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BRIEF COMMUNICATION

A transcobalamin gene polymorphism and the risk of venous thrombosis. The BRATROS (Brazilian Thrombosis Study)

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Introduction

Venous thromboembolism is a multifactorial disorder in which genetic and acquired risk factors may interact to determine the occurrence of the thrombotic event. Some abnormalities are categorized as genetic risk factors for VT (for instance, factor V Leiden (FVL), factor (FII) G20210A, and inherited deficiencies of antithrombin, protein C and protein S), whereas others are acquired risk indicators of thrombosis, such as cancer, antiphospholipid syndrome, surgery, trauma, oestrogen use, pregnancy, and puerperium. A third group of risk factors for VT is referred to as "mixed", because both inherited and environmental components determine their occurrence. Hyperhomocysteinemia is a classical example of a mixed risk factor for VTE [1–4].

Several cross-sectional and case-control studies have indicated that mild-to-moderate hyperhomo-

Abbreviations: VT, Venous thrombosis; TC, Transcobalamin; MTHFR, Methylenetetrahydrofolate reductase; CBS, Cystathionine-β-synthase; OR, Odds ratio; CI95, 95% confidence interval; FVL, Factor V Leiden; VTE, Venous thromboembolism; BRATROS, Brazilian Thrombosis Study; ARMS, Amplification-refractory mutation system.

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cysteinemia is associated with an increased risk of arterial and venous thrombosis [5—12]. High levels of homocysteine may result from acquired causes, such as deficiencies of folate, vitamin B12 and vitamin B6, renal dysfunction and old age, but genetic factors may also interfere with homocysteine metabolism and homocysteine plasma levels. The main genetic variants related to hyperhomocysteinemia are characterized by a decreased activity of enzymes that participate in metabolic pathways of the homocysteine metabolism [13]. A prevalent C to T substitution at nucleotide 677 in the gene encoding the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) is associated with reduced enzyme activity and mild hyperhomocysteinemia. The role of this polymorphism in venous thrombosis is controversial [14—18]. Two additional polymorphisms, A1298C in the gene encoding MTHFR and an insertion (844ins68) in the cystathionine-β-synthase (CBS) gene, do not seem to be associated with hyperhomocysteinemia or thrombotic risk. However, when these two gene variations are co-inherited with MTHFR C677T they may further impair homocysteine homeostasis [19—22], and some studies claimed that this co-inheritance increases thrombotic risk [23].

Transcobalamin (TC) is the plasma transporter of vitamin B12, being responsible for cellular availability of this vitamin, which is an important enzymatic cofactor of the homocysteine remethylation pathway. A polymorphism at nucleotide 776 in the TC gene, (C776G) has been described, and it seems to interfere with TC and homocysteine plasma levels [24]. This gene variation has a biallelic distribution in Caucasian populations and predicts an Arginine to Proline amino-acid substitution at codon 259 (P259R), which affects the protein phenotype. The allele 776C is associated with higher apo-TC plasma concentrations than the allele 776G. Thus, it has been speculated that the 776C allele could be associated with higher vitamin B12 availability and lower homocysteine levels. Heterozygotes C/G individuals were found to have higher homocysteine plasma levels [24]. Thus, this polymorphism seems to interfere with homocysteine metabolism and its plasma concentrations, but its role in influencing the risk of venous thrombosis is still uncertain.

We have sought to evaluate, in a case-control study, the contribution of the C776G polymorphism in TC gene to the risk of VT, on its own and when co-inherited with other common gene variations linked to homocysteine metabolism (MTHFR C677T, MTHFR A1298C and CBS 844ins68). In addition, we further investigated the role of the TC polymorphism as a determinant of homocysteine levels in plasma.

Materials and methods

Subjects

This study is part of the BRATROS (*Bra*zilian *Th*rombosis *S*tudy), a case-control study whose overall aim is to investigate the prevalence of risk factors for venous thrombosis in the Brazilian population. Details on the description of the BRATROS have been given elsewhere [25]. Briefly, cases enrolled came from three Brazilian University Hospitals: School of Medicine of Ribeirão Preto (University of São Paulo, USP), School of Medicine of Botucatu (State University of São Paulo, UNESP) and Federal University of São Paulo (UNIFESP). Included were four hundred thirty-four patients, consecutively admitted between October 1996 and August 2001, with an objectively verified episode of VT in a deep site (351 patients with lower limb VT, 23 patients with upper limb VT, 4 patients with intra-abdominal thrombosis, 40 with central VT, and 7 with thrombosis in other sites. Nine patients had VT in more than one site). Subjects aged more than 70 years or with malignant disease were excluded. Data on exposure to acquired risk factors for VT, i.e. immobilisation, surgery, trauma, use of oral contraceptives and hormone replacement therapy, were collected. Four hundred thirty-four controls (unrelated healthy blood donor candidates without a personal history of thrombosis) were matched with the patients for gender, age (±4 years), race, and time of recruitment. The general characteristics of patients and controls are presented in Table 1. In the present investigation, the prevalence of polymorphisms in genes related to homocysteine metabolism was determined in patients and controls from the BRATROS.

Table 1 BRATROS: general characteristics of patients with VT and controls

Variable	Patients (n = 434)	Controls (n = 434)
Median age (years)	41 (2—70)	41 (2—70)
Male / female ratio	0.7	0.7
Race		
Whites	85%	85%
Blacks and Mulattos	14.7%	14.7%
Asians	0.3%	0.3%
Family history of VTE	20%	—
Spontaneous VT	53%	—
Acquired risk factor for VT[a]	47%	—
Site of thrombosis		
Genetic risk factor for VT		
Factor V Leiden	10.1%	2.3%
Factor II G20210A	6.2%	0.9%
Inherited inhibitor deficiency[b]	9.2%	—

To investigate the role of the C776G TC polymorphism as a determinant of homocysteine levels, samples from one hundred and thirty-two healthy blood donors (aged between 18 and 60 years) were used to measure plasma homocysteine levels. For that purpose, these subjects were selected based on their previously known TC genotype (48 individuals were C/C homozygotes, 50 were C/G heterozygotes and 34 were G/G homozygotes).

The Institutional Ethical Committee approved this study and informed consent was obtained from all participants.

Methods

Genomic DNA was obtained from white blood cells using standard methods. Genotyping for FVL and FII G20210A was performed using methods previously described [26,27]. Genotyping for the polymorphism C776G in TC gene was performed by an amplification-refractory mutation system (ARMS) using forward primers containing the nucleotide substitution at the 3′ end [24]. Genotyping of the C677T and A1298C polymorphisms was done by PCR amplification and restriction enzyme analysis using *Hinf*I and *Mbo*II respectively, as previously reported [14,28]. The 844ins68 polymorphism in CBS gene was investigated by PCR amplification as previously described [29].

Blood collection for homocysteine plasma measurements was done in the morning, after overnight fasting. Plasma was separated from red cells immediately after collection, and stored at −80 °C until use. Total homocysteine determination was performed by the high-performance liquid chromatography (HPLC) method (Chromsystems, München, Germany) and quantified by fluorescence.

Statistical analysis

Allele frequencies were calculated by gene counting. Odds ratios (OR) as a measure of relative risk and 95% confidence intervals (CI95) were calculated by standard methods. One-way ANOVA was used to test for differences in total homocysteine levels between the three TC genotypes. A P value ≤ 0.05 was taken as statistically significant.

Results

The results of genetic analyses for the C776G polymorphism in patients and controls are shown in Table 2. The TC 776C/G and 776G/G genotypes (i.e., homozygous and heterozygous genotypes combined) were present in 279/434 patients (carrier frequency 64.3%) and in 272/434 controls (carrier frequency 62.7%). The frequency of the allele 776G was 0.406 among patients and 0.381 among controls. The distribution of these TC alleles was in accordance with Hardy—Weinberg equilibrium.

Table 2 TC C776G polymorphism and the risk of VT

Genotype	Patients (n = 434)	Controls (n = 434)	OR (CI95)
C/C	155 (35.7%)	162 (37.3%)	1[a]
C/G	206 (47.5%)	213 (49.1%)	1.0 (0.75—1.35)
G/G	73 (16.8%)	59 (13.6%)	1.29 (0.86—1.94)
C/G+G/G	279 (64.3%)	272 (62.7%)	1.07 (0.81—1.41)

[a] Reference category.

The OR calculated for association analyses between TC polymorphism and VT, represented in Table 2, demonstrated neutral risk. The OR for VT calculated for the heterozygote C/G genotype was 1.0 (CI95 : 0.75—1.35); for the homozygote G/G genotype the OR was 1.29 (CI95 : 0.86—1.94); and the overall OR (for homozygotes and heterozygotes) was 1.07 (CI95 : 0.81—1.41). The odds ratios were not substantially changed when recalculated taking into account VT site or specific location (proximal or distal, in the case of lower limb VT), or after exclusion of patients with transient risk factors.

Overall OR for VT associated with CBS 844ins68 was 1.05 (CI95 : 0.77—1.43); for homozygotes the OR was 0.40 (CI95 : 0.13—1.30), and for heterozygotes the OR was 1.12 (CI95 : 0.81—1.54). The overall OR for VT associated with MTHFR C677T was 1.04 (CI95 : 0.79—1.36); for homozygotes T/T the OR was 1.04 (CI95 : 0.68—1.58) and for heterozygotes C/T the OR was 1.04 (CI95 : 0.78—1.38). Taken together, homozygotes and heterozygotes for the MTHFR A1298C polymorphism yielded an OR for VT of 1.17 (CI95 : 0.90—1.53); for the homozygotes the OR was 1.29 (CI95 : 0.74—2.25); and for heterozygotes the OR was 1.15 (CI95 : 0.87—1.53).

We also performed stratified analyses to calculate the OR for VT in the presence of combined polymorphisms. The OR calculated for the concurrent presence of TC C776G and MTHFR C677T, MTHFR A1298C and CBS 844ins68 were shown in Table 3 and do not indicate interactions between any genotypes on VT risk (homozygotes and heterozygotes for each polymorphism combined). The OR for the association of the TC polymorphism with MTHFR C677T was 1.11 (CI95 : 0.75—1.66). For the associations of the TC polymorphism with MTHFR A1298C and CBS 844ins68, the OR for VT was 1.25 (CI95 : 0.86—1.83) and 1.15 (CI95 : 0.75—1.76), respectively.

The overall OR for VT associated with FVL was 4.66 (CI95 : 2.31—9.41), and for FII G20210A the OR

Table 3 Polymorphisms in genes related to homocysteine metabolism and the risk for VT

		Patients ($n=434$)	Controls ($n=434$)	OR (CI95)
TC C776G	MTHFR C677T			
–	–	68	73	1[a]
+	–	116	115	1.08 (0.71–1.65)
–	+	87	89	1.05 (0.67–1.63)
+	+	163	157	1.11 (0.75–1.66)
TC C776G	MTHFR A1298C			
–	–	86	102	1[a]
+	–	147	147	1.19 (0.82–1.71)
–	+	69	60	1.36 (0.87–2.14)
+	+	132	125	1.25 (0.86–1.83)
TC C776G	CBS 844ins68			
–	–	117	121	1[a]
+	–	210	210	1.03 (0.75–1.42)
–	+	38	41	0.96 (0.57–1.59)
+	+	69	62	1.15 (0.75–1.76)

(+) denotes presence of the mutant allele and (–) indicates the wild type genotype.
[a] Reference category.

was 7.13 (CI95: 2.47–20.57) (prevalence of both mutations in patients and controls are shown in Table 1). Co-inheritance of TC C776G with FVL or FII G20210A did not substantially change the risk of VT associated to these genetic risk factors for VT (data not shown in tables).

Results of plasma total homocysteine levels in healthy subjects with the three different TC genotypes are given in Table 4. Mean (±SD) total homocysteine levels in homozygotes C/C were 11.9 ± 5.6 μmol/L; for heterozygotes C/G mean homocysteine levels were 11.4 ± 6.7 μmol/L, and for homozygotes G/G mean levels of 11.9 (±6.1) were verified. No significant differences in homocysteine levels were observed between the three genotype groups ($P=0.52$).

Discussion

We investigated the C776G polymorphism in the TC gene as a candidate risk factor for VT. We did not find a significant relation between the presence of this polymorphism and the risk for VT. We also calculated the OR for VT when TC C776G was co-inherited with other prevalent molecular variations known to influence homocysteine metabolism (MTHFR C677T, MTHFR A1298C and CBS 844ins68). When associated, these polymorphisms did not significantly modify thrombotic risk. Furthermore, in carriers of FVL and FII G20210A, the two most prevalent genetic risk factors associated with VT, the 776G allele did not significantly alter thrombotic risk. Although the stratified analyses were performed with a relatively small number of carriers of associated defects, the numbers do not point to the possibility of an interactive effect between the tested mutations.

Several previous investigations suggested that, at least isolated, MTHFR C677T, MTHFR A1298C and CBS 844ins68 are not independent risk factors for VT [15,17,20,21,30]. In the present study, we evaluated a larger number of patients with VT and of controls than we had examined previously, and still no significant effect of these common polymorphisms on thrombotic risk could be verified [20,21]. In addition, the risk was not substantially changed when the variations were co-inherited (data not shown).

In agreement with the results above, measurements of homocysteine levels in healthy subjects revealed that the TC gene polymorphism is not a determinant of homocysteine concentrations in plasma, since we did not find differences in homocysteine levels between the three TC genotypes. Four common phenotypes (X, S, M and F) for TC, with different isoelectric points, have been described [31]. Subsequently, it has been reported that this phenotypic variability is multifactorial, and that the TC C776G polymorphism contributes to the existence of this difference [24]. Additionally, investigations reported an interference of this polymorphism on vitamin B12 metabolism by affecting holo-TC and apo-TC concentrations, and by interfering with homocysteine level [24,35]. In contrast to these findings, in another investigation the TC genotype was not found to be associated with the development of hyperhomocysteinemia in elderly volunteers [32]. The age of the volunteers

Table 4 Plasma total homocysteine levels in blood donors in relation to TC genotypes

TC genotype	Total homocysteine levels[a] (Mean ± SD)	Mean age (range)
C/C ($n=48$)	11.9 ± 5.6	35.3 years (18–59)
C/G ($n=50$)	11.4 ± 6.7	38.9 years (18–60)
G/G ($n=34$)	11.9 ± 6.1	34.8 years (18–58)

[a] Values are given in micromole per liter. No significant differences in homocysteine levels were observed between the three genotype groups ($P=0.52$, one-way ANOVA test).

was questioned as a possible factor interfering with this result [33]. In the present study, the age factor likely did not interfere with the results, since age distribution in the three TC genotype groups was similar, and none of the participants was older than 60.

The TC polymorphism presented a biallelic distribution in the population originally evaluated by Namour et al. [24] and the prevalence of the three TC genotypes observed in our population is similar to the frequencies reported in Caucasian populations previously investigated [24,32,34,36].

A possible confounding factor that may interfere with the results of case-control studies is ethnicity. When we designed this investigation we dealt with this point by matching cases and controls also for "race". We should acknowledge that the efficacy of such strategy—for the purpose of minimising ethnicity as a confounding—is unknown, and probably questionable. On the other hand, we believe that the best matching we have is the result of 500 years of intense miscegenation of the Brazilian population, as we have no reason to believe that this racial admixture took place in a significantly different way in the group of patients and of controls in our study. In fact, the power of the study to identify relevant risk factors for VT may be appreciated by analysing the significantly higher prevalences of prothrombotic genetic conditions in the patient group in comparison with the control group, including the FVL and FII G20210A mutations, which are known to exhibit a heterogeneous ethnic and geographic distribution. Because of these considerations, we believe that the results here obtained may reliably be taken as indicative of absence of a causative relation between the TC polymorphism and VT.

In spite of hyperhomocysteinemia to be considered a risk indicator of thrombotic diseases, none of the gene polymorphisms evaluated in this study, isolated or in combination, appears to influence venous thrombotic risk. With regards to the TC polymorphism, our negative finding is in line with the results previously reported by Zetterberg et al., in an investigation evaluating 86 patients with VT and 180 controls [37]. Those authors also did not find the TC polymorphism to be a risk factor for VT.

The absence of a causative relationship between TC polymorphism and thrombosis is further corroborated by the finding that the polymorphism did not influence homocysteine plasma levels in the present investigation. Such data, in addition to other published studies, indicate that gene variations related to homocysteine metabolism should not be assessed in patients with thrombophilia. In fact, it is our view that there is no solid evidence, derived from this or other studies, pointing to clinical utility of widespread homocysteine-related gene screening in the investigation of venous thrombotic diseases. Given the importance of avoiding unnecessary gene screening in multifactorial diseases, "negative" results as reported here may be as relevant as "positive" results, in association studies dealing with candidate genetic risk factors for VTE.

Summary

Hyperhomocysteinemia is considered to be a risk factor for venous thrombosis (VT). Transcobalamin (TC) is a plasma transporter of vitamin B12, and has an important role in metabolism of homocysteine. The polymorphism C776G in *TC* gene has been claimed to interfere with homocysteine plasma levels. We investigated the contribution of this polymorphism to the risk of VT, isolated and when co-inherited with other common gene variations (MTHFR C677T, MTHFR A1298C and CBS 844ins68). We also assessed the influence of the TC polymorphism on homocysteine levels. Genotyping for the four polymorphisms was carried out in 434 patients with VT and in 434 matched controls, and homocysteine levels were determined in plasma samples from 132 healthy blood donors and correlated with TC genotypes. The findings do not support the C776G TC polymorphism as a determinant of homocysteine levels or as a risk factor for VT.

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