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Prevalence of variants that confer risk for venous thromboembolism in an elderly population of northeastern Brazil

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ABSTRACT. Venous thromboembolism (VTE) is an important cause of morbidity and mortality stemming from cardiovascular disease. It is a multifactorial disease caused by a combination of acquired risk factors, of which advanced age is the most significant, and genetic factors, including the variants *FV* G1691A, *FII* G20210A, and *MTHFR* C677T. We estimated the prevalence of these genomic variants in an elderly population of northeastern Brazil. The study included 188 elderly persons (65-93 years), of which 68 (36.2%) were men and 120 (63.8%) were women. Variants were detected by polymerase chain reaction-

restriction fragment length polymorphism analysis, and subsequent electrophoresis on an 8% polyacrylamide gel stained with silver nitrate. The study population was in Hardy-Weinberg equilibrium for the 3 loci. Of the individuals analyzed, none carried variants of *FV* or *FII* (0%), and 24.7% had the *MTHFR* C677T polymorphism: 59 subjects (31.4%) were heterozygous (CT) and 17 subjects (9%) were homozygous (TT). Based on the analysis of these particular genes, we conclude that the study population does not present an increased risk for the development of VTE. Faced with a growing aging population worldwide, similar studies in other countries will help in the prevention of VTE in older individuals.

Key words: Genetic polymorphisms; Venous thromboembolism; *FV* G1691A; *FII* G20210A; *MTHFR* C677T; Elderly

INTRODUCTION

Cardiovascular disease is the leading cause of death worldwide. Among the various types, venous thromboembolism (VTE) is ranked third highest in the number of deaths, after stroke and ischemic heart disease (Goldhaber and Bounameaux, 2012). It is estimated that the annual incidence of VTE in the general population is from 1 to 2 cases per 1000 inhabitants. Incidence rates increase exponentially with age: the incidence of VTE in persons aged over 70 years is 3 times that of persons aged from 45 years to 69 years, which, likewise, is 3 times that of persons aged between 20 years and 44 years (Naess et al., 2007; Wong and Baglin, 2012). Furthermore, episodes of pulmonary embolism, the most serious clinical complication of VTE, are more common among the elderly, resulting in a higher fatality rate in this group (Nizankowska-Mogilnicka et al., 2003; Silverstein et al., 2007).

VTE is a typical example of a multifactorial disease, as it is caused by a combination of genetic and environmental risk factors that together increase the procoagulant tendency of blood (Ehrenforth et al., 2004). Owing to the fact that a variety of genes can be involved in the etiology of VTE, interindividual differences in genetic susceptibility to the disease exist. The most common genetic risk factors for the development of VTE include the transitions G1691A in coagulation factor V (*FV*) and G20210A in coagulation factor II (*FII*) (also known as prothrombin gene) (Rosendorff and Dorfman, 2007). The C677T polymorphism in the gene encoding the enzyme methylenetetrahydrofolate reductase (*MTHFR*) may contribute to the etiology of VTE; study results have been inconsistent (Nizankowska-Mogilnicka et al., 2003; Almawi et al., 2005; Torres et al., 2006; Alfirevic et al., 2010; He et al., 2010).

The *FV* G1691A mutation results in an *Arg*-to-*Glu* substitution at codon 506, which makes the coagulation protein resistant to proteolytic inactivation by activated protein C and, subsequently, increases fibrin formation (Bertina et al., 1994). Carriers of this mutation have a 4- to 12-fold greater risk of developing VTE (Torres et al., 2006; Bezemer et al., 2008; Alfirevic et al., 2010). Furthermore, the risk of VTE recurrence in heterozygous persons is approximately 40% higher than it is in persons without the mutation (Marchiori et al., 2007).

The *FII* G20210A mutation results in increased mRNA stability, followed by a subsequent increase in thrombin production (Poort et al., 1996). Alone, this mutation results in a 3- to 7-fold greater risk of VTE development (Nizankowska-Mogilnicka et al., 2003; Bouaziz-

Borgi et al., 2006). When associated with the *FV* G1691A allele, the risk and recurrence of thrombotic disease increases significantly (Ehrenforth et al., 2004; Marchiori et al., 2007).

The *MTHFR* C677T polymorphism results in an *Ala*-to-*Val* substitution at codon 222, which creates a thermolabile enzyme with reduced activity. Previous studies have shown that decreased levels of serum folate are a downstream result of this protein variation. Since serum folate is required for the conversion of homocysteine to methionine, these lowered levels ultimately result in a state of hyperhomocysteinemia, a condition that favors the formation of thrombi and stems from lesion formation in the vascular endothelium and the activation of the coagulation cascade (Dionisio et al., 2010; He et al., 2010).

Although there are a considerable number of acquired risk factors that increase the likelihood of VTE development (e.g., pregnancy, surgery, fractures, prolonged immobilization, and cancer), advanced age is critical among them as aging is naturally and irreversibly accompanied by characteristics that lead to decreased blood flow: reduced mobility, decreased muscle tone, and the aging of veins and arteries. Moreover, the aged individual has had a lifetime worth of exposure to risk factors (Engbers et al., 2010).

Understanding the prevalence of genetic factors that predispose people to VTE will help in the identification of populations at high risk, maximize the prevention of this serious disease, and help in defining follow-up strategies for special groups of patients (Cushman, 2007). Although VTE mainly affects aged individuals, most studies still focus on younger populations when investigating the etiology of the disease, especially in the case of genetically based thrombophilia (Rosendaal et al., 2007). It is clear, therefore, that if preventative strategies against the majority of thrombotic events are to be developed, research should focus on the elderly. The aim of the present study was to estimate the prevalence of the variations *FV* G1691A, *FII* G20210A, and *MTHFR* C677T in an elderly population in northeastern Brazil.

MATERIAL AND METHODS

Study population and DNA extraction

Representing the general population of the city of Parnaíba, PI, Brazil, 188 elderly subjects were recruited from the largest volunteer project on frailty in the elderly, developed in Brazil, the FIBER Network (Network of Research on Frailty in Elderly Brazilians). Subjects were informed about the study purpose and the experimental procedures involved, and signed consent forms prior to being enrolled. The study was approved by Universidade Federal do Piauí Ethics Committee in Research. To obtain genomic DNA, venipuncture was used to collect 4 mL peripheral blood into vacuum tubes containing EDTA. Genomic DNA was subsequently extracted from peripheral blood leukocytes with the Wizard® Genomic DNA Purification kit (Promega Inc., USA) according to manufacturer specifications.

Variant detection

Genomic variants were identified by polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP). PCRs contained 10 ng DNA, 1X buffer (100 mM Tris-HCl and 500 mM KCl, pH 8.5), 1.5 mM $MgCl_2$, 0.4 mM of each primer, 0.2 mM dNTP, 1.5 U Taq DNA polymerase, and distilled H_2O in a total reaction volume of 25 µL.

The program for PCR was an initial denaturation performed for 3 min at 95°C, followed by 35 cycles of 95°C for 45 s, 45-54°C for 60 s, and 72°C for 45 s, and a final extension of 7 min at 72°C

Identification of the *FV* G1691A variant

The *FV* G1691A mutation was detected by amplification of a 204-bp exon 10 fragment of the *FV* gene using the primers 5'-GCCCAGTGCTTAACAAGACC-3' and 5'-CCATTATTTAGCCAGGAGACC-3' (forward and reverse, respectively) and the annealing temperature was 45ºC. Approximately 5 µL resultant PCR product was digested with 2 U restriction enzyme *Mnl*I, subjected to polyacrylamide gel electrophoresis (8%), and, lastly, visualized with silver nitrate. In wild-type homozygous individuals, 3 DNA fragments (100, 66, and 37 bp in size) were generated by enzyme digestion, as *Mnl*I recognizes 2 cleavage sites within the amplicon. The presence of the *FV* G1691A mutation alters a restriction site in such a way that the second cut is prevented and, subsequently, 4 fragments (138, 100, 66, and 37 bp in size) and 2 fragments (138 and 66 bp in size) were generated in heterozygous and homozygous individuals, respectively.

Identification of the *FII* G20210A variant

A 345-bp fragment from the 3'-untranslated region of the *FII* gene was amplified using the primers 5'-TCTAGAAACAGTTGCCTGGC-3' and 5'-ATAGCACTGGGAGCATTGA AGC-3' (forward and reverse, respectively) and the annealing temperature was 47°C. The presence of a cleavage site for the restriction enzyme *Hin*dIII in the mutant allele enabled genotyping by digestion of 10 µL PCR product with 10 U enzyme. Digestion, therefore, generated 3 DNA fragments (345, 322, and 23 bp in size) and 2 fragments (322 and 23 bp in size) in heterozygous and homozygous individuals, respectively, as analyzed by polyacrylamide gel electrophoresis (8%) and silver nitrate visualization.

Identification of the *MTHFR* C677T polymorphism

A 251-bp exon 4 fragment of the *MTHFR* gene was amplified using the primers 5'-CTGACTGTCATCCCTATTGGCA-3' and 5'-CCTCACCTGGATGGGAAAGAT-3' (forward and reverse, respectively) and the annealing temperature was 54ºC. A 10-µL sample of the resultant PCR product was digested with 4 U *Hin*fI, which recognizes a restriction site that is only present in the polymorphic allele. Digestion, therefore, generated 3 fragments (251, 148, and 103 bp in size) and 2 fragments (148 and 103 bp in size) for heterozygous and homozygous individuals, respectively. Fragments were analyzed on an 8% polyacrylamide gel following electrophoresis and silver nitrate visualization.

Statistical analysis

Genotypic and allelic frequencies were determined by simple counting. To test whether the population was in Hardy-Weinberg equilibrium for the loci in question, genotype frequencies were first calculated from allele frequencies, and then their deviation from the

number of observed genotypes was determined by the chi-square test. We adopted a significance level of 5% and used the Fisher exact test to compare allele frequencies in this study with those of other populations. The BioEstat 5.0 program was used for the statistical evaluation of data.

RESULTS

The mean age of the 188 elderly study participants (aged 65-93 years) was 72.75 ± 7.44 years. Of those, 68 (36.2%) were men and 120 (63.8%) were women. The genotypic and allelic frequency distributions for the 3 loci investigated are presented in Table 1. The genotypic frequencies for all 3 loci were found to be within Hardy-Weinberg equilibrium. All subjects were homozygous wild type (GG) for coagulation factor gene mutations, giving a frequency of 0% for the *FV* G1691A and *FII* G20210A mutations. In contrast, the frequency of the *MTHFR* C677T polymorphism was considerable: of the 188 study subjects, 59 (31.4%) and 17 (9%) were homozygous (TT) and heterozygous (CT) for the polymorphic allele, respectively. The total allele frequency was 24.7%, with no statistically significant differences observed between the genders.

Table 1. Allelic frequencies of the *FV* G1691A, *FII* G20210A, and *MTHFR* C677T variants in the study population (N = 188).

Locus	Genotypic frequency						Allelic frequency	
	Genotype	Frequency (%)	Genotype	Frequency (%)	Genotype	Frequency (%)	Allele	Frequency (%)
FV G1691A	GG	188 (100)	GA	0 (0)	AA	0 (0)	A	0.0
FII G20210A	GG	188 (100)	GA	0 (0)	AA	0 (0)	A	0.0
MTHFR C677T	CC	112 (59.6)	CT	59 (31.4)	TT	17 (9)	T	24.7

DISCUSSION

VTE is an important cause of morbidity and mortality from cardiovascular disease in many countries. The incidence rate of VTE varies among ethnic populations, and is lowest in Asians and Hispanics. In this case, the low incidence of VTE may, therefore, be a reflection of the low prevalence of genetic risk factors associated with VTE in these populations. The *FV* G1691A mutation, for instance, is present in 5% of the Caucasian population, while it is found in only 0.5% of Asian populations (White et al., 2004). Moreover, although the prevalence of the *FV* G1691A mutation in Hispanics is higher than in African-Americans (2 *vs* 1%), the incidence of VTE is higher in the latter, suggesting that other prothrombotic variants may exist with an increased prevalence and/or that environmental stimuli may be associated with thrombosis in populations of African descendant (Cushman, 2007; Shaheen et al., 2012).

Despite the aging global population, few studies have investigated the prevalence of mutations associated with the development VTE in elderly populations. Here, we present the allelic and genotypic frequencies of 3 genomic variants critical in the etiology of VTE, *FV* G1691A, *FII* G20210A, and *MTHFR* C677T, in an elderly population in northeastern Brazil.

Our results identified an absence of the *FV* G1691A and *FII* G20210A mutations in the population of Parnaíba, PI: none of individuals analyzed (N = 188) carried a variant allele. In contrast, the variant *MTHFR* C677T allele was found at a frequency of 24.7%. A comparison between the frequencies found in this study with those observed in other populations is presented in Tables 2 and 3.

Table 2. Comparison of the allelic frequencies of the *FV* G1691A and *FII* G20210A in this study and other study populations.

Population	N	*FV* G1691A (%)		P	*FII* G20210A (%)		P	Reference
		G	A		G	A		
Brazilian (Parnaíba, Northeast region)	188	100	0.0	-	100	0.0	-	This study
Italian (Calabria, Southern region)	105	95.2	4.8	0.029*	97.1	2.9	0.123	Sottilotta et al., 2009
Ukrainian (Kiev, Central-North region)	172	98.3	1.7	0.248	98.5	1.5	0.248	Tatarskyy et al., 2010
Greek (Different regions)	140	95.0	5.0	0.029*	97.0	3.0	0.123	Gialeraki et al., 2008
French (Different regions)	6064	98.1	1.9	0.248	98.5	1.5	0.248	Mazoyer et al., 2009
Spanish (Valencia, Eastern region)	493	99.0	1.0	0.500	97.4	2.6	0.123	Francès et al., 2006
Polish (Malopolska, Southern region)	100	98.5	1.5	0.248	99.5	0.5	0.500	Nizankowska-Mogilnicka et al., 2003
Chilean (Talca, South-Central region)	1200	99.0	1.0	0.500	99.0	1.0	0.500	Palomo et al., 2009
Colombian (Medellin, Northern region)	114	99.6	0.4	0.500	100	0.0	1.00	Torres et al., 2006
Brazilian (Espírito Santo, Southern region)	100	95.2	4.8	0.029*	98.0	2.0	0.123	Stur et al., 2012
Brazilian (Belém, Northern region)	127	98.4	1.6	0.248	99.2	0.8	0.500	Yoshioka et al., 2006
Costa Rican (Tribes of American Indians)	729	99.6	0.4	0.500	99.86	0.14	0.500	Herrmann et al., 2004
Lebanese (Different regions)	697	92.1	7.9	0.003*	97.1	2.9	0.123	Almawi et al., 2005
Tunisian (Túnis, Northeast region)	198	96.8	3.2	0.060	98.7	1.3	0.248	Bouaziz-Borgi et al., 2006
Palestinian (West Bank)	303	88.6	11.4	<0.001*	95.0	5.0	<0.001*	Hussein, 2012
Chinese (Changchun, Northern region)	102	100	0.0	100	100	0.0	1.00	Jun et al., 2006

*Statistically significant difference.

Table 3. Comparison of the allelic frequencies of the *MTHFR* C677T polymorphism in this study and other study populations.

Population	N	*MTHFR* C677T		P	Reference
		C	T		
Brazilian (Parnaíba, Northeast region)	188	75.3	24.7	-	This study
Greek (Different regions)	140	60.0	40.0	0.017*	Gialeraki et al., 2008
Ukrainian (Kiev, Central-North region)	172	71.0	29.0	0.316	Tatarskyy et al., 2010
Croatian (Zagreb, Northwest region)	104	64.0	36.0	0.062	Alfirevic et al., 2010
Italian (Sicília, Southern region)	468	54.7	45.3	0.002*	Wilcken et al., 2003
Slovac (Kosice, Eastern region)	290	74.8	25.2	0.564	Behunova et al., 2010
Colombian (Medellín, Northern region)	114	56.5	43.5	0.003*	Torres et al., 2006
Brazilian (Belém, Northern region)	127	66.1	33.9	0.107	Yoshioka et al., 2006
Argentinian (Different regions)	418	63.0	37.0	0.046*	Genoud et al., 2000
Afro-American (Atlanta, Southeast region)	298	87.4	12.6	0.023*	Wilcken et al., 2003
Brazilian (Different regions)	240	71.0	29.0	0.316	Zanrosso et al., 2005
Mexican (Unknown region)	500	43.0	57.0	<0.001*	Wilcken et al., 2003
Korean (Seoul, Southern region)	100	56.0	44.0	0.003*	Woo et al., 2009
Japanese (Nagoya, East of Tokyo)	500	59.4	40.6	0.012*	Matsuo et al., 2004
Palestinian (West Bank)	303	92.9	7.1	<0.001*	Hussein, 2012
Lebanese (Different regions)	697	69.1	30.9	0.215	Almawi et al., 2005

*Statistically significant difference.

Although the prevalence of prothrombotic genetic alterations exhibits extensive ethnic and geographic variation, the presence of the *FV* G1691A and *FII* G20212A mutations specifically is mainly restricted to Caucasian populations. The Brazilian population is one of the most heterogeneous in the world owing to 5 centuries of interethnic crosses among populations from 3 continents, including the European settlers, in particular the Portuguese, African slaves, and indigenous Amerindians (Parra et al., 2003). Thus, considerable heterogeneity is expected in the frequency distribution of genetic variants within Brazilian territory. For example, while in a northern Brazilian population the frequency of the *FV* G1691A and *FII* G20212A variants was 1.6 (P = 0.248) and 0.8% (P = 0.5), respectively, (Yoshioka et al., 2006) in a southern Brazilian population of strong European descent, the frequency for those

same variants was 4.8 (P = 0.029) and 2% (P = 0.123) (Stur et al., 2012). The former northern findings did not differ significantly from the frequencies found in this study.

In Europe, the distribution of the *FV* G1691A allele shows significant heterogeneity between countries. In eastern Spain, the mutation is observed in approximately 1% of the general population (Francès et al., 2006), whereas in the general population of Greece and southern Italy, for example, the mutation frequency is approximately 5% (Gialeraki et al., 2008; Sottilotta et al., 2009). The latter finding differs from that observed in the present study (P = 0.029), wherein an absence of the *FV* G1691A mutation was identified in a population from northeastern Brazil. Nonetheless, our results are not significantly different from those found in most European countries (Nizankowska-Mogilnicka et al., 2003; Mazoyer et al., 2009; Tatarskyy et al., 2010). Moreover, the frequency of the *FII* G20212A mutation in southern Poland (0.5%) (Nizankowska-Mogilnicka et al., 2003), which differs from the 3% frequency found in Greece (Gialeraki et al., 2008), does not significantly differ from the frequency found in our study (0%).

The frequency of the *FV* G1691A and *FII* G20212A mutations in Latin countries is lower than that in the Caucasian populations of Europe. Similar to the frequency findings of this study, in Colombia, the *FII* G20212A mutation was not detected at all (0% frequency) and only 0.43% of the subjects in that study had the *FV* G1691A mutation (Torres et al., 2006). Likewise, in a study conducted in Chile (N = 1200), only 1% of the population had any variant alleles (Palomo et al., 2009). These variants are also rare in African, Chinese, and American Indian populations (Herrmann et al., 2004; Jun et al., 2006). On the other hand, they are common in Arab countries, with frequencies as high as approximately 11% for *FV* G1691A and 5% for *FII* G20212A (P < 0.001), as observed by Hussein (2012) in a Palestinian population.

The relationship between *MTHFR* C677T and the development of VTE remains unclear because, despite the failure of several studies to show an association (Nizankowska-Mogilnicka et al., 2003; Almawi et al., 2005; Torres et al., 2006; Alfirevic et al., 2010), in a meta-analysis of 8364 subjects, Den Heijer et al. (2005) concluded that the presence of the TT genotype poses a 20-fold greater risk for VTE than the CC genotype. One consequence of the *MTHFR* C677T polymorphism is a resultant increased plasma level of homocysteine, a condition considered to be an independent risk factor for VTE. The effect of this polymorphism however, is, modulated by the interaction of *MTHFR* with other genes involved in folate metabolism and environmental factors, of which dietary folate and vitamin B12 are the most important. Individuals homozygous for the 677T allele, therefore, only have elevated homocysteine levels if their diets lack those nutrients (Friso and Choi, 2005).

Unlike the *FV* G1691A and *FII* G20210A mutations, the *MTHFR* C677T polymorphism is not just concentrated within European populations but presents with a more varied geographical distribution. In Brazil, the frequency of the *MTHFR* C677T polymorphism in the general population is approximately 29%. In the present population studied of northeastern Brazil, approximately 25% of individuals had the mutant *MTHFR* C677T allele, similar to findings of most other regions of Brazil (Zanrosso et al., 2005). This frequency, however, is lower than that in European populations, where the allele frequency ranges from 25 to 45% (Table 3). The frequency of the TT genotype in Europe reaches as high as 20% in some regions, such as southern Italy (Wilcken et al., 2003). Despite being higher than in African-Americans (Wilcken et al., 2003), the allelic frequency of the *MTHFR* C677T polymorphism in our study was still significantly lower (P < 0.05) than that of other American populations (Genoud et al.,

2000; Wilcken et al., 2003; Torres et al., 2006). In Mexico, for instance, the highest recorded frequency of 677T is 57%. Through analysis of indigenous tribes of Costa Rica, Herrmann et al. (2004) found that the 677T allele was present in 69% of the population, the current highest observed prevalence of this polymorphism in the world. The high frequency of this polymorphism in the Asian countries Japan (40%) and Korea (44%) (Matsuo et al., 2004; Woo et al., 2009) were significantly different from the frequency found in this study ($P = 0.003$ and $P = 0.011$, respectively). Among the Arab countries, the frequency of this polymorphism ranges from 7 to 31% (Almawi et al., 2005; Hussein, 2012).

CONCLUSION

The present study revealed that the *FV* G1691A and *FII* G20210A mutations most frequently associated with VTE are rare in the general population of northeastern Brazil. The frequency of the *MTHFR* C677T polymorphism (25%), however, was similar to that observed in other regions of Brazil, but was lower than that found in European, Asian, and American populations. Therefore, with respect to the genetic factors investigated, we conclude that the current study population does not present with an increased risk for the development of VTE. As we are currently faced with a growing aging population worldwide, similar general population studies within other countries are needed to aide in the development of preventative measures against this disease in older individuals.

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