

Factor V Leiden (G1691A), Factor V R2 (A4070G), and Prothrombin (G20210A) Genetic Polymorphisms in Macedonian Patients with Occlusive Artery Disease and Deep Vein Thrombosis

Igor Spiroski¹, Sashko Kedev¹, Olivija Efinska-Mladenovska²

¹University Clinic of Cardiology, Faculty of Medicine, Ss Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia; ²Institute of Immunobiology and Human Genetics, Faculty of Medicine, Ss Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia

Abstract

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Correspondence: Dr. Igor Spiroski. University Clinic of Cardiology, Faculty of Medicine, Ss Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia. E-mail: ispiroski@gmail.com

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AIM: The aim was to analyze association of *Factor V Leiden (G1691A)*, *Factor V R2 (A4070G)*, and *Prothrombin (G20210A)* Genetic Polymorphism in Macedonian Patients with Occlusive Artery Disease (OAD) and Deep Vein Thrombosis (DVT).

METHODS: Investigated groups consists of 82 healthy, 76 patients with OAD, and 67 patients with DVT. Blood samples were collected after written consent, and DNA was isolated from peripheral blood leukocytes. Identification of *Factor V Leiden (G1691A)*, *Factor V R2 (A4070G)*, and *Prothrombin (G20210A)* Genetic Polymorphism was done with CVD StripAssay (ViennaLab, Labordiagnostica GmbH, Austria). The population genetics analysis package, PyPop, was used for analysis of the data. Pearson's *P*-values, crude Odds Ratio and Wald's 95% CI were calculated.

RESULTS: The frequency of *G* allele for *Factor V Leiden* was 0.976 for healthy participants, 0.954 for OAD, and 0.948 for DVT. The frequency of *A* allele for *Factor R2* is highest in healthy participants (0.951), smaller in patients with DVT (0.918), and smallest in the patients with OAD (0.908). *G* allele frequency for prothrombin was 0.976 in healthy participants, 0.980 in patients with OAD, and 0.978 in patients with DVT. Test of neutrality (*F_{nd}*) showed positive value, but was not significantly different from 0. *Factor V Leiden (G1691A)*, *Factor R2 (A4070G)*, and *Prothrombin (G20210A)* genotypes in healthy participants and patients with OAD and DVT were in Hardy Weinberg proportions. Any association of *Factor V Leiden (G1691A)*, *Factor R2 (A4070G)*, and *Prothrombin (G20210A)* genetic polymorphism with OAD, and DVT in Macedonians was not found.

CONCLUSION: We conclude that significant association of *Factor V Leiden (G1691A)*, *Factor R2 (A4070G)*, and *Prothrombin (G20210A)* genetic polymorphism with occlusive artery disease or deep venous thrombosis in Macedonians was not found.

Introduction

The association between the inherited gene mutations of *factor V*, *prothrombin*, and homocysteine metabolism and venous thromboembolic events is accepted widely, but their influence on the arterial circulatory system remains controversial. There are a lot of published papers dealing with single or combined mutations of *factor V*, *factor V R2*, and/or *prothrombin*, but the most important are papers with meta-analysis [1-5].

Published case-control and cohort studies correlating the *factor V Leiden*, *prothrombin (PT)*

G20210A, and methylenetetrahydrofolate reductase (*MTHFR*) *C677T (TT* genotype) mutations with myocardial infarction, ischemic stroke, or peripheral vascular disease were included in meta-analysis. The association between inherited gene mutations and arterial ischemic events was modest. It was found that genetic abnormalities specific to *factor V*, *prothrombin*, and homocysteine metabolism increase the risk for myocardial infarction and ischemic stroke, particularly among younger patients and women [1].

Meta-analyses were done of 191 studies in relation to *factor V G1691A* (ie, *factor V Leiden*), *factor VII G10976A*, *prothrombin G20210A*, *plasminogen activator inhibitor-1 (PAI-1) [-675] 4G/5G*, and three platelet glycoprotein (GP) receptor

variants (*GPIa C807T*, *GPIbalpha T[-5]C*, *GPIIIa C1565T*), involving a total of 66 155 coronary disease cases and 91 307 controls. The *1691A* variant of the factor V gene and the *20210A* variant of the prothrombin gene, both of which increase circulating thrombin generation, might each be moderately associated with the risk of coronary disease [2].

Keijzer et al., 2007 performed a meta-analysis to investigate a possible interaction between factor V Leiden and hyperhomocysteinemia, including 825 subjects with venous thrombosis and 2,109 controls, for the risk of venous thrombosis [3]. The meta-analysis yielded no evidence for additive or multiplicative interaction between factor V Leiden and hyperhomocysteinemia. Both the meta-analyses of published studies and a large case-only study did not show evidence for interaction between factor V Leiden and hyperhomocysteinemia for risk of venous thrombosis.

Another systematic and comprehensive meta-analysis on all candidate genes to assess their genetic contribution to the aetiology of venous thromboembolism (VTE) (pulmonary embolism and deep venous thrombosis) in all ethnic groups was published. Meta-analyses included approximately 126 525 cases and 184 068 controls derived from 173 case-control studies, which included 21 genes (28 polymorphisms). Statistically significant associations with VTE were identified for factor V *G1691A*, factor V *A4070G*, prothrombin *G20210A*, prothrombin *G11991A*, *PAI-1 4G/5G*, and *alpha-fibrinogen Thr312Ala* all in Caucasian populations. The work supports a genetic aetiology to VTE disease and provides reliable risk estimates [4].

Marjot et al., identified 26 case-control studies investigating 6 polymorphisms in 6 genes and included 1 183 cerebral venous thrombosis (CVT) cases and 5 189 controls [5]. Statistically significant associations with CVT were found for factor V Leiden/*G1691A* and prothrombin/*G20210A*. After iterative analysis controlling for interstudy heterogeneity, *methylene tetrahydrofolate reductase/C677T* was also found to be significantly associated. Variants in the remaining 3 genes (*Janus kinase-2*, *plasminogen activator inhibitor-1*, and *protein Z*) were not significantly associated. It concluded that CVT has a genetic basis. Genes involved in the clotting cascade provide a greater level of thrombosis risk in the cerebral venous circulation compared with its arterial circulation, and a greater level of risk exists for adults compared with children [5].

Twelve mutations in the CVD genes (*MTHFR C677T*, *MTHFR A1298C*, *PAI-1 4G/5G (SERPINE1)*, *FV G1691A (Leiden)*, *FV H1299R (R2)*, *Prothrombin G20210A*, *Factor XIII V34L*, *β -Fibrinogen -455 G-A*, *GPIIIa L33P (HPA-1)*, *ACE I/D*, *Apo B R3500Q*, and *Apo E2/E3/E4*) in Macedonians were performed. We published association of *MTHFR -677*, and *-1289*

polymorphisms with occlusive artery disease (OAD) and deep vein thrombosis (DVT) in Macedonians and any association was not found, except for the protective association between *MTHFR/CA:CC* diplotype and AOD [6]. Plasma concentration of total homocysteine (tHcy) in patients with DVT in comparison with healthy respondents was significantly increased for normal:normal (*CC:AA*), normal heterozygote (*CC:AC*), and heterozygote: heterozygote (*CT:AC*) haplotypes [7]. Any association of *SERPINE1* polymorphisms with OAD, and DVT in Macedonians was not found [8]. There are no data on Macedonian population about the *Factor V Leiden (G1691A)*, *Factor V R2 (A4070G)*, and *Prothrombin (G20210A)* polymorphisms and their possible associations with different diseases.

The aim of this study was to analyze association of *Factor V Leiden (G1691A)*, *Factor V R2 (A4070G)*, and *Prothrombin (G20210A)* genetic polymorphisms with occlusive artery disease, and deep vein thrombosis in Macedonians in order to investigate its role as a part of candidate genes in different vascular diseases in Macedonians.

Methods

Investigated groups

The total studied sample consists of 225 examinees composed of three different groups: healthy individuals, patients with occlusive artery disease, and patients with deep venous thrombosis.

a) *Healthy individuals* (n=82), 39 female and 43 male, aged 40.7 ± 11.3 years, born in different parts of Macedonia attending Institute for Transfusion for blood donation. Inclusion of healthy individuals was random, if medical doctor declare their health as acceptable (on the basis of medical documentation, completed interview, and physical examination). From the investigation were excluded individuals with family history of blood vessel diseases.

b) *Occlusive artery disease* (n=76), 29 female and 47 male patients with proved and documented myocardial infarct (n=52), brain infarct (n=22), and peripheral artery thrombosis (n=2), aged 63.3 ± 9.6 years hospitalized the Institute of Heart Diseases, Faculty of Medicine, and Institute for Transfusion, Skopje for outpatient treatment were included.

c) *Deep vein thrombosis* (n=67), 45 female and 22 male patients (diagnosed by ultrasonography and/or venography), aged 57.7 ± 11.8 years attending the Institute of Heart Diseases, Faculty of Medicine, and Institute for Transfusion, Skopje for outpatient treatment were included.

All individuals are of Macedonian origin, and residents of different geographical areas of the

Republic of Macedonia. All patients and healthy individuals included in this study signed a written consent to participate in the study which was approved by the Committee of the Ministry of Education and Science from Republic of Macedonia (No 13-1672/4-02).

Genomic DNA isolation and storage

Blood samples were collected after written consent and DNA was isolated from peripheral blood leukocytes by the phenol-chloroform extraction method or with BioRobot EZ1 workstation (QIAGEN) [9]. The quality and quantity of DNA was analyzed by GeneQuant (Pharmacia). Isolated DNA samples were stored in Macedonian Human DNA Bank (hDNAMKD) [10].

Typing Methods

Assay for the identification of *Factor V Leiden* (G1691A), *Factor V R2* (A4070G), and *Prothrombin* (G20210A) genetic polymorphism is based on polymerase chain reaction (PCR) and reverse-hybridization with CVD StripAssay (ViennaLab Labordiagnostica GmbH, Austria). The procedure includes three steps: 1) DNA isolation, 2) PCR amplification using biotinylated primers, 3) hybridization of amplification products to a test strip containing allele-specific oligonucleotide probes immobilized as an array of parallel lines. Bound biotinylated sequences are detected using streptavidin-alkaline phosphatase and colour substrates [11]. The assay covers alleles of *Factor V Leiden* (G1691A), *Factor V R2* (A4070G), and *Prothrombin* (G20210A). The genotype of a sample is determined using the enclosed Collector sheet or using the software StripAssay Evaluator, ver. 2.0, ViennaLab Diagnostics GmbH.

Statistical Methods

The population genetics analysis package, PyPop, developed by the Biostatistics Core for the Workshop [12-14] was used for analysis of the *Factor V Leiden* (G1691A), *Factor V R2* (A4070G), and *Prothrombin* (G20210A) data for this report. Allele frequencies and expected Hardy Weinberg proportions (HWP) for each *Factor V Leiden* (G1691A), *Factor V R2* (A4070G), and *Prothrombin* (G20210A) allele were determined [15]. The exact test for genotype frequency deviation from HWP was calculated using the Arlequin implementation accessed via PyPop [16]. Those alleles that did not fit HWP were evaluated to determine whether there was an excess of homozygotes or heterozygotes, or if any particular genotypes were significantly different from expected frequencies by the chi square test. The Ewens-Watterson homozygosity test of neutrality (EWN) [17] with Slatkin's exact p-values (SEPV) [18,

19] was used to indicate any deviations from the hypothesis of neutral selection for each locus. Pearson's p-values, crude Odds Ratio (OR) and Wald's 95% confidence interval (CI) were calculated for associations' analysis between *Factor V Leiden* (G1691A), *Factor V R2* (A4070G), and *Prothrombin* (G20210A) alleles and blood vessel disease with GraphPad QuickCalcs: free statistical calculators (<http://www.graphpad.com/quickcalcs/>) with Bonferroni corrected p-value [20, 21]. *P* less than 0.05 were taken as significant.

Results

Factor V, *Factor V R2*, and *Prothrombin* Alleles and Genotypes

Frequencies of *Factor V*, *Factor V R2*, and *Prothrombin* alleles, test of neutrality with Fnd statistic (Ewens-Watterson test of neutrality), and Slatkin's Exact *P* value (SEPV) with *P* of F statistics in Macedonians are shown on the Table 1.

The frequency of G alleles for *Factor V* varies between 0.976 for healthy participants, 0.954 for occlusive artery disease, and 0.948 for deep vein thrombosis indicating dominance of this allele. The frequency of A allele is smallest in healthy participants (0.024), and is more frequent in patients with deep vein thrombosis (0.052), and with occlusive artery disease (0.046). Similar findings were found for *Factor V R2* alleles (A and G), as well as for *Prothrombin* alleles (G and A). For the all *Factor V*, *Factor V R2*, and *Prothrombin* alleles, test of neutrality showed positive value for F_{nd} statistic without significant *P* of F statistics (Table 1).

Table 1: Frequencies of *Factor V*, *Factor V R2*, and *Prothrombin* alleles, test of neutrality with Fnd statistic (Ewens-Watterson test of neutrality), and Slatkin's Exact *P* Value with *P* of F statistics in Macedonians

	n [*]	Alleles			Test of Neutrality (F)	
		Allele	Number	Frequency	EWN [†]	SEPV [‡]
Factor V						
Healthy	82	G	160	0.976	0.757	0.671
		A	4	0.024		
Occlusive artery disease (OAD)	76	G	145	0.954	0.530	0.552
		A	7	0.046		
Deep vein thrombosis (DVT)	67	G	127	0.948	0.487	0.541
		A	7	0.052		
Factor V R2						
Healthy	82	A	156	0.951	0.487	0.532
		G	8	0.049		
Occlusive artery disease (OAD)	76	A	138	0.908	0.057	0.411
		G	14	0.092		
Deep vein thrombosis (DVT)	67	A	123	0.918	0.177	0.447
		G	11	0.082		
Prothrombin						
Healthy	82	G	160	0.976	0.757	0.671
		A	4	0.024		
Occlusive artery disease (OAD)	76	G	149	0.980	0.824	0.729
		A	3	0.020		
Deep vein thrombosis (DVT)	67	G	131	0.978	0.817	0.721
		A	3	0.022		

^{*} n= number of participants; [†] EWN=Ewens-Watterson test of neutrality; [‡] SEPV=Slatkin's Exact *P* Value.

Observed versus expected frequency of *Factor V*, *Factor V R2*, and *Prothrombin* genotypes, Hardy Weinberg proportion (HWP), and Guo and Thompson Hardy Weinberg Output (GTHWO) in Macedonians is given on the Table 2.

Table 2: Observed vs. expected *Factor V*, *Factor V R2*, and *Prothrombin* genotypes for each investigated group, Hardy Weinberg proportions, and Guo and Thompson Hardy Weinberg Output in Macedonians

Investigated group	Genotype	Observed number	Expected number	P-value	HWP P-value	GTHWO [†] P-value
Factor V						
Healthy	GG	78 (95.1%)	78.0	0.996		
	GA	4 (4.9%)	3.9	&	0.991	1.000
	AA	0 (0.0%)	0.0	&		
Occlusive artery disease (OAD)	GG	69 (92.0%)	69.2	0.984		
	GA	7 (8.0%)	6.7	0.900	0.969	1.000
	AA	0 (0.0%)	0.2	&		
Deep vein thrombosis (DVT)	GG	60 (89.5%)	60.2	0.981		
	GA	7 (10.5%)	6.6	0.887	0.885	1.000
	AA	0 (0.0%)	0.2	&		
Factor V R2						
Healthy	AA	74 (90.2%)	74.2	0.982		
	AG	8 (9.8%)	7.6	0.887	0.964	1.000
	GG	0 (0.0%)	0.2	&		
Occlusive artery disease (OAD)	AA	64 (84.2%)	62.6	0.864		
	AG	10 (13.1%)	12.7	0.447	0.436	0.109
	GG	2 (2.7%)	0.6	&		
Deep vein thrombosis (DVT)	AA	56 (83.6%)	56.5	0.952		
	AG	11 (16.4%)	10.1	0.776	0.771	1.000
	GG	0 (0.0%)	0.5	&		
Prothrombin						
Healthy	GG	78 (95.1%)	78.0	0.996		
	GA	4 (4.9%)	3.9	&	&	1.000
	AA	0 (0.0%)	0.0	&		
Occlusive artery disease (OAD)	GG	73 (96.0%)	73.0	0.998		
	GA	3 (4.0%)	2.9	&	&	1.000
	AA	0 (0.0%)	0.0	&		
Deep vein thrombosis (DVT)	GG	64 (95.5%)	64.0	0.998		
	GA	3 (4.5%)	2.9	&	&	1.000
	AA	0 (0.0%)	0.0	&		

[†], HWP = Hardy Weinberg proportions; [†], GTHWO = Guo and Thompson Hardy Weinberg Output; &, Cannot be calculated because expected ≤ 5 , χ^2 test.

The most frequent *Factor V* genotype in healthy participants was GG with observed frequency of 95.1%, smaller frequency was found for GA genotype (4.9%), and zero (0.0%) frequency was found for AA genotype. The frequency of *Factor V*/GA genotype were slightly decreased in patients with occlusive artery disease and deep vein thrombosis (92.0% and 89.5%, respectively), but *Factor V*/AA was absent (0.0%). Similar results were found for *Factor V R2* genotypes with the highest observed frequency of AA genotype (90.2%) in healthy participants, and slightly lower frequency in patients with occlusive artery disease (84.2%) and deep vein thrombosis (83.6%). Homozygous *Factor V R2*/GG genotype was absent in investigated groups (0.0%), except in patients with occlusive artery disease (2.7%). The most frequent genotype of *Prothrombin* was GG with observed frequency of 95.1% in healthy participants, 95.5% in patients with deep vein thrombosis, and 96.0% in patients with occlusive artery disease. Heterozygous *Prothrombin*/GA genotype observed frequency was found in less than 5% in all investigated groups. Homozygous *Prothrombin*/AA genotype was absent in all investigated groups (0.0%). P-values and Hardy Weinberg proportions were no significant. P-value of Guo and Thompson Hardy Weinberg output was 1.000 for all investigated genes (*Factor V*, *Factor V R2*, and *Prothrombin*) in all investigated groups, except for *Factor V R2* in patients

with occlusive artery disease (0.109) (Table 2).

Association between *Factor V*, *Factor V R2*, and *Prothrombin* polymorphisms and blood vessels diseases

Association between *Factor V*, *Factor V R2*, and *Prothrombin* alleles and genotypes with occlusive artery disease is shown on the Table III. We did not found any significant association between *Factor V*, *Factor V R2*, and *Prothrombin* alleles and genotypes with occlusive artery disease (Pearson's P value greater than 0.05) (Table 3).

Table 3: Association between *Factor V*, *Factor V R2*, and *Prothrombin* alleles and genotypes with occlusive artery disease with Pearson's P-value, crude odds ratio, and Wald's 95% confidence interval in Macedonians

Allele or Genotype	OAD (n=75)	Healthy (n=82)	Pearson's P-value	Odds ratio	Wald's 95% CI [†]
Factor V					
G	145 (95.4%)	160 (97.6%)	0.294	0.518	0.148-1.805
A	7 (5.6%)	4 (2.4%)	0.294	1.931	0.554-6.732
GG	69 (92.0%)	78 (95.1%)	0.285	0.505	0.142-1.801
GA	7 (8.0%)	4 (4.9%)	0.285	1.978	0.555-7.048
AA	0 (0.0%)	0 (0.0%)	&	&	&
Factor V R2					
A	138 (90.8%)	156 (95.1%)	0.130	0.505	0.206-1.241
G	14 (9.2%)	8 (4.9%)	0.130	1.978	0.806-4.858
AA	64 (84.2%)	74 (90.2%)	0.254	0.577	0.222-1.498
AG	10 (13.1%)	8 (9.8%)	0.463	1.445	0.538-3.882
GG	2 (2.7%)	0 (0.0%)	&	&	&
Prothrombin					
G	149 (98.0%)	160 (97.6%)	0.779	1.242	0.273-5.640
A	3 (2.0%)	4 (2.4%)	0.779	0.805	0.177-3.658
GG	73 (96.0%)	78 (95.1%)	0.776	1.248	0.270-5.766
GA	3 (4.0%)	4 (4.9%)	0.776	0.801	0.173-3.703
AA	0 (0.0%)	0 (0.0%)	&	&	&

[†], OAD=occlusive artery disease; [†], CI=confidence interval; &, Cannot be calculated because expected ≤ 5 , χ^2 test.

Association between *Factor V*, *Factor V R2*, and *Prothrombin* alleles and genotypes with deep vein thrombosis is shown on the Table IV. We did not found any significant association between *Factor V*, *Factor V R2*, and *Prothrombin* alleles and genotypes with deep venous thrombosis (Pearson's P value greater than 0.05) (Table 4).

Discussion

In this manuscript we report *Factor V*, *Factor V R2*, and *Prothrombin* alleles and genotypes that exist in Macedonians, and possible association with

occlusive artery disease, as well as with deep venous thrombosis. The results did not show any significant association of *Factor V*, *Factor V R2*, and *Prothrombin* alleles and genotypes with occlusive artery disease or deep venous thrombosis.

Table 4: Association between *Factor V*, *Factor V R2*, and *Prothrombin* alleles and genotypes with deep vein thrombosis with Pearson's *P*-value, crude odds ratio, and Wald's 95% confidence interval in Macedonians

Allele or Genotype	DVT (n=66)	Healthy (n=82)	Pearson's <i>P</i> -value	Odds ratio	Wald's 95% CI [†]
Factor V					
G	127 (94.8%)	160 (97.6%)	0.205	0.454	0.130-1.585
A	7 (5.2%)	4 (2.4%)	0.205	2.205	0.631-7.698
GG	60 (89.5%)	78 (95.1%)	0.196	0.440	0.123-1.571
GA	7 (10.5%)	4 (4.9%)	0.196	2.275	0.636-8.131
AA	0 (0.0%)	0 (0.0%)	&	&	&
Factor V R2					
A	123 (91.8%)	156 (95.1%)	0.242	0.573	0.224-1.469
G	11 (8.2%)	8 (4.9%)	0.242	1.744	0.681-4.468
AA	56 (83.6%)	74 (90.2%)	0.225	0.550	0.208-1.459
AG	11 (16.4%)	8 (9.8%)	0.225	1.817	0.686-4.815
GG	0 (0.0%)	0 (0.0%)	&	&	&
Prothrombin					
G	131 (97.8%)	160 (97.6%)	0.910	1.092	0.240-4.965
A	3 (2.2%)	4 (2.4%)	0.910	0.916	0.201-4.166
GG	64 (95.5%)	78 (95.1%)	0.908	1.094	0.236-5.068
GA	3 (4.5%)	4 (4.9%)	0.908	0.914	0.197-4.234
AA	0 (0.0%)	0 (0.0%)	&	&	&

*, DVT=deep vein thrombosis; †, CI=confidence interval; &, Cannot be calculated because expected ≤ 5 , χ^2 test.

We found positive value for F_{nd} statistic, without significant *P* of *F* statistics in healthy participants and in patients with occlusive artery disease and deep venous thrombosis. We found also that *Factor V*, *Factor V R2*, and *Prothrombin* genotypes are in equilibrium with HWP in healthy participants and in patients with OAD, as well as in patients with DVT.

Our results are in accordance with the meta-analysis published by Keijzer et al., 2007 where evidence for interaction between factor V Leiden and hyperhomocysteinemia for risk of venous thrombosis was not found [3]. Other studies with meta-analysis have shown that there is either moderate [1, 2] or significant association of several candidate genes with venous thromboembolism and cerebral venous thrombosis [4, 5]. The differences may be partly dependent on the number of investigated controls and patients.

Diseases of the cardiovascular system are complex genetic traits which include hundreds of associated candidate genes [22]. The numbers of patients and controls of our study is very small: in the association studies, there are possibilities that some positive results might be spurious and some negative findings might be a consequence of low statistical power. It could be due to their small sample size or methodological shortcomings, such as the selection of an appropriate control group. Further studies are merited to assess these associations in greater detail (including any gene-gene and gene-environment interactions) and to determine any implications with regard to potential therapies designed to reverse patients' prothrombotic phenotype.

Compared with patients with both deep venous thrombosis and pulmonary embolism, isolated deep venous thrombosis patients more often had thrombi located distally and had a similar number of affected veins. Compared with isolated pulmonary embolism patients, isolated deep venous thrombosis patients had a similar time between provocation and diagnosis, and similar in vitro coagulation time and thrombus density. Although some effects were differential for FVL-carriers and noncarriers, and some were differential for pulmonary embolism and deep venous thrombosis patients, none of the potential mechanisms offered a clear explanation [23]. Because our study groups are heterogeneous, the results should be analysed with precaution, and we need more homogenous subgroups for definitive conclusion about association between *SERPINE1* gene and cardiovascular diseases

In summary, association of *Factor V*, *Factor V R2*, and *Prothrombin* polymorphisms with occlusive artery disease, and deep venous thrombosis in Macedonians was not found. The results can be used for population meta-analysis, as well as for association studies with different diseases.

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