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Original article

Prevalence of thrombophilia-associated genetic risk factors in blood donors of a regional hospital in southern Brazil

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ABSTRACT

Introduction: Thromboembolic events occur due to an imbalance in the hemostasis and some factors associated with this condition can be inherited. In order to evaluate the frequency of genotypes considered to be common hereditary risk factors for thrombophilia associated with venous thrombosis (g.1691G > A and g.20210G > A) and hyperhomocysteinemia (g.677C > T and g.1298A > C), samples from voluntary healthy blood donors at the *Hospital de Clínicas de Porto Alegre* were tested.

Methods: We examined 325 blood samples from blood donors collected from October 2017 to July 2018. Blood was collected on filter paper and the DNA was extracted for single nucleotide polymorphisms (SNPs) analysis using the qualitative real time polymerase chain reaction.

Results: The calculated frequencies of each genetic variant in heterozygosity were 4% for the *FV* gene (g.1691G > A), 4% for the *F2* gene (g.20210G > A) and 42% and 39% for methylenetetrahydrofolate reductase (*MTHFR*), g.677C > T and g.1298A > C, respectively. Only the genetic variants of *MTHFR* were found in homozygosity, with frequencies of 14% and 6% (g.677C > T and g.1298A > C), respectively.

Discussion: Altogether, these results describe the frequencies of genetic variants associated with venous thrombosis and hyperhomocysteinemia in the analyzed group and are important to enhance our current knowledge about the genetic profiles of Brazilian blood donors.

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Introduction

Thromboembolic events occur as a result of the imbalance between fibrinolysis and thrombosis. Factors associated with coagulation abnormalities can be acquired (by obesity, smoking or immobilization) or inherited (e.g., variations in factor V, prothrombin and methylenetetrahydrofolate reductase genes).[1–3] Thus, in the last five decades, the molecular bases of pro-coagulation and anticoagulation pathways have been well studied and some hereditary risk factors were considered responsible for venous thromboembolism (VTE). Factor V *(FV)* (g.1691G > A), factor II *(F2)* (g.20210G > A) and methylenetetrahydrofolate reductase (*MTHFR)* (g.677C > T and g.1298A > C) variants are the most common molecular biomarkers used to evaluate a possible tendency for venous thromboembolism.[1,3–5]

The g.1691G > A variant (rs6025) in the factor V gene (*FV*),[6,7] also known as factor V Leiden (FVL), is the leading cause of genetic thrombophilia[8] and is observed in 5% of the European population worldwide.[9] The relative risk for venous thrombosis is 3- to 10-fold higher for heterozygotes and 50- to 100-fold higher for homozygotes, when compared to wild type subjects.[8,10] It is characterized by poor anticoagulant response to activated protein C (APC) due to the substitution of glutamine by arginine at codon 506, which leads to a loss of the protein cleavage site, raising the risk of VTE by increasing the production of thrombin.[11] The second most frequent genetic prothrombotic factor in humans is the g.20210G > A variant (rs1799963) in the prothrombin or coagulation factor II gene (*F2*).[6,7,12] The prevalence in the European population is approximately 1–4%, and the frequency among patients with venous thrombosis is 5–7%.[12]

The MTHFR is a key enzyme in the folate metabolism that catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate, being the predominant form of circulatory folate responsible for remethylation of homocysteine (Hcy) to methionine.[13] Variants g.677C > T (rs1801133) and g.1298A > C (rs1801131) in the gene encoding *MTHFR* cause enzyme thermolability and decrease its activity by up to 40%,[14] which leads to low folate levels and increased plasma hyperhomocysteinemia (Hcy).[15] Hcy also leads to prothrombotic events and is related to the presence of these variants.[16–18] Previous studies have reported an association between hemorrhagic (677TT and 677TT/1298AA genotypes) and ischemic stroke (1298CC and 677TT/1298CC genotypes), in cases of homozygosity or heterozygosity.[19]

In Brazil, the genotypic frequencies of the *FV* (g.1691G > A), *F2* (g.20210G > A) and *MTHFR* (g.677C > T) variants range from 0.7 to 4.8%, 0.7 to 3.6% and 35% to 44%, respectively.[20–27]

Robust evidence also exists with the association between non-O blood groups (e.g., A, B and AB) and a higher risk for VTE.[2,4,28] For this reason the ABO blood groups are frequently included in the panel of first-level laboratory tests for thrombophilia screening.

The objective of this study was to evaluate the prevalence of the *FV* (g.1691G > A) variant (rs6025), *F2* (g.20210G > A) variant (rs1799963) and *MTHFR* (g.677C > T rs1801133 and g.1298A > C rs1801131) variants in a healthy southern Brazilian population represented by voluntary blood donors at the *Hospital de Clínicas de Porto Alegre*.

Table 1 – SNPs identification and Assay ID.

Gene	SNP	rs number	Life Technologies Assay ID
FV	g.1691G > A	rs6025	C___11975250_10
F2	g.20210G > A	rs1799963	C___8726802_20
MTHFR	g.677C > T	rs1801133	C___1202883_20
MTHFR	g.1298A > C	rs1801131	C____850486_20

Methods

Sample

A convenience sample including 325 blood donors at the Blood Bank of the *Hospital de Clínicas de Porto Alegre* was collected from October 2017 to July 2018. The sample size was similar to other studies reported in the literature for the Brazilian population. The participants were self-classified as European-descendants and Afro-descendants. The study was approved by the *Hospital de Clínicas de Porto Alegre* Ethics Committee (IRB approval 17-0207) and all participants provided written informed consent.

DNA extraction

The DNA extraction protocol was adapted from Kato et al., 2014.[29] The DNA was isolated from capillary blood collected on filter paper (FP), and 3 disks of 3 mm were used. Samples were washed twice with ultrapure distilled water and vortexed 1 min in each wash. The supernatant was discarded, 75 μL of 10× TE Buffer was added, followed by incubation for 30 s. at 95 °C in a thermocycler (Applied Biosystems, Veriti). The DNA amount and quality were determined by spectrophotometry, using a NanoDrop 2000 (Thermo Scientific, Wilmington, DE). All samples were diluted to a final concentration of 5 ng/μL, stored at 4 °C and used within 1 month.

Genotyping

Genotyping was performed using TaqMan assays commercially available from Life Technologies. The variant, gene and assay numbers for the selected single nucleotide polymorphisms (SNPs) are described in Table 1.

Assays included primers and probes labeled with VIC and FAM dyes, each probe having been designed for a particular type of allele. Genotyping was performed according to the manufacturer's instructions. TaqMan reactions were performed using the StepOne Real-Time PCR System (Applied Biosystems, Life Technologies) following these PCR conditions: Pre-Read Stage at 60 °C for 30″, Hold Stage at 95 °C for 10′, PCR Stage with two steps of 60 cycles — step I at 95 °C for 15″/step II at 60 °C for 01′ and Post-Reading Stage at 60 °C for 30″.

Table 2 – Genotypic and allelic frequencies observed in the studied population.

	FV g.1691G > A	*F2* g.20210G > A	*MTHFR* g.677C > T	*MTHFR* g.1298A > C
Genotypic frequencies				
WT	96% (G/G)	96% (G/G)	44% (C/C)	55% (A/A)
HET	4% (G/A)	4% (G/A)	42% (C/T)	39% (A/C)
MUT	0% (A/A)	0% (A/A)	14% (T/T)	6% (C/C)
Allelic frequencies				
Allele 1	98.2% (G)	97.8% (G)	65.5% (C)	75% (A)
Allele 2	1.8% (A)	2.2% (A)	35.5% (T)	25% (C)

Genotypes: WT — wild type; HET — heterozygous; MUT — homozygous for the variant.

Statistical analysis

The genotypic and allelic frequencies for each SNP tested were calculated, considering that the sample was in Hardy–Weinberg equilibrium.

To compare the g.20210G > A, g.1691G > A, g.677C > T and g.1298A > C genetic variants between the groups of O and non-O blood types, the chi-square test of independence was used. All analyses were performed with the Statistical Package for Social Sciences version 25 software, with a level of statistical significance set at 5% (p-value <0.05), confidence interval (CI) = 95%.

Results

The analyzed group consisted of 131 males (40%) and 194 females (60%) and the mean age was 38.5 years (ranging from 18 to 76 years, standard deviation ±11.4). A total of 97% of the donors were self-classified as European-descendants (315) and 3%, as Afro-descendants (10). Blood type O was the most common, in 40% (130), followed by 29% of type A (95), 9% of type AB (28) and 4% of type B (13). Fifty-nine individuals (18%) were not considered eligible for blood donation due to their health history or hemoglobin level and, subsequently, blood typing was not performed. Genotypic and allelic frequencies are shown in Table 2, considering that the population was in Hardy-Weinberg equilibrium for the four variants evaluated. The most frequent variants found among the SNPs studied were the g.677C > T and g.1298A > C in the *MTHFR* gene. Heterozygous genotypes were present in 42% (137/325) and 39% (127/325) of blood donor samples tested for the g.677C > T and g.1298A > C, respectively, while homozygous mutant genotypes were present in 13.5% (44/325) and 5.5% (18/325), respectively. However, less frequent variants were observed in the *FV* (g.1691G > A) and in the *F2* (g.20210G > A) and these variants were present only in the heterozygous state (4% for both variants, being 12/325 for g.1691G > A and 14/325 for g.20210G > A).

Previous studies have shown evidence that non-O blood groups (A, B and AB) could be associated with a higher risk of VTE[2,4,28,30] and thus, we analyzed 266 blood donors from the studied population who had their blood typing available. We performed the distribution of genotyping between the two groups of blood type O and non-O blood type (including types A, B and AB), as presented in Table 3.

Analyzing the distribution in the blood groups, we observed that the proportions between O and non-O blood groups remained very similar, not statistically significant, except for the heterozygous group of the g.677C > T variant ($p = 0.040$ and the immunochromatographic (IC) test = 95%, $p < 0.05$).

Discussion

Available literature on the prevalence of the g.1691G > A and g.20210G > A variants is strongly influenced by geographic location. Prothrombotic mutations are extremely rare among non-Europeans (African descendants, Chinese, Japanese, Native Americans of North and South Americas, or Inuits of Greenland). In general, the Brazilian heterozygotes prevalence for the *F2* variant g.20210G > A is 0.8% on average (0.7–1.6%),[31] while the heterozygosity for the Leiden factor V variant occurs in 3–8% of the USA and European populations in general.[32] Niewiadonski and colleagues observed a heterozygous frequency of 1.2% for the *FV* variant and 0.5% for the *F2* variant in blood donors from São Paulo.[33]

One of the striking characteristics of the southern region of Brazil concerns its colonization, which began in the mid-seventeenth century by the Portuguese. The south of Brazil had majority of immigrants from the Azores, Spain, Germany, Italy, Poland and the Netherlands, among other European countries. Nonetheless, this contributed to the formation of the Brazilian society of the 19th century, of mostly European ethnicity.[34] This region received a small number of African slaves.

A study conducted in Somalia showed that these common genetic risk factors, most known for VTE, are absent or less frequent in this group, when compared to other ethnic populations.[35] Our population had very few individuals of Afro-descendant origin (3%), which could explain the higher prevalence of these variants. However, this study was limited by its sample size.

In the present study, we verified that homozygous genotypes for the g.1691G > A and g.20210G > A alterations are completely absent, in agreement with the great majority of studies. However, the heterozygous genotypes of both variants were higher than the previous reports in healthy Brazilian individuals,[20–22] much closer to the prevalence of these variants in studies of the healthy European population, such as Italy (2.3–5.7%) and Spain (3.1–6.5%).[31,36–38] Therefore, we can consider that the colonization origins still maintain genetic traits in the region.

Table 3 – Genotype O and non-O blood groups (A, B and AB) distribution for the studied population.

	WT	HET	MUT
FV g.1691G>A			
A	91 (35%)	4 (50%)	0 (0%)
B	13 (5%)	0 (0%)	0 (0%)
AB	28 (11%)	0 (0%)	0 (0%)
O	126 (49%)	4 (50%)	0 (0%)
	Type non-O 51%	Type non-O 50%	Type non-O 0%
	Type O 49%	Type O 50%	Type O 0%
F2 g.20210G>A			
A	90 (35%)	5 (55.5%)	0 (0%)
B	13 (5%)	0 (0%)	0 (0%)
AB	28 (11%)	0 (0%)	0 (0%)
O	126 (49%)	4 (44.5%)	0 (0%)
	Type non-O 51%	Type non-O 55.5%	Type non-O 0%
	Type O 49%	Type O 44.5%	Type O 0%
MTHFR g.677C>T			
A	35 (29%)	48 (44%)	12 (30%)
B	6 (5%)	6 (5%)	1 (3%)
AB	14 (12%)	11 (10%)	3 (7%)
O	65 (54%)	45 (41%)	25 (60%)
	Type non-O 46%	Type non-O 59%	Type non-O 39%
	Type O 54%	Type O 41%	Type O 60%
MTHFR g.1298A>C			
A	59 (40%)	31 (30%)	5 (31%)
B	4 (3%)	8 (8%)	1 (6%)
AB	16 (11%)	10 (10%)	2 (13%)
O	69 (46%)	53 (52%)	8 (50%)
	Type non-O 54%	Type non-O 48%	Type non-O 50%
	Type O 46%	Type O 52%	Type O 50%

Genotypes: WT — wild type; HET — heterozygous; MUT — homozygous for the variant.

It is, however, important to highlight that the blood donor candidate population is invariably biased. Health-related events and comorbidities tend to discourage blood donation, so that individuals presenting to a blood bank seeking to donate are usually "healthier" than the average population. This could influence findings from this study, especially homozygosity rates. Furthermore, analyses performed only on donation-eligible donors (those that eventually were considered able to donate blood), such as ABO blood type stratification in the present study, are even more prone to this bias.

Although we found that some subjects carry the mutant allele, they may not express the disorder due the reduced (or incomplete) penetrance showed by some autosomal dominant genes, such as the *FV*5 g.1691G>A. Genotyping studies of apparently healthy individuals may be an approach to understanding the penetrance of pathological variants.[39,40] Furthermore, genetic mutation is only one risk factor predisposing the carriers to venous thromboembolic disease and clinical thrombophilia is the consequence of multiple gene and/or environment interactions.

The FVL has a prevalence of carriers among Europeans of 5–10%.[9] Among patients with VTE, it is found in 20–30%, and in around 50% of patients with familial thrombophilia. The risk of thrombosis is increased 5-fold in heterozygotes and 50-fold in homozygotes.

The g.20210G>A variant in the *F2* gene is less prevalent (around 2%) and almost only found in Europeans. Carriers have a 2- to 3-fold increased risk of venous thrombosis, and the variant is found in approximately 6% of patients with VTE. Homozygosity for this variant is rarer than homozygosity for the g.1691G>A variant. However, the risk for VTE is high and has been reported to be 30 times increased.

As both FVL and the *F2* g.20210G>A are common, compound heterozygotes are not extremely rare among individuals with deficiencies of antithrombin, protein C, and protein S who may have up to 30–100 times increased risk for VTE.[41] However, these risks are estimated from family studies and might constitute an overestimation of the actual VTE risk. In studies among unselected patients with VTE, the risk associated with these deficiencies appears lower than in selected thrombophilic families.

The European Society of Cardiology (ESC) 2019 guidelines recommend thrombophilia screening for young VTE patients below 50 years of age and unprovoked VTE, especially in the presence of a family history of VTE.[42]

The *MTHFR* g.677C>T variant has a relatively high frequency worldwide, and the geographical pattern of allelic frequency supports the hypothesis that it is a risk factor for vascular diseases and neural tube defects.[35] A possible explanation for this high frequency could be a mutant heterozygous or homozygous selective advantage. Prevalence found for the *MTHFR* in this study were consistent with the results obtained from previous studies in Brazilian children and other control groups.[20–24,43,44] A comparison of the results obtained in the present study with those obtained in a recent prevalence study in blood donors in the central-southern region of Italy revealed that the cohort used in this study presented a higher

number of healthy individuals with heterozygotes for the g.677C > T and g.1298A > C.[45] None of the subjects tested had a 677TT/1298CC-associated genotype. Several publications have highlighted that the heterozygous or homozygous mutation of the *MTHFR* g.677C > T, which in the past was considered, has not been confirmed as a risk factor for the first VTE or for relapse (either alone or in combination with the FV Leiden).[3,46] Nevertheless, others have found the opposite confirming a correlation with VTE, thus making it difficult to come to a definite conclusion.[2,47,48]

A recent meta-analysis including 99 genetic association studies focusing on the relationship between the *MTHFR* gene polymorphisms and the risk of venous thromboembolism, revealed a significant association for the g.677C > T polymorphism in specific ethnic groups, such as Caucasians, East Asians and West Asians.[49] The authors attributed the failure to detect a significant correlation between the rs1801131 (g.1298A > C) polymorphism and VTE in overall analyses to its relatively weaker influence on the activity of the *MTHFR* compared to that of the rs1801133 (g.677C > T) polymorphism. Recently, an equivalent meta-analysis has come to similar results over the g.677C > T and g.1298A > C mutations, especially in patients of Asian ethnicity.[50] Nevertheless, these results should not be taken without care because the lack of individual patient data should be a strong limitation for those studies, as a result of the impossibility of incorporating other important acquired risk factors for VTE (age, gender and comorbid conditions) as confounding variables. In addition, the pathogenic mechanism of VTE is highly complex and hence, it is unlikely that a single *MTHFR* polymorphism can significantly contribute to its development. Accordingly, many societal and governmental guidelines[51–54] on thrombophilia screening do not recommend the *MTHFR* mutation testing in patients presenting VTE due to the lack of evidence of a higher recurrence risk in these patients. Therefore, the *MTHFR* screening does not seem to change the management of patients with VTE.

A larger sample size screening would be ideal for more accurate determination of the allele frequencies in the southern Brazilian population, since a difference in the frequency of the SNPs in our sample was observed, when compared with groups of other Brazilian regions, but very similar to that of the European population. It would also help to determine whether the non O-blood group status could be associated with heterozygosity for the g.677C > T variant in our sample, as we did not have sufficient power to ascertain this hypothesis. Although ABO blood groups have been shown to be associated with increased risks of venous thromboembolic disease, the reported magnitude of this association is inconsistent and is based on evidence from small-scale studies. A study using the SCANDAT2 (Scandinavian Donations and Transfusions) database of healthy population blood, non-O blood groups explained >30% of venous thromboembolic events. Although ABO blood groups may potentially be used with available prediction systems for identifying at-risk individuals, its clinical utility requires further comparison with other risk markers.[55]

Karasu et al. studied the combined effect of the ABO blood group and the presence of either the factor V Leiden or prothrombin g.20210G > A mutation, with wildtype carriers of the factor V Leiden and prothrombin G20210A mutation with blood group O as the reference category. Individuals carrying either the prothrombotic variant and blood group O had a 2.3-fold increased risk of VT (95% CI, 0.–5.9) and wildtype carriers of the factor V Leiden and prothrombin g.20210G > A mutation with blood group non-O had a 1.3-fold increased risk of VT (95% CI, 1.0–1.8). Those with both blood group non-O and a prothrombotic variant had a similar risk as those with blood group O and a prothrombotic variant.[56]

We also intend to compare the frequencies found here with those observed in women with recurrent miscarriage and in patients with the clinical condition of thrombophilia in our population to evaluate if there is an increased risk for VTE in those groups.

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Conflicts of interest

The authors declare no conflicts of interest.

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