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C677T substitution in the methylenetetrahydrofolate reductase gene as a risk factor for venous thrombosis and arterial disease in selected patients

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Abstract

Background and Objective. **Hyperhomocysteinemia, due to a combination of genetic and environmental factors, is considered to be a risk factor for vascular disease. Individuals with the thermolabile variant of methylenetetrahydrofolate reductase (MTHFR), due to homozygous C677T MTHFR gene mutation, have significantly raised plasma levels of homocysteine and may be at increased risk of vascular disease. However, it is still controversial a direct association between C677T homozygosity and the occurrence of vascular disease is still controversial.**

Design and Methods. **To clarify the contribution of C677T MTHFR mutation in arterial occlusive disease (AOD) or venous thromboembolism (VTE), we performed a case-controlled study including 160 cases with AOD and 180 cases with VTE attending our referral center and compared them with 200 matched healthy controls. MTHFR gene mutation was evaluated by PCR and odds ratios (OR) and the 95% confidence intervals (CI) were used to estimate the risk for venous or arterial thrombosis.**

Results. **There was a high prevalence of homozygotes for the mutated MTHFR allele among the whole group of cases with arterial disease (OR=2.35, *p*=0.001). Considering the AOD cases with and those without associated risk factors for arterial disease separately the difference remained significant only in the latter group (*p*=0.168 and P<0.001 respectively). In contrast, the prevalence of mutated homozygotes among the whole group of cases with VTE was not significantly different from that in the control group (OR=1.67; *p*=0.070). Excluding VTE cases with inherited thrombophilia or with circumstantial risk situations the value increased in both subgroups (OR=2.26; *p*=0.006 and OR=2.03; *p*=0.033 respectively). Considering only VTE cases with neither inherited thrombophilia nor circumstantial risk situations the risk increased further (OR=2.57; *p*=0.017).**

Interpretation and Conclusions. **These data suggest that in selected patients homozygosity for the MTHFR mutation increases the risk of both arterial and venous thromboses and that differences in selection criteria for the patient group may be responsible in part for the controversial association of the MTHFR mutation and vascular disease.**

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Mild or moderate hyperhomocysteinemia is a known risk factor for arterial and venous thrombophilia as single disorders or in combined defects.[1-4] Hyperhomocysteinemia is due to a combination of genetic and environmental factors and several mutations or combined defects affecting the homocysteine pathway have been reported.[5-7] A polymorphism, C677T in the methylenetetrahydrofolate reductase (MTHFR) gene, is responsible for the Ala223Val substitution in a highly conserved residue of the molecule.[8] The substitution renders the enzyme thermolabile and has been recognized as a cause of intermediate hyperhomocysteinemia.[9] Recent findings reveal that subjects carrying the MTHFR mutation in homozygous condition have significantly higher homocysteine levels than heterozygotes or normal homozygotes particularly when folate levels are in the low normal range.[10,11] These subjects may be at increased risk of cardiovascular disease.[8,12,13] We recently reported an association between C677T mutation and venous thrombosis in patients without known prothrombotic defects and that the association of the homozygous MTHFR mutation with low folate levels can be considered the main determinant of mild hyperhomocysteinemia in normal and thrombotic subjects.[14] However, not all studies have reported a direct association between C677T mutation and venous thromboembolism[15-18] or arterial vascular disease.[15,19-21] The relationships between C677T mutation and venous thromboembolism or arterial vascular disease are, therefore, still somewhat controversial. Moreover, the frequency of the mutated allele is quite high and it has a significantly heterogeneous distribution among different ethnic groups considered,[22-24] with a lower prevalence in black races than in white.[25,26] In the present study

we evaluated the prevalence of the C677T MTHFR gene mutation in two groups of patients, one group with venous thrombosis and one with arterial vascular disease, demonstrating an increase in thrombotic risk when inherited thrombophilia and/or acquired risk situations were excluded.

Design and Methods

Subjects

A total of 340 unrelated patients suffering from AOD or VTE were selected from among the 500 examined and enrolled in the study. Of these 340 patients, 160 had documented AOD (M/F 78/82; median age 48.5 yrs, range 24-65) and 180 had confirmed VTE (M/F 89/91; median age 49.5 yrs, range 24-65). Both groups attended our Center for investigation of their hypercoagulability state and/or to monitor anticoagulant therapy and had been free of thrombotic episodes for at least three months prior to the study.

In the VTE group, the ratio between pulmonary embolism (confirmed by pulmonary scintigraphy) and deep vein thrombosis (confirmed by phlebography or compression ultrasonography) as main diagnosis was 1/1.7. A total of 56 patients (31.1%) were identified as carriers of inherited thrombophilia: R506Q factor V Leiden mutation (n=32), G20210A factor II gene mutation (n=10), protein S deficiency (n=7), protein C deficiency (n=5), antithrombin deficiency (n=2). Venous thrombosis not associated with circumstantial risk situations (cancer, surgery, contraceptives therapy, recent child birth or trauma) accounted for 87 of the total events (48.3%).

In the group of patients with arterial occlusive disease, 63 cases (39.3%) were survivors of myocardial infarction (confirmed by electrocardiogram and coronary arteriography) and the remaining patients suffered from cerebral (n=50) or peripheral (n=47) occlusive disease (confirmed by computerized tomography and arteriography respectively). The cerebral occlusive disease was manifested as ischemic strokes, any form of transient ischemic attack having been excluded. One or more of the following known risk factors for arterial disease were present in 84 (52.5%) of the cases: hyperlipoproteinemia, hypertension, diabetes and smoking status.

The control group (n=200, M/F 1/1; median age 49.0, range 24-65) were blood donors, not smokers, from the same geographic region as the patients (Emilia Romagna, Northern Italy) and without a familial history of thrombophilia. Among the control group, a total of 11 subjects (5.5%) carried thrombophilic defects: R506Q factor V Leiden mutation (n=5), G20210A factor II gene mutation (n=4), protein S deficiency (n=1), protein C deficiency (n=1), antithrombin deficiency (n=0).

For all groups, age >65 or <24 and lack of informed consent were also considered as exclusion criteria.

Coagulation findings

To screen defects in the protein C pathway, including specialized assays for protein C, protein S, and for activated protein C resistance, we utilized a modification of the ProC® Global test (Dade-Behring, Milan Italy) as previously described.[27,28] Antithrombin activity (Chromogenix AB Mölndal, Sweden) was measured according to the supplier's instructions.

DNA analysis

Detection of the C677T MTHFR gene mutation

The Ala223Val substitution, due to the C677T transition in the MTHFR gene, creates an additional *HinfI* restriction site in the PCR amplified fragment as described by Frosst *et al.*[8] The PCR amplification cycles were modified as follows: 5 min initial at 94°C, followed by 30 cycles of 40 sec of denaturation at 94°C, 4 sec of annealing at 56°C and 12 sec of extension at 72°C in a Perkin Elmer DNA Thermal Cycler apparatus. A fragment of 198 bp was obtained and one tenth of the PCR product was digested by 0.5U of restriction enzyme *HinfI*.

Detection of the factor V Leiden (R506Q) and factor II (G20210A) gene mutation

For the one-step determination of factor V Leiden and prothrombin G20210A variant, we utilized multiplex PCR-mediated site-directed mutagenesis, creating a neo-site for TaqI endonuclease in both the factor V gene and prothrombin gene amplified fragments as recently described by Ripoll *et al.*[29]

Statistical analysis

The statistical significance of the differences of the genotype distribution between cases and controls was calculated by the Chi-square test. When appropriate, Yates' correction or Fisher's exact test was applied. *p* values less than 0.05 were considered statistically significant. Odds ratios (OR) and their 95% confidence intervals (CI) were used to estimate the risk for venous or arterial thrombosis. An OR was considered to be statistically significant when the lower limit of the 95% CI was > 1.0.

Results

As shown in Tables 1 and 2, among the whole group of control subjects investigated (n=200), the frequency of homozygotes for the mutated allele (TT) in the MTHFR gene was 15%, of homozygotes for the normal allele (CC) 34% and of heterozygotes (CT) was 51%.

Among all cases with VTE (n=180) the prevalence of mutated homozygotes was 22.7% which was not significantly different from that in the control group (p=0.070). This gave an odds ratio of 1.67 (CI 95% 0.99-2.81). After exclusion of 56 cases identified as carriers of prothrombotic defects, the risk for venous thrombosis increased and became statistically significant (OR=2.14; CI 95% 1.23-3.72; p=0.01). It would be better to compare this latter subgroup with controls not carrying prothrombotic defects. After exclu-

Table 1. Distribution of the MTHFR genotype and associated thrombotic risk in the patients with venous thrombosis.

	n	*MTHFR genotype*			*Odds ratio*	*p*
		CC (%)	CT (%)	TT (%)	(CI 95%)	
Total cases (VTE)	180	53 (29.4)	86 (47.7)	41 (22.7)	1.67 (0.99-2.81)	0.070
VTE without risk situations*	87	24 (27.5)	40 (45.9)	23 (26.4)	2.03 (1.10-3.76)	0.033
Total controls	200	68 (34.0)	102 (51.0)	30 (15.0)	--	--
VTE without inherited thrombophilia°	124	31 (25.0)	59 (47.6)	34 (27.4)	2.26 (1.28-3.99)	0.006
VTE without inherited thrombophilia and risk situations	50	11 (22.0)	24 (48.0)	15 (30.0)	2.57 (1.24-5.33)	0.017
Controls without thrombophilia°	189	62 (32.8)	100 (52.9)	27 (14.3)	--	--

**After exclusion of 93 cases associated with circumstantial risk situations; °respectively after exclusion of 56 cases and 11 controls with prothrombotic defects. Statistical significance was taken as p<0.05.*

Table 2. Distribution of the MTHFR genotype and associated thrombotic risk in the patients with arterial occlusive disease.

	n	*MTHFR genotype*			*Odds ratio*	*p*
		CC (%)	CT (%)	TT (%)	(CI 95%)	
Total cases (AOD)	160	32 (20.0)	81 (50.6)	47 (29.4)	2.35 (1.40-3.94)	0.001
AOD without risk factors associated*	76	14 (18.4)	34 (44.7)	28 (36.8)	3.30 (1.80-6.06)	<0.001
AOD with risk factors associated	84	18 (21.4)	47 (55.9)	19 (22.6)	1.65 (0.87-3.14)	0.168
Controls	200	68 (34.0)	102 (51.0)	30 (15.0)	--	--

**After exclusion of 84 cases with risk factors for arterial disease. Statistical significance was taken as p<0.05.*

sion of such subjects (n=11; see methods) the calculated risk value was substantially unaltered (OR=2.26; CI 95% 1.28-3.99; *p*=0.006); this value is reported in Table 1. The MTHFR genotype distribution in the controls after exclusion of carriers of inherited thrombophilia did not differ significantly from that of the whole group of controls (*p*=0.931). Increasing risk, with an appreciable significant value, was also obtained comparing normal controls with the cases not associated with circumstantial risk situations (n=87) (OR=2.03; CI 95% 1.10-3.76; *p*=0.033). Moreover, among VTE cases without defined prothrombotic defects, those cases not associated with circumstantial risk situations (n=50) showed the highest risk value (OR=2.57; CI 95% 1.24-5.33; *p*=0.017).

Among total cases with AOD (n=160) the prevalence of the mutated homozygotes was 29.4%. This was significantly different from that in the control group and gave an odds ratio of 2.35 (CI 95% 1.40-3.94; *p*=0.001). Considering the cases which had (n=84) and those which did not have (n=76) associated risk factors for arterial disease separately, the odds ratio value was higher in the latter group (OR=3.30; CI 95% 1.80-6.06; p<0.001). Although there was a difference between AOD cases having associated risk factors and controls (22.6% vs 15%) this was not statistically significant (p=0.168).

Discussion

The thermolabile variant of MTHFR, due to the C677T homozygous mutation in the MTHFR gene, is the most frequent inherited defect of homocysteine metabolism.[8-14] The defect may lead to hyperhomocysteinemia, a condition included among the disorders of venous and arterial occlusive disease.[1,2,30,31] Nevertheless, a direct association between C677T mutation and vascular diseases is still somewhat controversial.[14-21]

We hypothesized that the controversy was in part due to different criteria adopted in the recruitment of patients. Therefore we checked whether divergent results would be obtained by analyzing subsets of patients separately. We did not measure plasma homocysteine levels, because the aim of the study was to identity a possible positive association of the C677T mutation with venous or arterial diseases in subsets of selected cases of thrombotic patients.

The allele frequency and the MTHFR genotype distribution in our control population were similar to those that we reported in a recent study[14] and they were also in accordance with those given for the Italian population.[22] Our finding that in unselected cases of VTE homozygosity for the C677T MTHFR genotype is not significantly associated with thrombotic risk (*p*=0.070) is in agreement with previous studies in Italian patients.[17,32] Contrariwise, the statistical significance we found in VTE cases without coexisting prothrombotic defects (p=0.006), although in accordance with our previous report,[14] contrasts with other published data, in that although a higher risk was found in patients without inherited thrombophilia this was not significantly different from that in controls.[32,33]

Increasing risk for VTE, only when C677T mutation was associated to factor V Leiden[34] or independently from its coexistence,[16,35] was also reported. We also found appreciable risk values (p=0.033) considering VTE not dependent on circumstantial risk situations; on the one hand, this agrees with data reported for hyperhomocysteinemia by Ridker *et al.* who found an increased risk of VTE only in idiopathic cases,[36] on the other it is in contrast with results of a recent study in which the risk associated with the MTHFR mutation increased in subjects with predisposing circumstantial risk factors for venous thrombosis.[16] Finally, a study in the Brazilian population showed a positive association between C677T mutation and venous thrombosis with a lower risk when cases with inherited prothrombotic defects were excluded.[15] The controversial association remains also considering arterial disorders. When we evaluated the cases with arterial disease not associated with acquired risk factors, the risk was about three-fold greater than in controls (p<0.001) decreasing to non-significant values (p=0.168) when only cases with risk factors were calculated. This agrees with previous reports in which similar classes of arterial diseases were considered.[15,20] In addition, we did not find significant differences in the prevalence of the 677TT MTHFR genotype among patients with the different categories of arterial disease investigated, nor differences in their respective associated risk value (data not shown). However, some reports have failed to demonstrate a direct association between MTHFR mutation and cerebral arterial disease or myocardial infarction or premature vascular disease[19,21,37,38] stating that the risk enhancement, if any, seems to be very slight. The high prevalence of the MTHFR mutation in the Italian population[14,22] could be the result of a low selection pressure for the mutated allele because of the higher intake of folic acid with the Mediterranean diet that could play a specific protective role against hyperhomocysteinemia associated with homozygous MTHFR mutation.

In conclusion, our data show that the 677TT MTHFR genotype can be considered an independent risk factor for both arterial and venous thrombosis in selected cases of patients and we suggest that other variables, such as differences in the genetic background and in the features related to the population investigated or in the nutritional intake of folate, could be responsible in part for the controversy over the association of MTHFR mutation and vascular disease.

Contributions and Acknowledgments

DG, MLS and GLS were responsible for the conception and design of the study and analyses of the data. GLS was also responsible for funding. CT and SF performed coagulation and DNA analysis. All the authors contributed to the analysis and writing of the paper. The order in which the names appear is based on the fraction of the total work performed. The last name is that of the principal clinician involved and the senior author.

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Disclosures

Conflict of interest: none.

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