# Investigation of *SERPINE1* genetic polymorphism in Macedonian patients with occlusive artery disease and deep vein thrombosis

Igor Spiroski[1], Sashko Kedev[1], Slobodan Antov[1], Dejan Trajkov[2], Aleksandar Petlichkovski[2], Sloboda Dzhekova-Stojkova[3], Stojanka Kostovska[4], Mirko Spiroski[2]

[1] Clinic of Cardiology, Faculty of Medicine, Ss. Cyril and Methodius University, Skopje, Republic of Macedonia
[2] Institute of Immunobiology and Human Genetics, Faculty of Medicine, Ss. Cyril and Methodius University, Skopje, Republic of Macedonia
[3] Institute of Medical and Experimental Biochemistry, Faculty of Medicine, Ss. Cyril and Methodius University, Skopje, Republic of Macedonia
[4] Institute of Transfusion, Skopje, Republic of Macedonia

## Abstract

**Background:** Raised *SERPINE1* plasma levels are related to a 1-bp guanine deletion/insertion (*4G5G*) polymorphism in the promoter of the *SERPINE1* (plasminogen activator inhibitor 1 – PAI1) gene. Evidence suggested that the plasma levels of *SERPINE1* modulate the risk of coronary artery disease; furthermore, that the *4G5G* polymorphism affects the expression of the *SERPINE1* gene.

**Aim:** To analyse association of *SERPINE1* polymorphism with occlusive artery disease (OAD) and deep venous thrombosis (DVT) in Macedonians in order to investigate its role as a part of candidate genes in different vascular diseases in Macedonians.

**Methods:** Investigated groups consisted of 82 healthy patients, 75 with OAD, and 66 with DVT. Blood samples were collected after written informed consent was obtained, and DNA was isolated from peripheral blood leukocytes. Identification of *SERPINE1* polymorphism was done with CVD StripAssay (ViennaLab, Labordiagnostica GmbH, Austria). The population genetics analysis package, PyPop, was used for analysis of the *SERPINE1* data. Pearson's P-values, crude odds ratio and Wald's 95% CI were calculated with Bonferroni corrected p value.

**Results:** The frequency of 4G allele for *SERPINE1* was 0.538 for DVT, 0.555 for healthy participants, and 0.607 for OAD. The frequency of 5G allele for *SERPINE1* was the smallest in patients with OAD (0.393) and was higher in healthy participants (0.445), and patients with DVT (0.462). Test of neutrality (Fnd) showed negative value, but was significantly different from 0 for *SERPINE1* in healthy participants (p of F = 0.041) and in patients with DVT (p of F = 0.030). *SERPINE1* genotypes in healthy participants and patients with OAD were not in Hardy Weinberg proportions (p = 0.019 and 0.001, respectively). No association between *SERPINE1* polymorphisms and OAD or DVT was found.

**Conclusion:** There is no significant relationship between *SERPINE1* polymorphisms and occlusive artery disease or deep venous thrombosis in Macedonian population.

**Key words:** *SERPINE1* polymorphism, occlusive artery disease, deep venous thrombosis, Macedonians

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## Introduction

Plasminogen activator inhibitor-1 (PAI-1) is the principal inhibitor of tissue plasminogen activator (tPA) and urokinase (uPA), the activators of plasminogen and hence fibrinolysis (the physiological breakdown of blood clots). It is a serine protease inhibitor (serpin) protein (SERPINE1). The other PAI, plasminogen activator inhibitor-2 (PAI-2), is secreted by the placenta and is only present in significant amounts during pregnancy. In addition, protease nexin acts as an inhibitor of tPA and urokinase. PAI-1, however, is the main inhibitor of the plasminogen activators [1]. The *SERPINE1* gene is located on 7q21.3-q22.1, with DNA size of 11.85 Kb, and nine exons [2].

Raised SERPINE1 plasma levels are related to a 1-bp guanine deletion/insertion (*4G5G*) polymorphism in the promoter of the *SERPINE1* gene [3]. Evidence has suggested that the plasma levels of *SERPINE1* modulate the risk of coronary artery disease, and furthermore, that the *4G5G* polymorphism affects the expression of the *SERPINE1* gene [4].

**Address for correspondence:**
Igor Spiroski MD, PhD, Clinic of Cardiology, Faculty of Medicine, Ss. Cyril and Methodius University, Blvd. Krste Petkov Misirkov bb, 1000 Skopje, Republic of Macedonia, tel.: +38 92 329 32 93, e-mail: ispiroski@gmail.com

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Investigations of the relationship between the *SERPINE1 4G5G* polymorphism in 1,179 healthy employees and the occurrence of coronary artery disease in their first-degree relatives have shown that the group with a first-degree relative who had suffered from a coronary ischaemic episode had a higher number of homozygotes for the deleted allele (*4G4G*) of the *SERPINE1* gene compared with subjects without such a family history (odds ratio = 1.62). The frequency of the *4G* allele was abnormally high in these individuals as well. The individuals with a positive family history were older and exhibited a higher body mass index and total cholesterol levels than those without [5].

Defects in *SERPINE1* are the cause of plasminogen activator inhibitor-1 deficiency (PAI-1 deficiency) [MIM:173360], characterised by abnormal bleeding due to SERPINE1 defect in the plasma [6, 7]. High concentrations of SERPINE1 have been associated with thrombophilia (MIM:188050), an autosomal dominant disorder in which affected individuals are prone to develop serious spontaneous thrombosis [8].

We investigated the association of *MTHFR -677*, and *-1289* polymorphisms with occlusive artery disease (OAD) and deep vein thrombosis (DVT) in Macedonians and no association was found, except for the protective association between *MTHFR/CA* : *CC* diplotype and OAD [9]. 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 [10]. There are no data on the Macedonian population about the *SERPINE1* polymorphism and its possible associations with different diseases.

The aim of this study was to analyse the association of *SERPINE1* polymorphism with OAD or DVT in Macedonians in order to investigate its role as one of the candidate genes in different vascular diseases in this population.

## Methods

### Investigated groups

The study group consisted of 223 subjects composed of three different groups: healthy individuals, patients with OAD, and patients with DVT.

a) Healthy individuals (n = 82, 40 females and 42 males, aged 40.7 ± 11.3 years), born in different parts of Macedonia, attending the Institute for Transfusion for blood donation, were included. They had no history of any chronic disease, normal medical documentation and physical examination. Inclusion of healthy individuals was random, if a medical doctor declared their health as acceptable (on the basis of medical documentation, completed interview, and physical examination). Subjects with a family history of blood vessel diseases were excluded.
b) Occlusive artery disease [n = 75, 29 female and 46 male patients with documented myocardial infarction (n = 52), brain infarction (n = 22), and peripheral artery thrombosis (n = 2), aged 63.3 ± 9.6 years], hospitalised at the Institute of Heart Diseases, Faculty of Medicine, and the Institute for Transfusion, Skopje for outpatient treatment, were included.
c) Deep vein thrombosis [n = 66, 45 female and 24 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 were 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 form to participate in the study, which was approved by the Committee of the Ministry of Education and Science from the Republic of Macedonia (No. 13-1672/4-02).

### Genomic DNA isolation and storage

Blood samples were collected and DNA was isolated from peripheral blood leukocytes by the phenol-chloroform extraction method or with BioRobot EZ1 workstation (QIAGEN) [11]. The quality and quantity of DNA were analysed by GeneQuant (Pharmacia). Isolated DNA samples were stored in the Macedonian Human DNA Bank (hDNAMKD) [12].

### Typing methods

Assay for the identification of *SERPINE1* polymorphism is based on polymerase chain reaction (PCR) and reverse-hybridisation with CVD StripAssay (ViennaLab Labordiagnostica GmbH, Austria). The procedure includes three steps: 1) DNA isolation, 2) PCR amplification using biotinylated primers, 3) hybridisation of amplification products to a test strip containing allele-specific oligonucleotide probes immobilised as an array of parallel lines. Bound biotinylated sequences are detected using streptavidin-alkaline phosphatase and colour substrates [13]. The assay covers 2 alleles: *SERPINE1/4G*, and *SERPINE1/5G*. 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 analysis

The population genetics analysis package PyPop, developed by the Biostatistics Core for the Workshop [14-16], was used for analysis of the *SERPINE1* data for this report. Allele frequencies and expected Hardy Weinberg proportions (HWP) for each *SERPINE1* allele were determined [17]. The exact test for genotype frequency deviation from HWP was calculated using the Arlequin

implementation accessed via PyPop [18]. Those alleles that did not fit HWP were evaluated to determine whether there was an excess of homozygotes or heterozygotes, or if any specific genotypes were significantly different from expected frequencies by the chi square test. The Ewens-Watterson homozygosity test of neutrality (EWN) [19] with Slatkin's exact p-values (SEPV) [20, 21] 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 analysis of associations between *SERPINE1* alleles and blood vessel disease with GraphPad QuickCalcs free statistical calculators (http://www.graphpad.com/quickcalcs) with Bonferroni corrected p value [22]. A p value < 0.05 was considered significant.

## Results

### *SERPINE1* alleles and genotypes

Frequencies of *SERPINE1* 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 in Table I.

The frequency of *4G* alleles for *SERPINE1* varied between 0.538 for DVT, 0.555 for healthy participants, and 0.607 for OAD, indicating common 'wild type' allele. The frequency of *5G* allele was the lowest in patients with OAD (0.393), and was higher in healthy participants (0.445), and DVT (0.462). For all the *SERPINE1* alleles, the test of neutrality showed a negative value for the $F_{nd}$ statistic, with significant p of F statistics in healthy participants (p = 0.041) and in patients with DVT (p = 0.030) (Table I).

The observed versus expected frequency of *SERPINE1* genotypes, Hardy Weinberg proportion (HWP), and Guo and Thompson Hardy Weinberg Output (GTHWO) in Macedonians is given in Table II.

The most frequent *SERPINE1* genotype in healthy participants was *4G5G* with observed frequency of 62.2%, lower frequency was found for *4G4G* genotype (24.4%), and the lowest frequency was found for *5G5G* genotype with 13.4%. The frequencies of *SERPINE1*/*4G4G*, and /*4G5G* genotypes were slightly increased in patients with OAD (28.0% and 65.3%, respectively), but *SERPINE1*/*5G5G* was decreased (6.7%). In contrast, *SERPINE1*/*4G5G* and /*5G5G* genotypes in patients with DVT were lower (50.0% and 21.3%, respectively) with higher *SERPINE1*/*4G4G* (28.8%) genotype. All genotypes in healthy participants and in patients with OAD showed significant deviation from the HWP (p = 0.019 and p = 0.001, respectively) and only

**Table I.** Frequencies of *SERPINE1* 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

| | | Alleles | | | Test of neutrality (F) | |
|---|---|---|---|---|---|---|
| | n | allele | number | frequency | EWN Fnd | SEPV p of F |
| Healthy | 82 | *4G* | 91 | 0.555 | –1.907 | 0.041 |
| | | *5G* | 73 | 0.445 | | |
| Occlusive artery disease | 75 | *4G* | 91 | 0.607 | –1.792 | 0.080 |
| | | *5G* | 59 | 0.393 | | |
| Deep venous thrombosis | 66 | *4G* | 71 | 0.538 | –1.885 | 0.030 |
| | | *5G* | 61 | 0.462 | | |

*Abbreviations: n – number of participants, EWN – Ewens-Watterson test of neutrality, SEPV – Slatkin's Exact p value*

**Table II.** Observed vs. expected *SERPINE1* 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 |
|---|---|---|---|---|---|---|
| Healthy | *4G4G* | 20 (24.4%) | 25.2 | 0.296 | 0.019 | 0.026 |
| | *4G5G* | 51 (62.2%) | 40.5 | 0.099 | | |
| | *5G5G* | 11 (13.4%) | 16.2 | 0.193 | | |
| Occlusive artery disease | *4G4G* | 21 (28.0%) | 27.6 | 0.209 | 0.001* | 0.002* |
| | *4G5G* | 49 (65.3%) | 35.8 | 0.027 | | |
| | *5G5G* | 5 (6.7%) | 11.6 | 0.053 | | |
| Deep venous thrombosis | *4G4G* | 19 (28.8%) | 19.1 | 0.982 | 0.963 | 1.000 |
| | *4G5G* | 33 (50.0%) | 32.8 | 0.974 | | |
| | *5G5G* | 14 (21.3%) | 14.1 | 0.980 | | |

*Abbreviations: HWP – Hardy Weinberg proportions, GTHWO – Guo and Thompson Hardy Weinberg Output, cannot be calculated because expected ≤ 5, $\chi^2$ test; * statistically significant after Bonferroni adjustment (p value x number of genotypes) < 0.05*

genotypes from the patients with DVT showed a good fit with HWP expectations (p = 0.963). After Bonferroni adjustment, only genotypes in OAD showed significant deviation from the HWP (Table II).

### Association between *SERPINE1* polymorphism and blood vessel diseases

The association between *SERPINE1* alleles and genotypes with OAD is shown in Table III. We did not find any significant association between *SERPINE1* alleles or genotypes and OAD (Pearson's p value greater than 0.05).

The association between *SERPINE1* alleles and genotypes with DVT is shown in Table IV. We did not find any significant association between *SERPINE1* alleles or genotypes and DVT (Pearson's p value greater than 0.05).

## Discussion

In this manuscript we report *SERPINE1* polymorphisms that exist in Macedonians, and a possible association with OAD, as well as with DVT. The results did not show any significant association of *SERPINE1* polymorphisms with OAD or DVT.

We found a negative value for the $F_{nd}$ statistic, with significant p of F statistics in healthy participants and in patients with DVT (but not in patients with OAD), which indicates balancing selection operating on the alleles at that cluster. We also found that *SERPINE1* genotypes are not in equilibrium with HWP in healthy participants and in patients with OAD, contrary to the genotypes in patients with DVT, which are in equilibrium with HWP.

After Bonferroni correction of p-values, only genotypes in OAD showed significant deviation from the HWP. Several types of multiple testing corrections are used: i) Bonferroni; ii) Bonferroni Step-down (Holm); iii) Westfall and Young Permutation; and iv) Benjamini and Hochberg False Discovery Rate [22, 23]. The methods are listed in order of their stringency, with the Bonferroni being the most stringent, and the Benjamini and Hochberg FDR being the least stringent. The more stringent a multiple testing correction, the fewer the false positive genes are allowed. The trade-off of stringent multiple testing corrections is that the rate of false negatives is very high.

Among 14 916 men 40 to 84 years old participating in the Physicians' Health Study who provided baseline blood samples for DNA analysis, 374 suffered first myocardial infarction and 121 had venous thromboembolism during 8.6 years of follow-up. Obtained data indicate that the *4G5G* polymorphism in the promoter of the *SERPINE1* gene is not a major pathogenetic risk factor for arterial or venous thrombosis among middle-aged men [24], which is similar to our results.

However, in patients with acute myocardial infarction compared to patients with stable coronary artery disease (SCAD), *SERPINE1 4G4G* genotype was found to be an independent predictor for development of myocardial infarction. *SERPINE1 4G4G* genotype had a protective effect against development of high grade stable coronary stenoses [25]. In another study it was concluded that there is a close relationship of the *SERPINE1 4G5G* polymorphism

**Table III.** Association between *SERPINE1* alleles and genotypes with occlusive artery disease with Pearson's p value, crude odds ratio, and Wald's 95% CI in Macedonians

| Allele or genotype | OAD (n = 75) | Healthy (n = 82) | Pearson's p value | Odds ratio | Wald's 95% CI |
|---|---|---|---|---|---|
| *4G* | 91 (60.7%) | 91 (55.5%) | 0.353 | 1.237 | 0.789-1.940 |
| *5G* | 59 (39.3%) | 73 (44.5%) | 0.353 | 0.185 | 0.515-1.267 |
| *4G4G* | 21 (28.0%) | 20 (24.4%) | 0.607 | 1.206 | 0.591-2.459 |
| *4G5G* | 49 (65.3%) | 51 (62.2%) | 0.683 | 1.146 | 0.597-2.199 |
| *5G5G* | 5 (6.7%) | 11 (13.4%) | 0.163 | 0.461 | 0.152-1.395 |

*Abbreviations: OAD – occlusive artery disease, CI – confidence interval*

**Table IV.** Association between *SERPINE1* alleles and genotypes with deep venous thrombosis with Pearson's p value, crude odds ratio, and Wald's 95% CI in Macedonians

| Allele or genotype | DVT (n = 66) | Healthy (n = 82) | Pearson's p value | Odds ratio | Wald's 95% CI |
|---|---|---|---|---|---|
| *4G* | 71 (53.8%) | 91 (55.5%) | 0.770 | 0.934 | 0.589-1.480 |
| *5G* | 61 (46.2%) | 73 (44.5%) | 0.770 | 1.071 | 0.676-1.697 |
| *4G4G* | 19 (28.8%) | 20 (24.4%) | 0.546 | 1.253 | 0.602-2.609 |
| *4G5G* | 33 (50.0%) | 51 (62.2%) | 0.137 | 0.608 | 0.315-1.173 |
| *5G5G* | 14 (21.3%) | 11 (13.4%) | 0.208 | 1.737 | 0.730-4.135 |

*Abbreviations: DVT – deep venous thrombosis, CI – confidence interval*

with its plasma level in DVT in the Chinese Han ethnic group, although the lack of association between this genetic variation and risk of DVT suggests no major cause--effect pathogenic role of this polymorphism by itself [26]. Another study also identified a significant interaction between the *SERPINE1* gene and smoking status, indicating that *SERPINE1 4G* allele increased the risk of coronay heart disease among non-smokers in Chinese [27]. Eleven SNPs capturing the common genetic variation of the *SERPINE1* gene were genotyped in the HIFMECH study [28]. In the 510 male cases and their 543 age-matched controls, a significant gene-smoking interaction was observed. *SERPINE1* haplotypes are mildly associated with plasma levels of PAI-1 and with the risk of MI in non-smokers [29]. Some results indicate that the 4G/4G *PAI-1* genotype might be strongly associated with high PAI-1 levels regardless of metabolic syndrome-related variables and smoking status [30].

Several meta-analysis studies have been published on the *SERPINE1* gene associations with cardiovascular diseases. Results from meta-analyses are published which included 6359 cases and 13 805 controls derived from 55 case-control studies and included 12 genes (13 polymorphisms). Significant associations with haemorrhagic stroke were identified for those homozygous for the *5G* allele in the *SERPINE1 4G5G* polymorphism [29]. Seventy-six studies were included in the meta-analyses, which were all performed in mainland China and referred to 6 candidate genes and 7 polymorphisms. Among the gene polymorphisms tested in that study, an association of gene polymorphisms with increasing risk of ischaemic stroke was confirmed for 6 polymorphisms, including *SERPINE1 4G5G* [31]. In a case-control study of 190 hospital cases of first-ever ischaemic stroke and 185 community-based controls the findings did not indicate any association between the *SERPINE1* or *t-PA* polymorphisms and risk of stroke. Adding the *SERPINE1* results to previous studies in a meta-analysis indicated a strong association between this polymorphism and ischaemic stroke ($p = 0.0002$) with the *SERPINE1 4G5G* locus [32]. Meta-analyses on seven haemostatic genetic variants, for which the available evidence on each comprises at least 5000 coronary disease cases and at least 5000 controls, were performed. Combined analyses of studies of the *SERPINE1 4G* variant yielded a per-allele relative risk for coronary disease. However, there was an indication of publication bias in these studies [33].

The platelet *SERPINE1* mRNA levels correlated significantly with the PAI-1 antigen content, but there was no association between the polymorphism and mRNA levels, or protein levels in platelets. Also, plasma levels of PAI-1 antigen were not associated with homozygosity of the *4G5G* polymorphism, but as expected BMI and triglycerides emerged as significant predictors of plasma PAI-1 levels. The importance of the *4G5G* polymorphism on PAI-1 levels is controversial and although levels of platelet mRNA are related to its content of PAI-1 protein, there is no association between the *4G5G* promoter polymorphism and platelet *SERPINE1* mRNA or protein expression [34].

Diseases of the cardiovascular system are complex genetic traits which include hundreds of associated candidate genes [35]. Our results with only one gene (*SERPINE1*) can be only a part of the complex investigation of candidate genes for cardiovascular diseases in Macedonians. The numbers of patients and controls of our study was 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 the 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 DVT and pulmonary embolism, subjects with isolated DVT more often had thrombi located distally and had a similar number of affected veins. Compared with isolated pulmonary embolism patients, isolated DVT 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 non--carriers, and some were differential for pulmonary embolism and DVT patients, none of the potential mechanisms offered a clear explanation [36]. Because our study groups were heterogeneous, the results should be analysed with caution, and we need more homogeneous subgroups for a definitive conclusion about an association between the *SERPINE1* gene and cardiovascular diseases.

In summary, an association of *SERPINE1* polymorphisms with OAD or DVT 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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# Występowanie polimorfizmu genu *SERPINE1* u osób z miażdżycą zarostową tętnic kończyn dolnych lub zakrzepicą żył głębokich w populacji macedońskiej

**Igor Spiroski[1], Sashko Kedev[1], Slobodan Antov[1], Dejan Trajkov[2], Aleksandar Petlichkovski[2], Sloboda Dzhekova-Stojkova[3], Stojanka Kostovska[4], Mirko Spiroski[2]**

[1] Klinika Kardiologii, Uniwersytet Świętych Cyryla i Metodego, Skopie, Macedonia

[2] Instytut Immunobiologii i Genetyki, Uniwersytet Świętych Cyryla i Metodego, Skopie, Macedonia

[3] Instytut Biochemii Medycznej i Eksperymentalnej, Uniwersytet Świętych Cyryla i Metodego, Skopie, Macedonia

[4] Instytut Transfuzji, Skopie, Macedonia

## Streszczenie

**Wstęp:** Zwiększone stężenie *SERPINE1* jest związane z polimorfizmem delecji/wstawienia 1-bp guaniny (*4G5G*) do promotora genu *SERPINE1* (ang. *plasminogen activator inhibitor 1*, PAI1). Wykazano, że stężenie osoczowe *SERPINE1* ma wpływ na ryzyko wystąpienia choroby wieńcowej. Ponadto wiadomo, że polimorfizm *4G5G* wpływa na ekspresję genu *SERPINE1*.

**Cel:** Ocena związku pomiędzy występowaniem polimorfizmu genu *SERPINE1* a miażdżycą zarostową tętnic kończyn dolnych (OAD) lub zakrzepicą żył głębokich (DVT) u mieszkańców Macedonii.

**Metody:** Badaniami objęto grupę 82 zdrowych osób, 75 chorych z OAD oraz 66 chorych z DVT. Po uzyskaniu zgody pobierano krew i izolowano DNA z leukocytów krwi obwodowej. Polimorfizm genu *SERPINE1* badano za pomocą CVD StripAssay (ViennaLab, Labordiagnostica GmbH, Austria). Do analizy statystycznej polimorfizmu SERPINE1 użyto populacyjnego programu PyPop.

**Wyniki:** Częstość występowania allela *4G* dla *SERPINE1* wynosiła 0,538 w grupie DVT, 0,555 w grupie osób zdrowych oraz 0,607 w grupie OAD. Częstość występowania allela *5G* dla *SERPINE1* była najniższa w grupie OAD (0,393), pośrednia u osób zdrowych (0,445), a najwyższa w grupie DVT (0,462). Test neutralności (Fnd) miał wartości ujemne, ale był istotnie różny od 0 dla genu *SERPINE1* u osób zdrowych (p dla testu F = 0,041) i chorych z DVT (p dla testu F = 0,030). U osób zdrowych i chorych z OAD genotypy *SERPINE1* nie układały się w proporcji Hardy'ego-Weinberga (odpowiednio, p = 0,019 i 0,001).

**Wnioski:** Nie znaleziono związku pomiędzy polimorfizmem genu *SERPINE1* a OAD lub DVT u mieszkańców Macedonii.

**Słowa kluczowe:** polimorfizm genu *SERPINE1*, polimorfizm genu *PAI1*, miażdżyca zarostowa tętnic kończyn dolnych, głęboka zakrzepica żylna, Macedonia

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**Adres do korespondencji:**

dr n. med. Igor Spiroski, Clinic of Cardiology, Faculty of Medicine, Ss. Cyril and Methodius University, Blvd. Krste Petkov Misirkov bb, 1000 Skopje, Republic of Macedonia, tel.: +38 92 329 32 93, e-mail: ispiroski@gmail.com

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