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RESEARCH ARTICLE

Large-scale screening for factor V Leiden (G1691A), prothrombin (G20210A), and MTHFR (C677T) mutations in Greek population

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Abstract

Background and aims: To provide a fair estimate of the prevalence of factor V Leiden (FVL) (G1691A), prothrombin (G20210A), and MTHFR (C677T) mutations in the Greek population.

Methods: We genotyped a representative sample of 974 apparently healthy Greek adults by the method of real-time PCR and we calculated the allele frequencies of factor V Leiden (FVL) (G1691A), prothrombin (G20210A), and MTHFR (C677T) mutations. In addition, we determined the frequency of co-occurrence of FVL (1691A) and prothrombin (20210A), FVL (1691A) and MTHFR (677T), prothrombin (20210A) and MTHFR (677T) mutations.

Results: The career frequencies of FVL (1691A), prothrombin (20210A), and MTHFR (677T) alleles were 7.5%, 4.5%, and 49.3% while the allele frequencies were 4%, 2.25%, and 39.5%, respectively. The coexistence of the allele frequencies combinations of two, FVL (1691A) and Prothrombin (20210A), FVL (1691A) and MTHFR (677T), prothrombin (20210A) and MTHFR (677T) was found in 1 (0.9%), 29 (3.5%), and 22 (3%) samples, respectively. Triple heterozygous carriers were not found.

Conclusion: Allele frequencies of the two (FVL and MTHFR) mutations are higher compared with published data. The large sample size of our study enhances the validity of our results and suggests a biological affinity of Greek population with Southern Italian populations.

KEYWORDS
FVL, Greece, MTHFR, prothrombin, thrombophilia

1 | INTRODUCTION

Patients with thrombophilia run a greater risk of deep vein thrombosis (DVT) or pulmonary embolism, collectively referred as venous thromboembolism (VTE). Venous thromboembolism is a common disease with an annual incidence of about 1 per 1000 adults resulting in more than 50.000 deaths in the United States.[1] It is considered a multifactorial disease caused by environmental risk factors (age, obesity, oral contraceptives, and immobility) and inherited risk factors, that is, genetic polymorphisms in specific genes.[2] Among the most common

polymorphic alleles investigated in the last few decades are the G1691A factor V Leiden (FVL) mutation,[3,4] the G20210A prothrombin mutation,[4] and the C677T mutation of MTHFR gene.[5] The frequencies of these mutations also vary according to geographic and ethnic background.

Blood coagulation is downregulated by the protein C anticoagulant system. Protein C is activated on the endothelium by the thrombomodulin-thrombin complex and together with protein S degrades the activated forms of factor V and VIII.[6] Activated protein C (APC) inherited resistance is one of the major causes of venous thrombosis.[7] Factor V Leiden (FVL) is the most common inheritable cause of resistance to APC and refers to a point mutation at nucleotide 1691 of exon 10 of FVL gene (G to A transition) that leads to a procoagulant state due to the loss of one of the activated protein C (APC) cleavage sites.[8] The factor V Leiden allele frequency is high in Europeans (2%-8%) and virtually absent in African and Asian populations,[9] while Lebanon exhibits the highest prevalence of FV allele (14.4%).[10]

Prothrombin, coagulation factor II, is proteolytically cleaved to form thrombin in the clotting process, which converts fibrinogen to fibrin leading to the activation of platelets. A substitution of G to A at position 20 210, located in the 3′UTR region, of prothrombin has been found to be associated with increased levels of prothrombin and an approximately 3-fold higher risk of venous thrombosis.[11] Carriers of both FVL 1691A and prothrombin 20210A mutations have a 16-fold greater risk of venous thrombosis.[12] The 20210A prothrombin variant is more common in Southern Europe (3%) followed by Northern Europe (1.7%), while it is very rare in Asian and African populations (0%).[13]

A common mutation in the methylenetetrahydrofolate reductase gene (MTHFR), an enzyme that catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is the methyl donor in the conversion of homocysteine to methionine, has been implicated with hyperhomocystinemia.[14] Elevated levels of homocysteine have been associated with an increased risk of venous thrombosis.[15] The MTHFR (C677T) mutation refers to the replacement of C to T at nucleotide 677 of MTHFR gene, which results in the substitution of valine to alanine at amino acid position 222. The mutant enzyme exhibits decreased activity, and MTHFR (C677T) homozygous individuals have a 3-fold higher risk of premature cardiovascular diseases.[16] The prevalence of 677T MTHFR allele has been found to be high in Europe with the lowest allele frequency in Finland (25.1%) and Netherland (27.4%), and the highest in Italy (45%; 47.3% in Sicily), France (34%-36%), Hungary (33.7%), and Spain (33%).[17,18] The frequency of 677T MTHFR allele is significantly lower in Africa and African Americans populations (3%) compared with other ethnic groups.[17]

The aim of this study was to investigate the frequencies of (G1691A) factor V Leiden (FVL), prothrombin (G20210A), and MTHFR (C677T) mutations, separately and in combination, in a large sample size of Greek adults in order to have an accurate analysis for the population of Greece.

TABLE 1 Demographic characteristics of the study cohort

	Factor V G1691A			Prothrombin G20210A			MTHFR C677T			All
	GG (n = 875)	GA (n = 71)	AA (n = 2)	GG (n = 724)	GA (n = 34)	AA (n = 0)	CC (n = 303)	CT (n = 417)	TT (n = 125)	(n = 974)
Sex, n (%)						N/A				
Male	164 (18.7)	19 (26.7)	0	119 (16.5)	6 (16.7)	N/A	31 (10.3)	57 (13.7)	21 (16.7)	189 (19.4)
Female	711 (81.3)	52 (73.3)	2 (100)	605 (83.5)	28 (83.3)	N/A	272 (89.7)	360 (86.3)	104 (83.3)	785 (80.6)
Age (mean ± SD) (years)	35.76 ± 5.75	43.21 ± 6.88	39.48 ± 5.32	35.48 ± 5.43	36.67 ± 4.04	N/A	34 ± 4.50	36.30 ± 6.43	37.57 ± 4.04	36.62 ± 5.97

Abbreviation: N/A, not applicable.

2 | MATERIAL AND METHODS

A total of 974 blood samples were collected from healthy unrelated individuals at the - Molecular Department of Locus Medicus Center in Athens. We obtained a written consent from all participants, following the guidelines of the Greek Bioethical Committee.

2.1 | DNA extraction

The analysis was performed in 974 blood samples of the Laboratory of Cellular Biology and Immunology of Locus Medicus center located in Athens. All blood samples were derived from apparently healthy unrelated individuals of Greek origin and were collected into tubes containing K2 or K3 EDTA. DNA extraction was performed using QIAamp DNA Blood Mini Kit (Qiagen Inc., Germany), according to the manufacturer's instructions. The samples were stored at −20°C for further processing. The quality and quantity of the extracted DNA were tested using a spectrophotometer.

2.2 | Real-time PCR: Detection of V Leiden G1691A, PT G20210A, and MTHFR C677T polymorphisms

To determine the prevalence of the three mutations, samples were genotyped of the (G1691A) factor V Leiden, prothrombin (G20210A), and MTHFR (C677T) mutations using commercially available kits from Tib Molbiol (Berlin, Germany): The Light Mix Factor V (Leiden) kit, the Light Mix Factor II G20210A kit, and the Light Mix MTHFR C677T kit. Mutation screening was performed by real-time PCR using a Light Cycler instrument (Roche Applied Science). Based on melting curve analysis of PCR products, gene polymorphisms were detected. The probe melting temperature for wild-type factor V was 59°C, whereas for factor V Leiden was 51°C. The probe melting temperatures for prothrombin (G20210A) and MTHFR (C677T) polymorphisms were 50°C and 58°C, respectively. Positive and negative controls were included in each reaction.

2.3 | Statistical analyses

All statistical analyses were performed using SPSS 23.00 (SPSS Inc., Chicago, Illinois). We evaluated the Hardy-Weinberg equilibrium for each SNP through Pearson's x 2. The prevalence of each mutation was analyzed using descriptive statistics and was expressed in percentages. The prevalence of combined mutations was examined through cross-tabulation analysis.

3 | RESULTS

The demographic characteristics of our cohort are presented in Table 1. From the entire cohort, 785 participants were females (80.6%) and 189 participants were males (19.4%). Age ranged from 15 to 77 with a mean age of 36.62 (± 5.97). There were no homozygous for prothrombin G20210A mutation (Table 1). The observed allele frequencies of all SNPs did not differ from the expected frequencies under the Hardy-Weinberg equilibrium (Table S1).

3.1 | Allele frequencies of V Leiden G1691A, prothrombin G20210A, and MTHFR C677T mutations

The 974 DNA samples were analyzed for the presence of some of the following mutations: G1691A in factor V (FVL), G20210A in prothrombin, and C677T in MTHFR. Of the 974 samples, 948 were tested for the presence of FVL mutation, 758 for the G202101 prothrombin mutation, and 845 for C677T mutation in MTHFR. As shown in Table 2, from the 948 samples examined for FVL mutation, 71 subjects were heterozygous (7.5%) and 2 homozygous mutants (0.2%). Thirty-four samples (4.5%) from the 758 examined were heterozygous for the 20210A mutation in prothrombin, whereas no homozygous mutant was found. The C677T mutation in MTHFR gene was found in the heterozygous state in 417 subjects out of the 845 samples examined (49.3%) and in 125 individuals in the homozygous state (35.9%). Overall, the career frequencies of FVL, 20210A prothrombin, and 677T MTHFR alleles were 7.5%, 4.5%, and 49.3%, while the allele frequencies were 4%, 2.25%, and 39.5%, respectively.

3.2 | Coexistence of V Leiden G1691A, prothrombin G20210A, and MTHFR C677T mutations

The 741 samples were examined of the coexistence of FVL G1691A and prothrombin G20210A mutations and the 835 samples of the co-presence of FVL G1691A and MTHFR C677T mutations. The 725 samples were examined of the combination of prothrombin G20210A and MTHFR

TABLE 2 Genotype and allele frequencies of G1691A Factor V, G20210A prothrombin, and C677T MTHFR mutations

	Factor V G1691A	Prothrombin G20210A	MTHFR C677T
Heterozygous	71 (7.5%)	34 (4.5%)	417 (99.3%)
Homozygous mutant	2 (0.2%)	0 (0%)	125 (14.8%)
Wild-type homozygous	875 (92.9%)	724 (95.5%)	303 (35.9%)
Total	948	758	845
Career frequency	7.5%	4.5%	49.3%
Allele frequency	4%	2.25%	39.5%

TABLE 3 Prevalence of combined mutations of prothrombotic risk factors

Compound carriers	Frequency (%)
Factor V Leiden and PT G20210A (n = 751)	1 (0.1%)
Factor V Leiden and MTHFR C677T (n = 835)	29 (3.5%)
PT G20210A and MTHFR C677T (n = 725)	22 (3%)
FVL and PT G20210A and MTHFR C677T	0 (0%)

C677T mutations. As shown in Table 3, only one sample was found to be the carrier of FVL G1691A and prothrombin G20210A mutations (0.1%), 29 samples of FVL G1691A and MTHFR C677T mutations (3.5%) and 22 samples of prothrombin G20210A and MTHFR C677T mutations (3%). Triple heterozygotes mutations were not found.

4 | DISCUSSION

Venous thromboembolism (VTE) is a major disease, which affects approximately 1 in 1000 persons per year. VTE is common among Europeans, while it appears to be rare among other ethnic groups (Africa).[19] This disease may occur from genetic or environmental factors or both. Therefore, it is important to investigate the prevalence of genetic thrombotic risk factors among different ethnic groups to prevent episodes of thrombotic events.

In the present study, the allele frequency of (1691A) factor V Leiden (FVL) in a large sample size of apparently healthy Greek donors was found to be 4%, with carrier frequency 7.5%. Similar allele frequency has been reported in the population of Southern Italians (4.8%).[20] Previous studies conducted in healthy Greek population have reported 2.5% of FVL (1691A) allele frequency.[21,22] The 1.5% observed difference might be explained by the divergence in the sample size of studies; our study examined 974 samples while the previous one 160 and 200.[21,22] In Greek Cypriots, previous studies have found a higher prevalence of FVL (1691A) allele frequency (12%), in part because that population is not identical of the Hellenic population of Mainland Greece.[23] The highest career prevalence of FVL (1691A) mutation has been reported in Lebanon (37.1%).[10]

The prevalence of prothrombin (20210A) allele was found to be 2.3% and career frequency 4.5%, in line with the previously reported 2.2% of the Greek population.[21] A similar frequency of (20210A) prothrombin allele (2.9%) has been found in the population of Southern Italians.[20] In Greek-Cypriot population, the allele frequency of this variant is higher (7.5%)[23] and this discrepancy may be attributed to variations of regional genetic differences. The highest allele prevalence of prothrombin mutation was found in Palestinians living in Israel (6.5%-11.7%).[24] It has been reported that prothrombin mutation is more prevalent in Southern Europe than in northern (allele frequency, 3% vs 1.7%), especially in the Mediterranean region, and it is virtually absent among Africans and Americans.[25,28] The founder effect theory has been proposed to explain the different geographical distribution of this allele. This theory suggests that the mutation occurred 4 thousand years ago after the divergence of Africans from Non-Africans and Caucasoid from Mongoloids.[1,13]

The MTHFR (677T) allele frequency was found to be 39.5% with carrier frequency of 49.3%, higher than the previously reported 35.3%[21] and 35%.[22] The prevalence of MTHFR (677T) has been found to be slightly higher in Greek Cypriots (40%).[23] Epidemiological studies have found a higher incidence rate of this variant among Europeans (24.1%-64.3%) compared with Africans (0%-35.5%). As far as the European region is concerned, a higher allele frequency has been observed in Southern than in Northern Europe.[17] This led to the hypothesis that the prevalence of MTHFR (677T) allele is influenced by the adequacy of folic intake with increased frequency of this allele to be observed in countries with sufficient folic intake, giving a selective advantage to 677T homozygotes against colon cancer and acute leukemia.[26,27] The protective effect is probably related to the reduced MTHFR activity, which causes a diversion of 5,10-MnTHF toward thymidine synthesis, preventing dUMP misincorporation into DNA, which can induce double strands breaks.[25]

Compound carriers of FVL (1691A) and prothrombin (20210A), and of FVL (1691A) and MTHFR (677T) allele frequencies were accounted 0.1% and 3.5% of the total sample, respectively. The combination of MTHFR (677T) and prothrombin (20210A) allele frequencies was 3% of the total sample. The triple heterozygous allele frequencies of the three mutations were not found.

The large sample size of our study consolidates the accurate allele frequency of the three prothrombotic mutations in the healthy Greek population, which is 4% of FVL (1691A), 2.3% of prothrombin (20210A), and 39.5% of MTHFR (677T). Our results suggest a high biological affinity of Greek population with the population of Southern Italians, while it seems that the Greek-Cypriots population is genetically different from the Greek Mainland population. These data complement the existing epidemiological studies and may be useful in the development and implementation of strategies to prevent venous thromboembolism.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Conceptualization: Thaleia A. Bei, Apostolos Beloukas, Vassilis Tsilivakos.

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All authors have read and approved the final version of the manuscript.

Dr Thalia Bei had full access to all of the data in this study and takes complete responsibility for the integrity of the data and the accuracy of the data analysis.

TRANSPARENCY STATEMENT

Dr Thalia Bei affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

DATA AVAILABILITY STATEMENT

We carefully documented data, methods, and materials used to conduct the research in the article. We will share anonymized data at the request of other qualified investigators for purposes of replicating procedures and results.

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SUPPORTING INFORMATION

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