in vivo *26*: 1095-1102 (2012)

Prevalence of Thrombosis-related DNA Polymorphisms in a Healthy Greek Population

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Abstract. *Genetic association studies have revealed a correlation between DNA variations in genes encoding factors of the haemostatic system and thrombosis-related disease. This study investigated the prevalence of 13 such genetic risk factors in a sample (N=400 alleles) of the Hellenic population of Greece. Some of these polymorphisms [coagulation factor V (F5) Leiden, coagulation factor II (F2) G20210A, 5,10-methylene tetrahydrofolate reductase (MTHFR) C677T, coagulation factor XIII A1 subunit (F13A1) Val34Leu, serpine1 (SERPINE1) 4G/5G, angiotensin I-converting enzyme (ACE) I/D, angiotensinogen (AGT) Met325Thr, integrin A2 (ITGA2) C807T] have been previously studied in Hellenic populations of Greece and Cyprus, while others such as coagulation factor XII (F12) C46T, plasma carboxypeptidase B2 (CPB2) C1040T, platelet glycoprotein Ib α polypeptide (GP1BA) VNTR, thrombomodulin (THBD) -A33G and protein Z (PROZ) -A13G have not. Most of the allelic frequencies observed are similar to those reported for other Southern European populations. Knowledge of the prevalence of these variations in a given population may assist in the design of effective preventive measures against cardiovascular disease.*

Several hereditary and lifestyle-related factors are known to influence individual susceptibility to de-regulation of the haemostatic system and cause vascular diseases from birth and until late adult life (1-3). In recent years, genetic association studies have revealed links between genetic variations in certain factors of the haemostatic system and risk of venous and arterial thrombosis (4-7). These polymorphic variations are located in the genes of a wide variety of factors involved in coagulation, fibrinolysis, platelet activity and other thrombosis-related functions (2, 7). Most of these genetic variations are single-nucleotide polymorphisms (SNPs) that have a functional role and influence either gene expression or protein activity, thereby influencing haemostatic mechanisms either in a quantitative or in a qualitative fashion, respectively (7).

It is widely accepted that certain polymorphic alleles are correlated to venous thrombosis and may also confer a modest risk for arterial thrombosis, *e.g. coagulation factor V* (*F5*) Leiden (G1691A), *coagulation factor II* (*F2*) G20210A, and *5,10-methylene tetrahydrofolate reductase* (*MTHFR*) C677T (2, 5, 6, 8). *F5* Leiden and *F2* G20210A are found almost exclusively in Caucasian populations, with frequencies ranging between 1-2.5% and 2-4% respectively (9, 10). On the contrary, *MTHFR* C677T is distributed throughout the world, with variant allele frequency ranging between 5-60% (11).

Polymorphisms in several other haemostasis-related factors have been associated with risk of thrombosis, but further research is required in order to unequivocally establish these correlations (2, 7). Some of the thrombosis-related polymorphic alleles are very common worldwide, such as *serpine1* (*SERPINE1*) 4G/5G, which ranges between 42-54% in Caucasians, while others differ among populations, such as *thrombomodulin* (*THBD*) -G13A which ranges between 8-10% in East Asians and it is extremely rare in Caucasians (<1%) (12-18).

Since there are differences in the prevalence of polymorphic alleles among populations, as with all inherited genetic variations, genetic profiling of each population is required in order to collect data that could identify individuals at risk and influence local policies for disease prevention. The International Haplotype Mapping (HapMap) Project has determined the genotypes of several hundred individuals from populations in Africa, Asia and Europe (19).

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Key Words: Cardiovascular risk factors, coagulation factors, DNA polymorphisms, genetic predisposition, Greece, single-nucleotide polymorphisms, thrombophilia.

The HapMap research aims to create a public genome-wide database of common SNPs and, consequently, enable systematic studies of most common SNPs for their potential role in human disease. HapMap is expected to facilitate the development of molecular diagnostic tools and enhance capability of prevention through early intervention.

In this report, we describe the distribution frequency of gene polymorphisms of several haemostatic factors in a representative sample of the Hellenic population of Greece, which allows evaluation of risk for thrombosis and comparison with the distribution in other ethnic populations. Some of these polymorphisms have been previously studied in samples of the Hellenic population, while others such as *coagulation factor XII* (*F12*) C46T, *plasma carboxypeptidase B2* (*CPB2*) C1040T, *platelet glycoprotein Ib α polypeptide* (*GP1BA*) VNTR, *thrombomodulin* (*THBD*) -A33G and *protein Z* (*PROZ*) -A13G, to our knowledge, have not been previously examined by other groups (14, 20-25).

Materials and Methods

A total of 200 consecutive healthy blood donors of Hellenic origin without history of thrombosis were included in this study. All participating individuals were residents of the Athens metropolitan area but originated from several regions of Greece. Overall, the Greek population is genetically and culturally homogeneous and shares genetic similarities with other Caucasian populations (26). Blood samples were collected from participants, after written informed consent. The protocol of this study was examined and signed by the review board of the Department of Oral and Maxillofacial Surgery of the University of Athens. The majority of individuals were male (sex ratio 3:2) and their mean age was 58.4 years (SD=17.7). DNA was extracted from blood with the use of Nucleon™ kit (Amersham, Piscataway, NJ, USA).

Molecular detection of the polymorphisms in the respective genes was performed by allele specific PCR or restriction fragment length polymorphism typing, as previously described (9, 16, 24, 27-32). The following thrombosis-related polymorphisms were studied: a) coagulation factors: *coagulation factor V* (*F5*) Leiden (G1691A), *coagulation factor II* (*F2*, also known as *prothrombin*) G20210A, *coagulation factor XII* (*F12*) C46T and *coagulation factor XIII A1 subunit* (*F13A1*) Val34Leu; b) fibrinolysis related factors: *serpine1* (*SERPINE1*, also known as *plasminogen-activator inhibitor-1*) 4G/5G and *plasma carboxypeptidase B2* (*CPB2*, also known as *thrombin-activatable fibrinolysis inhibitor*) C1040T; c) thrombin-related factors: *thrombomodulin* (*THBD*) -A33G and *protein Z* (*PROZ*) -A13G; d) platelet-adhesion factors: *integrin A2* (*ITGA2*, also known as *glycoprotein Ia*) C807T and *platelet glycoprotein Ib α polypeptide* (*GP1BA*) variable number of tandem repeats (VNTR); e) other factors: *5,10-methylenotetrahydrofolate reductase* (*MTHFR*) C677T, *angiotensin converting enzyme* (*ACE*) insertion/deletion polymorphism (I/D) and *angiotensinogen* (*AGT*) Met325Thr. Several DNA samples were analyzed twice for verification of genotyping results.

Statistical analysis for the calculation of the exact 95% confidence intervals (CI) was performed using the SAS® software (version 9.0; SAS Institute Inc, Chicago, IL, USA). All observed genotypic frequencies were tested with GenePop version 4.1 (developed by M. Raymond and F. Rousset at Montpellier, France and freely available at http://kimura.univ-montp2.fr/~rousset/Genepop.htm), using the exact test for the evaluation of deviation from the Hardy-Weinberg (H-W) equilibrium. The number of alleles and the observed and expected heterozygosities under H-W equilibrium were computed for each polymorphism using the DOS version of GenePop 4.1. The *p*-values of Fisher's exact test for deviation from H-W equilibrium were computed, as previously reported, using the same version of GenePop with the following parameters for the Markov chain method: dememorization=10,000; batches=400; iterations per batch=5,000 (33).

Results

The detected genotypes, as well as the variant allelic and carrier frequencies in the studied sample of the Hellenic population are presented in Table I. For coagulation factors *F2, F5, F12* and *F13A1* the observed variant allelic frequencies were 2%, 2.5%, 37% and 22%, respectively. The variant allelic frequencies for the fibrinolysis-related factors *SERPINE1* and *CPB2* were 49% and 37% respectively. The prevalence of the *PROZ* -13G polymorphism was 6.3%, while no participants carrying the *THBD*-33A allele were identified. The observed frequencies for the remaining variant alleles were: *MTHFR* 667T: 35%; *ACE* I: 27.5%; *AGT* 235Thr: 36.5%; *ITGA2* 807T: 47% and *GP1BA* D: 8%. No VNTR-E variant was detected in *GP1BA*.

All the observed genotypic frequencies, with three exceptions, were compatible with the H-W equilibrium. The exceptions were those for the polymorphisms of two platelet-adhesion factors, *ITGA2* (expected percentage genotypes C/C: 28%, C/T: 50%, T/T: 22%) and *GP1BA* (C/C: 50%, C/D: 11%, D/D: 1%, B/C: 30%, B/D: 3%, B/B: 5%), as well as coagulation factor *F12* (C/C: 40%, C/T: 47%, T/T: 13%).

Discussion

Thrombosis is a disorder reliant on the complex interplay of genetic and environmental factors (2). Variations in genes encoding for certain factors of the haemostatic system, such as *F5* Leiden, have been shown to confer a significant pro-thrombotic phenotype, while emerging evidence has implicated the significance of DNA polymorphisms in other factors, such as *SERPINE1* and *F13A1* (2, 5, 8, 34).

In the present study, we evaluated the prevalence of polymorphisms in several thrombosis-related genes in healthy blood donors of Greek origin. Some of these polymorphisms have been previously studied in Hellenic populations of Greece and Cyprus, while others such as *F12 C46T, CPB2 C1040T, GP1BA* VNTR, *THBD* -A33G and *PROZ* -A13G have not (14, 21, 22, 25). Despite the small number of participants in the present study (n=200), important conclusions may be drawn.

Table I. *Frequencies of genotypes, alleles and carriers of the thrombosis-related polymorphisms in a sample of the Greek population (N=200 individuals). CI: Confidence interval (95%).*

Gene	Polymorphism	Genotype	Frequency	Carrier frequency	Variant allelic frequency	CI
Coagulation factors						
Coagulation factor II (F2)	G20210A	G/G	96%			
		G/A	4%	A: 4%	A: 2%	0.6-5%
		A/A	0%			
Coagulation factor V (F5)	G1691A	G/G	95%			
		G/A	5%	A: 5%	A: 2.5%	0.8-5.7%
		A/A	0%			
Coagulation factor XII (F12)	C46T	C/C	31%			
		C/T	64%	T: 69%	T: 37%	30.3-44.1%
		T/T	5%			
Coagulation factor XIII A1 subunit (F13A1)	Val34Leu	Val/Val	60%			
		Val/Leu	36%	Leu: 40%	Leu: 22%	16.5-28.4%
		Leu/Leu	4%			
Fibrinolysis-related factors						
Serpine1 (SERPINE1)	4G/5G	5G/5G	30%			
		4G/5G	41%	4G: 70%	4G: 49%	41.9-56.15%
		4G/4G	29%			
Plasma carboxypeptidase B2 (CPB2)	C1040T	C/C	39%			
		C/T	48%	T: 61%	T: 37%	30.3-44.1%
		T/T	13%			
Thrombin-related factors						
Thrombomodulin (THBD)	-G13A	G/G	100%			
		G/A	0%	A: 0%	A: 0%	0-1.49%
		A/A	0%			
Protein Z (PROZ)	-A33G	A/A	87%			
		G/A	13%	G: 13%	G: 6.5%	3.5-10.9%
		G/G	0%			
Platelet-adhesion factors						
Integrin A2 (ITGA2)	C807T	C/C	16%			
		C/T	74%	T: 84%	T: 47%	39.9-54.2%
		T/T	10%			
Platelet glycoprotein Ib α polypeptide (GP1BA)	VNTR	C/C	54%			
		C/D	14%			
		D/D	1%	D: 15%	D: 8%	4.6-12.7%
		B/D	0%			
		B/B	11%			
		B/C	20%			
Other factors						
Methylene tetrahydrofolate reductase (MTHFR)	C677T	C/C	38%			
		C/T	54%	T: 62%	T: 35%	28.4-42.1%
		T/T	8%			
Angiotensin I-converting enzyme (ACE)	I/D	D/D	51%			
		D/I	43%	I: 49%	I: 27.5%	21.4-34.2%
		I/I	6%			
Angiotensinogen (AGT)	Met235Thr	Met/Met	39%			
		Met/Thr	49%	Thr: 61%	Thr: 36.5%	29.8-43.6%
		Thr/Thr	12%			

F5 Leiden has emerged as probably the most important hereditary pro-thrombotic factor in Caucasians, with homozygotes exhibiting 50-100 greater relative risk of venous thrombosis (11). This variant is virtually absent from non-Caucasian populations and is thought to have arisen approximately 21,000 to 34,000 years ago in Caucasians; it has a wide allelic frequency range (1-7%) in Europeans (11). In the present study, the *F5 Leiden* frequency (2.5%) was

consistent with those previously observed in Greeks (2-4.2%), as well as in other Southern Europeans (1.8-4.2%) (11, 14, 15, 20-23, 25, 35) and Central Europeans (3-3.9%) (12, 36-38). It should be mentioned that one study of several populations reported a higher allelic frequency, of 7% in Greek-Cypriots, although other studies of the same population found a range of 3.5-4%, which is closer to that of mainland Greece (10, 14, 19, 21-25). These differences in frequency could be due to diverse sampling procedures.

The allelic frequency for *F2* 20210A (2%), a polymorphism that influences prothrombin levels, is in accordance with previous observations in Hellenic populations of Greece and Cyprus (2.0-2.7%) (21, 25, 35, 39). Two studies in Greek women of reproductive age have yielded slightly different results (frequency of 1%), but this discrepancy is probably due to differences in the demographic composition of the studied populations (40, 41). An allelic frequency of 5% has been reported in a Greek-Cypriot population but this might be attributed to variations in participant selection and/or regional genetic differences (14). Overall, the allelic frequency matches those observed for other Southern Europeans (1.5%) (11). As with *F5* Leiden, *F2* 20210A variant is present almost exclusively in Caucasian populations, indicating a common origin (11).

On the contrary, the *MTHFR* 677T allele is distributed in populations with different genetic backgrounds (11). The presence of the T-allele renders the MTHFR enzyme thermolabile, therefore predisposing to hyperhomocysteinaemia, which is a pro-thrombotic condition (6, 42). The prevalence of the 667T allele has been found to be similar in Greeks and Greek-Cypriots (35-39%) and our results (35%) confirm the previous findings (14, 21, 25, 43). A comparable prevalence occurs in Germans (37.9%), but a considerable difference has been noted in Northern Italians (46.4% and 53%) due to the relatively large number of T/T homozygotes (20% compared to 8% in our study) (12, 15, 44).

Variations in other coagulation factors, such as F13, could have an effect on thrombosis (45). Studies have suggested that the *F13* 34Leu allele confers protection against deep vein thrombosis (45). The prevalence of this particular allele varies considerably between populations (45). Our findings (22%) are in accordance with the prevalence of this allele in Italians (20%) and Turks (19%), while there seems to be a difference when compared to other Europeans, such as Germans (27.1%) and Austrians (27.4%) (12, 15, 38). In addition, there seems to be a significant difference from a Greek-Cypriot sample (13%) (14).

Modulation of the fibrinolytic system can also influence susceptibility to thrombosis. Variants in fibrinolysis-related factors such as *SERPINE1* and *CPB2* have been shown, to a diverse extent, to have a possible association with risk of thrombotic events (46-49). In the case of *SERPINE1*, homozygosity of the 4G allele is considered a risk factor for venous thrombosis and myocardial infarction (46, 47). This study found that the variant 4G allele was marginally less common (49%) than the wild-type 5G allele. These results match those observed in two Greek-Cypriot studies (42% and 46%), and the frequency reported in Italians (47.4%), while a higher prevalence was observed in East Germans (53.6%) and Swedes (53%) (12, 14, 15, 25, 46).

The effect of the C1040T polymorphism on CPB2 functionality is well-established but its role in thrombosis remains unclear (48-50). The prevalence of this polymorphism in Caucasians (31-33%) is much higher than in populations from sub-Saharan Africa (11%) (50, 51). To our knowledge, no other study has examined the 1040T allelic frequency in the Hellenic population. The T-allele prevalence observed in this study (37%) is not substantially higher than that found in Southern Europeans and other Caucasians (49, 51).

Regarding the *ACE* polymorphism, we found a surprisingly high prevalence of the thrombosis-related D allele (72.5%), which was considerably increased compared to a previous study in Northern Greek normotensive individuals (58%) (24). This difference could be explained either by the exclusion of hypertensive participants in the previous study, which may have artificially underestimated the prevalence of the D allele, or alternatively due to regional genetic differences. A recent study in a more representative sample from mainland Greece found a D allelic prevalence of 64% (52). Data from other Southern European regions report a 63-67% D allelic frequency (53).

The prevalence of the thrombin-related *PROZ*-A33G (6.5%) and *THBD* -A13G (0%) were comparable to those found in other Caucasian populations (6% and <1%, respectively) (13, 54). In contrast, a considerable difference was observed between Italians and our sample of the Hellenic population, in regard to the prevalence of the *AGT* 235Thr allele (46.5% compared to 36.5%, respectively), a finding reflecting the existing variation in frequencies among Caucasian populations (31-50%) (55, 56).

All genotypic distributions, except for three polymorphisms, conformed to the H-W principle. Deviations from the H-W equilibrium in samples representing the general population may occur due to chance, the effects of genetic drift (in a small population), significant migration, selection pressure, new unidentified mutations and finally genotyping error (57). Deviation from the H-W equilibrium is frequently under-reported or mis-represented in many allelic prevalence and genetic association studies, but when identified, it is important to provide possible explanations (57-59).

In the case of *ITGA2* (T allele: 47%), the deviation resulted from a significantly lower frequency of T/T homozygotes and to a lesser extent of C/C homozygotes. Similar deviation has been reported in the German population, with an equivalent variant allele frequency (42%), while no deviation was observed in young Italians in whom this frequency was

considerably lower (37%) (12, 48). Interestingly, a previous Greek study also documented a deviation from the H-W equilibrium for *ITGA2*; the reported allelic frequency was considerably lower (36.4%) but the age range was much narrower (50.6±0.81 years) (20). Homozygosity of the variant T-allele is thought to promote platelet adhesion, and through this mechanism, may possibly increase the risk for certain vascular diseases (selection pressure) (20, 29). While errors in genotyping cannot be excluded, the surprisingly small number of T heterozygotes may be a result of our exclusion criteria since only individuals without a prior history of thrombotic disease were included in the present study, as well as in the German study (12). Of course, the role of this particular polymorphism in certain thrombotic diseases, such as coronary arterial disease and cerebrovascular disease, remains uncertain and a profound impact on our results seems to be speculative at present (7, 60).

Furthermore, the observed genotypes of the *GP1BA* VNTR polymorphism were also significantly different from those expected. The relatively large number of alleles (four) in this locus can amplify the impact of chance on genotypic distribution, especially in a modest sample size such as our own, and therefore, this deviation is most likely incidental. Carriers of the D-allele (15% in our study) are thought to have a greater risk of myocardial infraction (61).

Regarding the *F12* 46T allele, this first study of this polymorphism in the Hellenic population detected a substantially higher frequency compared to other Europeans. For example, when compared to samples of the Spanish population (18-20%), the prevalence of the T allele is almost double in our study (37%) (62, 63). A 46T allelic frequency of 26% was noted in Scots (64). The genotypic distribution for this polymorphism deviated from the expected distribution based on the H-W principle, mainly because the number of heterozygotes was significantly higher than expected. Nevertheless, deviation from H-W equilibrium was also observed in all aforementioned studies (62-64). The underlying cause for this phenomenon already seen in three populations remains unknown.

Large-scale epidemiological studies are needed in order to fully-understand the contribution of each thrombosis-related DNA polymorphism to vascular disease. Only then may population-wide screening be warranted for preventative purposes. At this stage, information about the prevalence of polymorphisms (as shown here) is needed for each population and might prove valuable in shaping local health policies.

Acknowledgements

The Authors thank all individuals who donated blood samples for this study. This work was co-funded by the European Social Fund and National Resources (EPEAEK II "Pythagoras" 70/3/7391) grant to E.V.

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Received May 2, 2012
Revised September 19, 2012
Accepted September 21, 2012