distribution of the control population. Two cut-points were obtained to define hyperhomocysteinemia according to the age group in both sexes: ⩽ 40 years, tHcy > 14.5 μmol/l in men ($n = 16$) and > 11.2 μmol/l in women ($n = 35$); > 40 years, tHcy > 22.6 μmol/l in men ($n = 18$) and > 11.3 μmol/l in women ($n = 22$).

Statistical analysis

The Statistical Package for the Social Sciences (SPSS Inc., Chicago, Illinois, USA) was employed for statistical analysis. Results are expressed as median and range. The significance of difference in medians was tested by Mann–Whitney U test. The chi-squared (χ^2) test was used to compare the frequency of categorical variables and, when appropriate, Yates' correction or Fisher's exact test were applied. Spearman's rank correlation coefficient (r_s) was calculated to test the association between two variables. The odds ratio (OR) was calculated as an approximation of relative risk with 95% confidence intervals (CI). Two-tailed $P < 0.05$ was considered statistically significant.

Results

Fasting tHcy in patients and controls

Median age was similar in patients (40 years; range, 9–82 years) and controls (38 years; range, 9–78 years) ($P = 0.406$). Median tHcy levels were significantly higher in patients than in controls and in men than in women (except for controls > 40 years), as shown in Table 1. Plasma tHcy levels

Table 1. Median total homocysteine (tHcy) levels (μmol/l) according to age and sex in patients and in controls

	Patients		Controls	
Median tHcy	7.4		6.9	
Range	3.6–137.0		3.2–22.6	
P (Mann–Whitney test)	0.033			
	Patients		Controls	
	Men	Women	Men	Women
Age ⩽ 40 years				
n	16	31	16	35
Median tHcy	8.7	6.0	6.9	5.5
Range	5.1–17.6	3.8–16.7	4.6–14.5	3.2–18.7
P (Mann–Whitney test)	0.002		0.010	
Age > 40 years				
n	18	26	18	22
Median tHcy	10.3	7.0	7.7	7.4
Range	6.4–137.0	3.6–20.5	3.9–22.6	4.1–11.4
P (Mann–Whitney test)	0.002		0.295	

increase with age in patients ($r_s = 26.9\%$, $P = 0.010$) and in controls ($r_s = 44.5\%$, $P < 0.001$). Median tHcy levels were not statistically different between patients with recurrent VTE (8.2 μmol/l; range, 4.0–137 μmol/l) and those with one episode (7.2 μmol/l; range, 3.6–58.6 μmol/l) ($P = 0.372$). Hyperhomocysteinemia was detected in 11 patients (12.1%) and in two controls (2.2%), yielding an OR for venous thrombosis of 6.1 (95% CI, 1.3–28.4). Four (11.8%) male patients and seven (12.3%) female patients ($P > 0.999$), no control men and two (3.5%) control women ($P = 0.526$) had hyperhomocysteinemia. Genetic risk factors for venous thrombosis were detected in 21 out of 91 patients (23.0%): three ATIII, one protein C and five protein S deficiencies, three heterozygous for factor V G1691A, five heterozygous for prothrombin G20210A, and four with combined defects (one factor V G1691A + prothrombin G20210A, one factor V G1691A + ATIII, and two prothrombin G20210A + protein S). Among the controls ($n = 90$), three were heterozygous for factor V G1691A and one for prothrombin G20210A mutation. After excluding subjects carrying known genetic risk factors for venous thrombosis, hyperhomocysteinemia was observed in 10 out of 70 patients (14.3%) and in two out of 86 controls (2.3%), yielding an OR for venous thrombosis of 7.0 (95% CI, 1.5–33.1).

The MTHFR C677T polymorphism in patients and controls

The MTHFR 677TT genotype was found in nine patients (9.9%) and in five controls (5.5%), with an OR for venous thrombosis of 1.8 (95% CI, 0.6–5.8). After excluding subjects with other known genetic risk factor for venous thrombosis, the OR remained almost the same (2.0; 95% CI, 0.6–6.7), and among those subjects the MTHFR 677TT genotype was detected in only one patient (heterozygous for prothrombin G20210A mutation). Two patients with recurrent VTE (5.7%) and seven with a single episode (12.5%) were homozygous for the MTHFR C677T mutation ($P = 0.473$). Patients with the MTHFR 677TT genotype had significantly higher levels of tHcy compared with the 677CC ($P = 0.010$) and the 677CT ($P = 0.016$) genotypes. In the control group, tHcy levels were only significantly higher in homozygous for the MTHFR C677T mutation compared with the 677CC genotype ($P = 0.030$), but not with the 677CT genotype ($P = 0.084$). Table 2 shows tHcy levels and the prevalence of hyperhomocysteinemia according to the different MTHFR 677 genotypes.

Discussion

In this study, 12.1% of the patients and 2.2% of the controls meet the criterion of hyperhomocysteinemia considering the 95th percentile of the sex-specific and age-specific tHcy distribution of the control population. In our results, tHcy concentrations were higher in men and increase with age, which is in agreement with a previous report [10]. Therefore, age-related and sex-related differences were important variables to define hyperhomocysteinemia.

Table 2. Total homocysteine (tHcy) levels (μmol/l) and hyperhomocysteinemia (Hhcy) according to methylenetetrahydrofolate reductase (MTHFR) 677 genotypes in patients and in controls

	MTHFR 677 genotype		
	677TT	677CT	677CC
Patients			
n (%)	9 (9.9%)	38 (41.8%)	44 (48.3%)
Median tHcy	15.0	7.1	7.6
Range	5.1–137.0	4.0–21.6	3.6–20.5
Hhcy (*n*)	4	4	3
Controls			
n (%)	5 (5.5%)	46 (51.1%)	39 (43.4%)
Median tHcy	7.8	7.2	6.6
Range	7.0–22.6	3.5–18.7	3.2–11.4
Hhcy (*n*)	0	1	1

We report a significant association between hyperhomocysteinemia and venous thrombosis, which persists when subjects with other known genetic risk factors for venous thrombosis are excluded. This is the first study that analyses hyperhomocysteinemia as a risk factor for thrombosis in Brazilians. Previous studies have also demonstrated that mild hyperhomocysteinemia is an independent risk factor for venous thrombosis [2,8,11].

We found no significant association between the MTHFR 677TT genotype and venous thrombosis, although subjects with this genotype have clearly higher tHcy levels. Other case–control studies also failed to demonstrate a significant association between the MTHFR 677TT genotype and venous thrombosis [7,8]. In a meta-analysis, the MTHFR 677TT genotype was associated with elevated homocysteine levels but does not increase the cardiovascular risk [12]. On the contrary, there are some reports showing a positive association between the MTHFR 677TT genotype and venous thrombosis [5,6]. Therefore, the effect of the MTHFR C677T mutation on the risk of venous thrombosis still remains controversial.

Homocysteine concentration depends on genetic–environmental interactions among enzymatic defects, age, sex, vitamin intake (vitamin B_{12}, vitamin B_6 and folate), smoking, and coffee consumption [1]. Probably, as the interaction among these factors increases, the impact on tHcy levels and on the risk of thrombosis becomes more evident.

We conclude that fasting hyperhomocysteinemia is a significant risk factor for venous thrombosis in patients without known acquired thrombophilia and other genetic risk factors for venous thrombosis, which reinforces the importance of homocysteine evaluation in thrombotic disease. Although tHcy levels are significantly higher in those homozygous for the MTHFR C677T mutation, our study does not show a significant association between this genotype and venous thrombosis.

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