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POVEZANOST POLIMORFIZAMA ZA FII PROTROMBIN, FV LEIDEN I MTHFR GEN SA VENSKIM TROMBOEMBOLIZMOM KOD CRNOGORSKIH PACIJENATA

Authors Sladjana Teofilov*, Zvonko Magić†, Tatjana Ostojic*, Milena Bulatović*, Olivera Miljanović*, Vojnosanitetski pregled (2019); Online First September, 2019.

UDC:

DOI: https://doi.org/10.2298/VSP190402086T

When the final article is assigned to volumes/issues of the Journal, the Article in Press version will be removed and the final version appear in the associated published volumes/issues of the Journal. The date the article was made available online first will be carried over.
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Gene polymorphisms and thrombosis

Sladjana Teofilov*, Zvonko Magic†§, Tatjana Ostojić*, Milena Bulatović*, Olivera
Miljanović*

* Center for Medical Genetic and Immunology, Clinical Center of Montenegro, Podgorica, Montenegro
† Institute for Medical Research, Military Medical Academy, Belgrade, Serbia;
§ Medical faculty, University of Defence, Belgrade, Serbia

Correspondence to:
Sladjana Teofilov, Ul. Zmaj Jovina, 54/57, Podgorica, Crna Gora
Phone: +382 69398870
Email: steofilova@yahoo.com
Abstract

**Background / Aim.** Polymorphisms of the factor V Leiden (FV G1691A), factor II Prothrombin (FII G20210A) and methylenetetrahydrofolate reductase (MTHFR C677T) genes are the most commonly investigated inherited risk factors for developing venous thromboembolism (VTE). Despite this fact, there is insufficient data regarding their clinical burden and distribution in Montenegrin population. Consequently, the present study aimed to determine the frequency of these polymorphisms in Montenegrin patients with VTE. **Methods.** This case-control study was conducted on 160 Caucasian subjects. The study group was composed of 80 patients (35 men and 45 women) with VTE. The control group consisted of 80 healthy individuals (32 men and 48 women), without previous thromboembolic episode. Genotyping of the FV G1691A, FII G20210A, and MTHFR C677T polymorphisms was performed by allele-specific polymerase chain reaction (PCR). **Results.** Our results demonstrated that the frequency of heterozygotes (HET) for FII G20210A and FV G1691A were significantly higher in VTE group compared to a healthy control group ($\chi^2 = 11.7; p = 0.001$ and $\chi^2 = 17.69; p < 0.001$ respectively). This study confirmed the association of FII G20210A and FV G1691A polymorphisms with an increased risk of VTE (OR 10.5; 95% CI = 2.34 to 47.27 and OR 14.8; 95% CI = 3.34 to 65.43; p < 0.001 respectively). Recessive homozygotes (RH) for FII G20210A and FV G1691A were not found in any of investigated group. As regarding MTHFR C677T, the difference between the frequency of HET and RH in the control and VTE group is not significant. **Conclusion.** Our study has shown that FII G20210A and FV G1691A polymorphisms are significantly associated with the VTE. Detection of above mentioned polymorphisms prior to VTE development can contribute to the prevention of further VTE occurrence, especially among patients' relatives who are carriers of these polymorphisms.

**Key words:** venous thromboembolism, gene polymorphisms, FII G20210A, FV G1691A, MTHFR C677T.
Apstrakt


Rezultati. Rezultati ove studije su pokazali da je učestalost heterozigota (HET) za FII G20210A i FV G1691A značajno veća u VTE grupi u poređenju sa kontrolnom grupom ($\chi^2 = 11.7; p = 0.001$ i $\chi^2 = 17.69; p < 0.001$). Ova studija je potvrdila povezanost polimorfizama za FII G20210A i FV G1691A sa povećanim rizikom od VTE (OR 10.5; 95% CI = 2.34 do 47.27; p = 0.001 i OR 14.8; 95% CI= 3.34 do 65.43; p < 0.001). Recesivni homozigoti (RH) za FII G20210A i FV G1691A nisu pronađeni ni u jednoj od ispitivanih grupa. Za polimorfizam MTHFR C677T, nije pronađena značajna razlika u učestalosti HET i RH između VTE grupe i kontrolne grupe. 

Zaključak. Naša studija je pokazala da su polimorfizmi za FII G20210A i FV G1691A značajno povezani sa VTE i njihovo pravovremeno otkrivanje može doprineti prevenciji VTE, posebno kod srodnika pacijenata koji su nosiovi ovih polimorfizama.

Ključne reči: venski tromboembolizam, polimorfizmi gena, FII G20210A, FV G1691A, MTHFR C677T.
Introduction

Venous thromboembolism (VTE) is a multifactorial disease that results from the interaction between acquired and genetic risk factors with an incidence of 1-2 per 1,000 persons annually. Most common clinical manifestation of VTE are deep venous thrombosis (DVT) and pulmonary thromboembolism (PTE). Under the age of 25 thrombosis is rarely diagnosed, but after the age of 40 shows an increasing tendency and it is often repeated and additionally complicated by pulmonary embolism.\(^1\)\(^-\)\(^3\)

Thrombophilia represents a group of inherited and acquired coagulation abnormalities associated with thrombosis. Although, thrombophilia itself is not a disease, it increases the risk of developing VTE in response to the provocation/perturbation by environmental factors.\(^4\)\(^,\)\(^5\) Numerous acquired and hereditary risk factors are known to be responsible for the occurrence of VTE.\(^5\)\(^,\)\(^6\) Genes encode proteins of the hemostasis system, affecting their synthesis and activity, and the acquired factors stimulate the tendency of hypercoagulability. Many studies suggested a multifactorial etiology of VTE. Therefore, the hereditary predisposition only represents an increased risk and does not determine if the disease will necessarily manifest in genetically affected population.\(^7\)\(^-\)\(^11\)

The most common single nucleotide polymorphisms (SNPs) tested in the genes associated with VTE are genes for factor II Prothrombin (\(FII\) G20210A), factor V Leiden (\(FV\) G1691A), and methylenetetrahydrofolate reductase (\(MTHFR\) C677T).\(^11\) Many previous studies have shown that the frequency of polymorphisms (recessive homozygotes-RH and heterozygotes-HET) of these genes was significantly higher in patients with VTE compared to the healthy population.\(^11\)\(^-\)\(^14\) Nevertheless, there are studies with controversial results, especially about the role of \(MTHFR\) gene polymorphisms in VTE.\(^15\)\(^-\)\(^16\) Besides, that the prevalence of genetic polymorphisms in the healthy population can vary in regards to different geographical regions, and differ among ethnic groups.\(^17\)\(^,\)\(^18\)

According to the literature, \(FV\) G1691A polymorphisms are the most important genetic risk factor for the manifestation of VTE. The gene for \(FV\) consists of 25 exons and is located on chromosome 1q23. \(FV\) G16961A mutation leads to the replacement of arginine for glutamine at position 506 in the protein resulting in reduced sensitivity of \(FV\) to the inhibitory effect of activated protein C (APC) and the balance of the hemostatic system moves to a state of hypercoagulability.\(^19\)
Coagulation factor V (FV) is an important protein (cofactor), with a double role in the maintenance of hemostatic balance due to the same influence in both procoagulation and the anticoagulation mechanism of blood clotting \(^1\). The relative risk of thrombosis in HET FV G16961A carriers is 3 to 7 times, and for the RH of FV A16961A carriers 50 to 80 times higher compared to non-carriers of these polymorphisms \(^2\). Prevalence of FV G16961A increases from west to east Europe and from north to south Europe. In a healthy European population HET for FV G16961A is present with a frequency of 5-7\%, and 15-50\% in VTE patients \(^18, 21\). This mutation is rarely found in the populations of Africa, Australia and South Asia \(^22, 23\). A large epidemiologic study conducted in the USA presented a 5.27\% incidence of HET for FVG1691A in European individuals, 2.21\% in Latinos, 1.23\% in Afro-Americans, 1.25\% in American Indians, and only 0.45\% in Asians \(^22\).

Another important genetic risk factor for VTE is the FII G20210A (Prothrombin). Prothrombin is the precursor of thrombin and has an important role in the formation of fibrin in the coagulation process. Substitution of guanine (G) for adenine (A) in FII gene at position 20210 is associated with an increase in prothrombin plasma concentrations. Gene for FII is located on chromosome 11p11, in a 3’- untranslated region \(^24\). The prevalence of polymorphism of FII G20210A in the European population is 2-4\% \(^25\). The prevalence of FII G20210A in Northern Europe is 1.7\%, and in the Mediterranean region is twice as high. In patients with VTE, HET for FII G20210A is present in 6-18\% \(^25\). This polymorphism was found to be very rare or even absent in African and Asian populations, as well as in American Indians and Australian Aborigines \(^25\). RH variant for FII G20210A is very rare. The risk becomes 50-fold higher among individuals with two copies of the 20210A allele \(^26\).

MTHFR is the key enzyme in the regulation of the metabolism of folate and homocysteine levels, that catalyzes the reaction of 5, 10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate, which functions as a methyl donor in the conversion of homocysteine to methionine. Increased level of homocysteine in the blood has a toxic effect on the vascular structure \(^27\). RH and HET of MTHFR C677T result in the reduction of synthesis of 5-methylenetetrahydrofolate, leading to an increased concentration of homocysteine in the plasma which increases the risk of arterial and venous thrombosis \(^27\). The gene for MTHFR is located on chromosome 1 at position 1p36.3 \(^28\). The prevalence
HET of MTHFR C677T gene in a European healthy population is high (30-50%) and it is not associated with an increased risk of thromboembolism. The prevalence of MTHFR C677T in Northern Europe is significantly lower than in southern Europe, so the MTHFR C677T polymorphism in Norway accounts for about 28%, in Italy about 44%. Prevalence of the RH in the healthy European population is 5-15% \(^{29,30}\).

The SNPs in FV G16961A, FII G20210A, and MTHFR C677T genes have been broadly investigated worldwide. Although the majority of studies confirmed the importance of these polymorphisms, as the most common inherited risk factors for the development of VTE, there are still some inconsistencies, especially regarding the different ethnic populations \(^{17,18,23}\). To our knowledge, there is no published data on these polymorphisms in patients with VTE and general population in Montenegro, although the similar investigations polymorphisms have been conducted on the population of pregnant women with adverse pregnancy outcomes and pregnant women with successful procreation \(^{31,32}\).

The aim of this case-control study was to determine the frequency of FV G16961A, FII G20210A and MTHFR C677T gene polymorphisms in both patients with VTE and healthy subjects in Montenegro, and to assess their association with VTE development. This is the first investigation of SNPs in genes for thrombophilia susceptibility (FII, FV, and MTHFR) in Montenegrin patients with VTE, and we believe that it could serve as a knowledgeable data for comparison with different populations from the region or broader.

**Methods**

This case-control study consists of 160 Caucasian subjects. The study group is composed of 80 patients (35 men and 45 women) after experiencing at least one clinically confirmed episode of VTE (DVT and/or PTE). The evidence of VTE had been documented in their medical records with appropriate diagnostic methods and specialists’ expertise.

The main criterion for inclusion of patients in the study group was one or more recurrent episodes of VTE without any of well-known comorbidity (cancer, diabetes mellitus). The control group (CG) consisted of 80 healthy persons (32 men and 48 women) who have not experienced the thromboembolic episode by the time they accepted the participation in the study. For CG we randomly selected 80 voluntary blood donors (32 men and 48 women).
with no history of the thromboembolic episode, who were as similar as possible to the VTE group regarding age and gender.

The ethics committee of Clinical Center of Montenegro approved this case-control, retrospective-study (Number: 03/01-5005/1). All participants provided their informed consent to take part in the research and study has been performed in accordance with the Declaration of Helsinki. The research was conducted at the Center for Medical Genetics and Immunology, Clinical Center of Montenegro, in the period from January 2015 to July 2017.

*Gene analysis*

Deoxyribonucleic acid (DNA) was isolated from peripheral blood collected in 4.5 ml tubes with Na-citrate (9N Coagulation sodium citrate 3.2%), and extracted with a commercial test QIA amp DNA Blood Mini Kit (Qiagen, Germany). Extracted DNA was dissolved in 200 µl buffer AE and stored at -20°C. SNPs for *FV* G1691A, *FII* G20210A, and *MTHFR* C677T were detected by allele-specific polymerase chain reaction (PCR). DNA amplification was performed by Attomol Quick type, factor II 20210 G>A, factor V G1691A and *MTHFR* 677C>T. HotStarTaq DNA polymerase provided by Qiagen. Heterozygous control template DNA for *FII* G20210A, *FV* G1691A, and *MTHFR* C677T were used as positive PCR controls. PCR was performed in a thermocycler (Mastercycler gradient Eppendorf) using the temperature regime: initial activation (15 min 95°C), 5 cycles (1 min 94°C; 1 min 63°C; 1 min 72°C), 30 cycles (30 s 94°C; 30 s 63°C, 30 s 72°C), final elongation synthesis (2 min 72°C). Amplified DNA samples were analyzed after electrophoresis (2.5% agarose gel, stained with ethidium bromide) and visualised on Ultra Violet (UV) transilluminator.

*Statistical analyses*

Statistical analyses were performed using the IBM SPSS software program (version 21.0). Descriptive statistics were used for the demographic characteristics. The significance of the differences in the distribution of HET and RH polymorphisms between the VTE group and
the controls group was investigated by chi-square test (χ2). Moreover, the odds ratio (OR) with their corresponding 95% CI (Confidence Interval) was used to represent the association between SNPs and VTE risk. The P values less than 0.05 were considered as statistically significant.

**Results**

Baseline demographic and clinical characteristics of participants are presented in Table 1. The VTE group includes 51 (63.75%) patients with DVT, 17 (21.25%) patients with a PTE, and 12 (15%) of patients with both DVT and PTE. In VTE group, the youngest patient who had DVT was 10 years old. Majority of patients from VTE group had the first episode of DVT and/or PTE before the age of 50 (69%).

The results of the allele and genotype frequencies for SNPs in *FV* G1691A, *FII* G20210A, and *MTHFR* C677T within the examined groups are presented in Table 2. The results obtained by comparing the VTE group with a CG showed that the frequency of HET for *FII* G20210A and *FV* G1691A were higher in VTE group (χ2 = 16.26; p = 0.001 and χ2 = 17.69; p< 0.001. RH for *FII* G20210A and *FV* G1691A were not found in any investigated group. Comparing to the control group the incidence of RH alleles A for *FII* G20210A and *FV* G1691A is higher in the VTE group. Risk estimate analyses showed that the risk for VTE is significantly higher in presence of *FII* G20210A (OR=10.5; 95% CI 2.34-47.27; p = 0.001) and *FV* G1691A (OR=14.8; 95% CI 3.34-65.43; p < 0.001. As regarding *MTHFR* C677T, a difference in the frequency of HET, RH and wild type (WT) between VTE and the control group is not significant (p=0.603). Recessive allele T for the *MTHFR* gene was approximately equally distributed in both examined groups.

Statistically significant difference was not found when comparing the distribution of examined genotypes between genders within each group (Results are not presented in the tables).

The distribution of individual and multiple polymorphisms of the investigated genes within the study groups demonstrated that only 18.75% of patients in the VTE group do not have any HET and RH for investigated polymorphisms as opposed to 36.25% in the control group (Table 3). Furthermore, the presence of two or more investigated polymorphisms were detected in a larger percentage of subjects in the VTE group comparing with CG (*FV*
G1691A with MTHFR C677T: 16.25% and 1.25% respectively; FII G20210A with MTHFR C677T: 8.75% and 2.5%, respectively).

Discussion

The occurrence of the thromboembolic disease is in the line with the simultaneous presence of a gene polymorphisms and environmental risk factors which can partly explain the inconsistent results of similar studies conducted in different geographic regions\textsuperscript{10,11,34}. The results of our study show that the presence of polymorphisms FII G20210A and FV G1691A is significantly higher in the VTE group compared to a healthy control group. Recessive homozygotes for these two coagulation factors are absent both in VTE group and in the controls group. A recent meta-analysis performed on a large sample, including 11000 cases and 21000 controls, shows a significant association of the HET for FV G1691A and FII G20210A with VTE\textsuperscript{11} and it is in concordance to our study. Literature data have shown that the combined effect of more than one genetic polymorphism for thrombophilia susceptibility can double or triple the risk for VTE\textsuperscript{9,33,34}. The combination of the most significant genetic risk factor, FV G1691A and FII G20210A, with HET and RH in the MTHFR gene, has been frequently found in patients with VTE\textsuperscript{11,33}. We had also found a higher presence of two or more polymorphisms in the VTE group compared to a controls group. In two patients, polymorphisms for all three examined genes were observed simultaneously.

In our study, the frequency of FV G1691A, in a healthy group of subjects is 2.5%, which is in correlation with the published data on the white European population. We found that the overall frequencies of FV G1691A polymorphisms are significantly higher in patients with VTE increasing the risk for venous thromboembolism in FV G1691A carriers for almost 15 times (OR=14.8; 95% CI 3.34-65.43; p < 0.001). This finding is consistent with the reported prevalence of FV G1691A for other countries in the region (Italy 15.3%, Croatia 16%, Macedonia 21.1%, Serbia 29.3%, Bulgaria 25%, Bosnia and Herzegovina 18% and Greece 31.9%)\textsuperscript{11,22}. Our study shows that allele A is present in 13.8% of subjects VTE versus 1.2% in healthy subjects.

Polymorphism FII G20210A leads to an increased prothrombin production, which may result with 30% to 70% higher levels of prothrombin in HET and RH comparing to the
production in the absence of this condition (25). In our study, 17 out of 80 patients (21.3%) with VTE were heterozygotes for the \textit{FII} G20210A polymorphism. In the control group, the \textit{FII} G20210A presented in 2.5% participants. Risk for development of VTE in \textit{FII} G20210A carriers was 10.5 times higher (OR=10.5; 95% CI 2.34-47.27; p = 0.001) than in wild-type carriers. The results of our research are in correlation with literature data, in which it is reported that the HET of FII 20210 is present in 6-18% of patients with VTE and 2-4% in the healthy European population\(^{24}\). Our results similarly correlate with the result of Serbia (11.6% patients VTE)\(^{13}\). Other studies from our region report different results in which heterozygotes for \textit{FII} 20210 were presented in patients with VTE with lower frequency (Croatia 4% and Bosnia and Herzegovina 2.7%)\(^{30,34}\).

The role of \textit{MTHFR} polymorphisms in the development of VTE is controversial: some authors have shown an association between the \textit{MTHFR} C677T polymorphism with VTE\(^{32,34}\), while others have proven the contrary\(^{11,36,37}\). Our results showed that HET and RH for \textit{MTHFR} C677T are present in a large and approximately equal percentage in both examined groups (53.8% and 18.8% vs 53.8% and 15% respectively). We have also reported previously that the frequency of HET and RH for \textit{MTHFR} C677T is similar in the population of healthy pregnant women and women with adverse pregnancy outcomes\(^{31,32}\). These results confirm previously published data of similar studies\(^{5,11,13,16}\). We did not observe the difference in the frequencies of \textit{FV} G1691A, \textit{FII} G20210A, and \textit{MTHFR} C677T polymorphisms in investigated genes between genders in the patients’ group or in the control group. Determining the distribution of these polymorphisms according to the gender is especially important in women because they undergo hemostatic changes during the pregnancy\(^{32}\).

It is estimated that 40-50% of the thrombosis results from thrombophilia, and therefore the genetic testing this condition is significant in the prevention and treatment of thrombosis, with an important role in determining the duration of secondary anticoagulant prophylaxis, especially in case of increased risk of thromboses such as surgery, pregnancy, immobilization, and trauma. Knowledge about the presence of these risk factors can support prevention of these diseases, especially among patients' relatives who are carriers of these polymorphisms.

Our study showed the significantly higher presence of \textit{FV} G16961A and \textit{FII} G20210A polymorphisms in patients with VTE, compared with healthy controls with no history of
VTE. In contrast, we did not detect any significant associations between the homozygous \textit{MTHFR} 677TT and heterozygous \textit{MTHFR} C677T genotype and VTE. This study included a small sample of patients and thus therefore the conclusions should be confirmed by future research in the field.

The multifactorial etiology of VTE implies a necessity of further research of more genes related to thrombophilia and their interplay with environmental risk factors, which should lead to a more comprehensive and reliable genetic counseling and prevention of VTE.

\textbf{Declaration of interest}

The authors declare that they have no competing interests.

\textbf{REFERENCES}


Table 1

Demographic characteristics of the VTE group and Control group

<table>
<thead>
<tr>
<th></th>
<th>VTE group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DVT</td>
<td>PTE</td>
</tr>
<tr>
<td>n patients (%)</td>
<td>51 (63.75%)</td>
<td>17 (21.25%)</td>
</tr>
<tr>
<td><strong>sex</strong></td>
<td></td>
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<tr>
<td>male</td>
<td>22 (43.14%)</td>
<td>8 (47.06%)</td>
</tr>
<tr>
<td>female</td>
<td>29 (56.86%)</td>
<td>9 (52.94%)</td>
</tr>
<tr>
<td><strong>age</strong></td>
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<tr>
<td>Mean±SD</td>
<td>42.88±14.34</td>
<td>46.35±14.22</td>
</tr>
<tr>
<td>median</td>
<td>43</td>
<td>51</td>
</tr>
<tr>
<td>age range</td>
<td>10-73</td>
<td>27-64</td>
</tr>
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</table>

The age of the first episode VTE

before 50 55 (69%)

after 50 25 (31%)

DVT- Deep vein thrombosis; PTE-pulmonary thromboembolisms
<table>
<thead>
<tr>
<th>genotype /allele (n/%)</th>
<th>VTE group</th>
<th>Control group</th>
<th>OR(95%CI)*</th>
<th>x²</th>
<th>p value</th>
<th>p value</th>
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<tbody>
<tr>
<td>WT (G/G)</td>
<td>58 (72.5%)</td>
<td>78 (97.5%)</td>
<td>14.8 (3.34-65.43)</td>
<td>17.69</td>
<td></td>
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<tr>
<td>FV G1691A</td>
<td></td>
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<td></td>
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<tr>
<td>HET(G/A)</td>
<td>22 (27.5%)</td>
<td>2 (2.5%)</td>
<td>&lt;0.001</td>
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<tr>
<td>RH(A/A)</td>
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<td>0</td>
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<td></td>
<td></td>
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<tr>
<td>allele G</td>
<td>138 (86.3%)</td>
<td>158 (98.8%)</td>
<td>12.6 (2.90-54.52)</td>
<td>16.26</td>
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<tr>
<td>allele A</td>
<td>22 (13.8%)</td>
<td>2 (1.2%)</td>
<td>&lt;0.001</td>
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<td>&lt;0.001</td>
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<tr>
<td>WT (G/G)</td>
<td>63 (78.8%)</td>
<td>78 (97.5%)</td>
<td>10.5 (2.34-47.27)</td>
<td>11.70</td>
<td></td>
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<tr>
<td>FII G20210A</td>
<td></td>
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<tr>
<td>HET(G/A)</td>
<td>17 (21.3%)</td>
<td>2 (2.5%)</td>
<td>0.001</td>
<td>0.001</td>
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<tr>
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<tr>
<td>allele G</td>
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<td>158 (98.8%)</td>
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<td>2 (1.2%)</td>
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<td>WT(C/C)</td>
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<td>25 (31.3%)</td>
<td>1.2 (0.60-2.36)</td>
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<tr>
<td>HET(C/T)</td>
<td>43 (53.8%)</td>
<td>43 (53.8%)</td>
<td>0.603</td>
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<tr>
<td>RH(T/T)</td>
<td>15 (18.8%)</td>
<td>12 (15.0%)</td>
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<tr>
<td>allele C</td>
<td>87 (54.4%)</td>
<td>93 (58.1%)</td>
<td>0.86 (0.55-1.34)</td>
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<td>allele T</td>
<td>73 (45.6%)</td>
<td>67 (41.9%)</td>
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</tbody>
</table>
**Wild-type vs. heterozygous+homozygous; WT-wild type; HET-heterozygous; RH-recessive homozygous; OR- Odds Ratio; 95% CI- 95% Confidence Interval; \(x^2\)- Chi squared test**

Table 3

Representation of individual and multiple polymorphisms in genes: *FV* G1691A, *FII* G20210A and *MTHFR* C677T within the study groups

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>VTE group (N=80)</th>
<th>Control group (N=80)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>FV</em> G1691A</td>
<td>4 (5%)</td>
<td>1 (1.25%)</td>
</tr>
<tr>
<td><em>FII</em> G20210A</td>
<td>5 (6.25%)</td>
<td>0</td>
</tr>
<tr>
<td><em>MTHFR 677</em></td>
<td>31 (38.75%)</td>
<td>47 (58.75%)</td>
</tr>
<tr>
<td><em>FV</em> G1691A</td>
<td><em>FII</em> G20210A</td>
<td>3 (3.75%)</td>
</tr>
<tr>
<td><em>FV</em> G1691A</td>
<td><em>MTHFR 677</em></td>
<td>13 (16.25%)</td>
</tr>
<tr>
<td><em>FII</em> G20210A</td>
<td><em>MTHFR 677</em></td>
<td>7 (8.75%)</td>
</tr>
<tr>
<td><em>FV</em> G1691A</td>
<td><em>FII</em> G20210A</td>
<td>2 (2.5%)</td>
</tr>
<tr>
<td>None†</td>
<td>15 (18.75%)</td>
<td>29 (36.25%)</td>
</tr>
</tbody>
</table>

* *MTHFR* C677T and *MTHFR* T677T;  
† There is not one of heterozygous and recessive homozygous