# ASSOCIATION BETWEEN INHERITED THROMBOPHILIA AND ISCHEMIC BRAIN DISEASE IN VOJVODINA

## *POVEZANOST NASLEDNE TROMBOFILIJE I ISHEMIJSKE BOLESTI MOZGA U VOJVODINI*

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**Abstract**

**Introduction.** The most common genetic causes of thrombophilia include deficiency of histidine-rich glycoprotein, proteins C and S, antithrombin III, as well as mutations in the factor V and factor II genes. Thrombophilia is primarily associated with an elevated risk of deep vein thrombosis and/or thromboembolism; however, thrombosis can also occur in atypical locations such as the retinal, cerebral, and splanchnic veins. Ischemic stroke can result from restricted blood flow to the brain. Some studies suggest a potential link between ischemic brain disease and inherited thrombophilia. **Material and Methods.** Venous blood samples were used to extract genomic DNA, and detection of allele types in patients and controls was performed using real-time polymerase chain reaction on Gentier 96R Real-Time PCR System (TianLong, China) with an allelic discrimination assay. **Results.** Among the risk factors evaluated, the most common one linked to the development of cerebral ischemia was resistance to activated protein C due to the presence of FV Leiden mutation. **Conclusion.** Further studies involving larger cohorts of patients with reported cases of cerebral ischemia are necessary to determine whether a significant association exists between inherited thrombophilia and cerebral ischemia.

**Key words:** Blood Coagulation Disorders, Inherited; Brain Ischemia; Thrombophilia; Risk Factors; Activated Protein C Resistance; Polymerase Chain Reaction; Mutation

**Sažetak**

**Uvod.** Najčešći genetički faktori koji uzrokuju trombofiliju su mutacije u genima za faktor V i faktor II, kao i deficit glikoproteina bogatog histidinom, antitrombina III, proteina C i proteina S. Trombofilija se najčešće dovodi u vezu sa trombozom dubokih vena i/ili tromboembolijom, ali se tromboza može dogoditi i na mestima kao što su splanhične, cerebralne i retinalna vena. Prekid protoka krvi do mozga može dovesti do ishemijskog moždanog udara. Istraživanja su ukazala na moguću povezanost između ishemijske bolesti mozga i nasledne trombofilije. **Materijal i metode.** Nakon ekstrakcije DNK iz uzoraka venske krvi, određen je tip alela metodom lančane reakcije polimeraze u realnom vremenu na instrumentu *Gentier 96R Real-Time PCR System (TianLong, China)*, metodom alelske diskriminacije. **Rezultati.** Među ispitivanim faktorima rizika za razvoj cerebralne ishemije najzastupljeniji je bio rezistencija na aktivirani protein C usled potvrđene *FV Leiden* mutacije. **Zaključak.** Potrebno je sprovesti ovakvo istraživanje na većem broju pacijenata sa dokumentovanom cerebralnom ishemijom kako bi se sa sigurnošću utvrdilo da li postoji značajna povezanost između nasledne trombofilije i ishemijske bolesti mozga.

**Ključne reči:** nasledni poremećaji koagulacije; ishemija mozga; trombofilija; faktori rizika; rezistencija na aktivirani protein C; PCR; mutacije

### Introduction

Hypercoagulability results from either an excessive activity of pro-coagulant factors or a deficiency in anti-coagulant factors [1]. Thrombophilia, characterized by an increased tendency for blood to clot, can be either inherited or acquired [2, 3], although thrombotic events typically result from the interaction of both genetic and environmental factors [4]. Genetic causes of thrombophilia include mutations in the factor V (FV) gene, antithrombin III deficiency, protein C or S deficiencies, histidine-rich glycoprotein deficiency, and prothrombin-related thrombophilia [2].

The most common form of inherited thrombophilia is the FV Leiden mutation, caused by a guanine-to-adenine substitution at nucleotide 1691 in the FV (proaccelerin) gene. This substitution leads to the replacement of arginine with glutamine at position 506 of the heavy chain, resulting in partial resistance to activated protein C (APC)-mediated proteolysis, thereby sustaining factor V's pro-coagulant activity [2, 5].

The second most common hereditary thrombophilia is prothrombin-related thrombophilia [2]. Factor II (FII), or prothrombin, is a vitamin K-dependent proenzyme produced by the liver produces. It plays a crucial role in converting fibrinogen into fibrin. Elevated FII levels are associated with adenine-to-gua-

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***Abbreviations***

FV – factor V
APC – activated protein C
FII – factor II
AT – antithrombin
CI – cerebral ischemia
EDTA – ethylenediamine tetraacetic acid
DNA – deoxyribonucleic acid
PCR – polymerase chain reaction
qPCR – real-time polymerase chain reaction
TIA – transient ischemic attack
RFU – relative fluorescence unit

nine replacement at position 20210. Mutations in the factor II gene are correlated with an increased risk of thrombosis in specific venous regions, such as the portal vein and intracranial veins [6].

The three major inhibitors of blood coagulation are antithrombin, protein C, and protein S [7]. Antithrombin (AT), a serine proteinase inhibitor, enhances the inhibitory process in the presence of the co-factor heparin. Antithrombin directly inactivates thrombin as well as factors IX, X, and XI through the formation of a covalent complex. Thrombin, in turn, activates protein C, which proteolytically inactivates factors Va and VIIIa, inhibiting thrombin production and subsequent coagulation. Protein S, a vitamin K-dependent glycoprotein synthesized in the liver, megakaryocytes, endothelial cells, and Leydig cells acts as a co-factor for protein C [3, 8].

Non-genetic factors also influence coagulation, including age, tissue damage, malignancy, pregnancy, oral contraception and hormone replacement therapy, physical inactivity, and obesity [9].

While deep venous thrombosis and/or thromboembolism are the most frequent manifestations of thrombophilia, thrombosis can also occur in uncommon locations, such as the retinal, cerebral, and splanchnic veins [2].

Given that approximately 25% of ischemic strokes are cryptogenic [10], genetic testing for thrombophilia is often sought to uncover possible stroke causes. Ischemic strokes, which account for about 87% of all stroke cases, are a leading cause of death or severe disability [11]. Ischemic strokes can be caused by a thrombosis, embolism, or systemic hypoperfusion, all of which restrict blood flow to the brain, depriving it of sufficient oxygen and glucose, and leading to cell death [12].

A 2019 study by Chiasakul et al. found that patients with arterial ischemic stroke were significantly more likely to have inherited thrombophilia, such as FV Leiden or the prothrombin G20210 mutation, or deficiencies in protein C and protein S [13].

Conversely, other studies have found limited evidence to support strong association between thrombophilia and cerebral ischemia (CI). Some argue that FV Leiden and prothrombin gene mutations are not significantly related to ischemic stroke, questioning the cost-effectiveness and utility of thrombophilia testing in various stroke scenarios [14–16].

The aim of this study is to evaluate the potential correlation between hereditary thrombophilia and the development of CI in a population from Vojvodina.

## Material and Methods

Genetic testing to detect point mutations in the factor II and factor V genes was conducted at the Center for Forensic Medicine and Laboratory Medicine Center of the University Clinical Center of Vojvodina from January 2022 to July 2024. This case-control study included 83 patients from the Neurology Clinic, University Clinical Center of Vojvodina, diagnosed with ischemic brain disease, and 112 healthy controls (volunteers with no known health conditions), matched by sex and age. Informed consent was obtained from all participants, and the study was approved by the Institutional Ethics Committee, in accordance with the Declaration of Helsinki. Venous blood samples were collected from each participant into ethylenediamine tetraacetic acid (EDTA) tubes, and dried blood spots were prepared from each sample. Genomic DNA was extracted from these dried blood spots using the Chelex100® Molecular Grade Resin reagent (Bio-Rad, Hercules, CA, USA), following the manufacturer's protocol. Diluted DNA samples were added to a reaction mix consisting of TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), along with the primers and specific probes (TaqMan SNP Genotyping Assay, Applied Biosystems, Foster City, CA, USA) to differentiate between wild-type and mutant alleles. Allele detection in patients and controls was performed using real-time polymerase chain reaction (qPCR) on the Gentier 96R Real-Time PCR System (TianLong, China), employing the allelic discrimination assay. Data on antithrombin III, protein C, protein S, lupus anticoagulant, and beta-2 glycoprotein 1 antibody levels were retrieved from patient's medical records. Statistical analysis was conducted using SPSS software, version 23.0 (Chicago, IL, USA). A p-value of $\leq 0.05$ was considered statistically significant. The data were analyzed using the $\chi^2$ test to examine individual predictors and binomial logistic regression analysis to assess the combined effect of all potential predictors on ischemic brain disease.

## Results

Of the 83 patients, 43 (51.81%) were male and 40 (48.19%) were female, resulting in a male-to-female

**Table 1.** Frequency of analyzed parameters in patients and controls

| Parameter | Patients N (%) | Controls N (%) | p |
|---|---|---|---|
| APC resistance with FV Leiden mutation | 17 (20.48%) | 1 (0.89%) | < 0.001 |
| FII G20210A | 9 (10.84%) | 1 (0.89%) | 0.05 |
| Antithrombin III | 1 (1.20%) | 0 (0%) | 0.16 |
| Protein S | 3 (3.61%) | 0 (0%) | 0.02 |
| Protein C | 1 (1.20%) | 0 (0%) | 0.17 |
| Lupus anticoagulant | 3 (3.61%) | 0 (0%) | 0.02 |
| Anti-beta-2-glycoprotein-1 antibodies | 1 (1.20%) | 0 (0%) | 0.16 |

ratio of 1.08:1. In the control group, 58 (51.79%) were male and 54 (48.21%) were female, with a similar male-to-female ratio of 1.07:1. The average age of patients was 47.91 years (95% CI: 43.58 – 48.74), while the average age of the controls was 46.16 (95% CI: 46.29 – 49.53). A small percentage of participants were in the older age group, with 5 patients (6.02%) and 6 controls (5.36%) classified as such, while the remaining participants fell into the middle-aged category (98.92%).

Among the inherited thrombophilias studied, the most common risk factor for developing CI in the patient group was APC resistance due to the presence of FV Leiden mutation (20.48%). This was followed by the prothrombin (FII) G20210A gene mutation (10.84%), protein S deficiency (3.61%), positive lupus anticoagulant test results (3.61%), protein C deficiency (1.20%), and a positive beta-2 glycoprotein-1 antibody test (1.20%) **(Table 1).** Combined thrombophilia was observed in 2.41% of patients, with one patient exhibiting APC resistance with protein C deficiency and another showing APC resistance with protein S deficiency. Both the FII G20210A and FV Leiden mutations were found in 0.89% of healthy controls. All patients with confirmed mutation had heterozygous genotype **(Figures 1 and 2),** and the remaining participants did not carry either factor II **(Figure 3)** or factor V **(Figure 4)** gene mutations.

The prevalence of the FII G20210A mutation, protein S deficiency, lupus anticoagulant, FV Leiden mutation, and APC resistance was significantly higher in patients with CI (p = 0.05, 0.02, 0.02, and < 0.001, respectively). However, the Phi and Cramer's V values

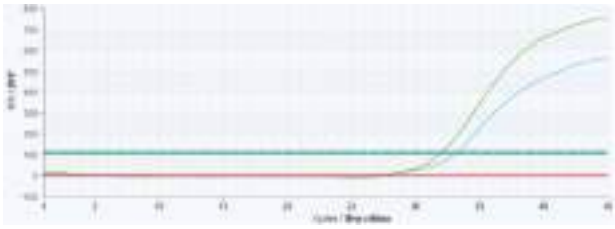

**Figure 2.** Sample amplification curve with heterozygous mutation in gene for factor II (prothrombin)
RFU – relative fluorescence unit

for the FII G20210A, protein S deficiency, and lupus anticoagulant were 0.14, 0.18, and 0.18, respectively, indicating weak associations. In contrast, the FV Leiden mutation and APC resistance showed moderate associations with CI, as reflected in the Phi and Cramer's V values of 0.31 and 0.32, respectively. No statistically significant associations were found between CI and antithrombin III, protein C deficiency, or the presence of anti-beta-2-glycoprotein antibodies (p = 0.16, 0.17, and 0.16, respectively). To account for potential confounding factors, binomial logistic regression was applied. The analysis confirmed that both the FII G20210A and the FV Leiden mutations were significantly associated with CI (p = 0.02 for both).

## Discussion

This study demonstrates a significant association between certain inherited thrombophilias and ischemic brain disease. These findings contradict much of the previous literature, which has largely failed to establish a clear causal link between inherited

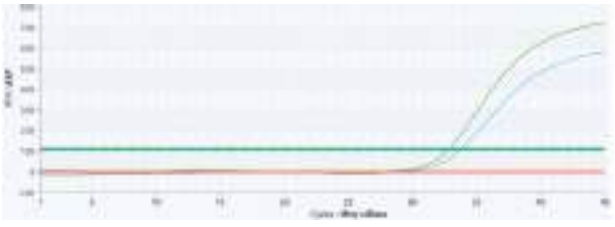

**Figure 1.** Sample amplification curve with heterozygous mutation in gene for Factor V
RFU – relative fluorescence unit

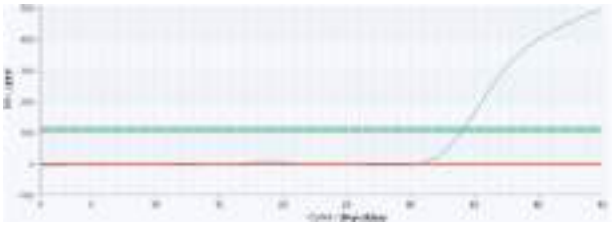

**Figure 3.** Sample amplification curve without mutation in gene for factor II (prothrombin)
RFU – relative fluorescence unit

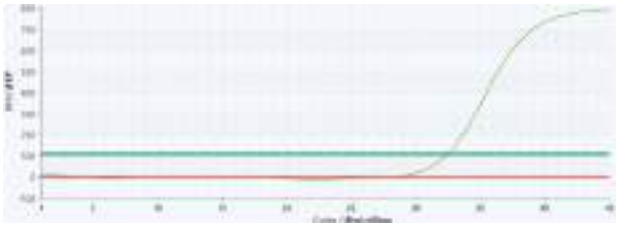

**Figure 4.** Sample amplification curve without mutation in gene for factor V
RFU – relative fluorescence unit

thrombophilia and stroke [14–16]. The most prominent thrombophilic risk factor identified in our study was APC resistance due to the FV Leiden mutation. Other thrombophilias, including the FII G20210A mutation, antithrombin III, protein C and protein S deficiencies, and the presence of a lupus anticoagulant or anti-beta-2-glycoprotein-1 antibodies, were only weakly associated with CI. Our results align with those reported by Chiasakul et al. [13], where 10% of 1,900 acute ischemic stroke patients were tested for thrombophilia markers, including APC resistance, FII G20210A mutation, homocysteine levels, factor VIII, antithrombin, protein S, protein C, anti-beta-2-glycoprotein-1 antibodies, anticardiolipin antibodies, and lupus anticoagulant. Of these patients, 72.1% had at least one abnormal test result [17]. Similarly, a 2018 retrospective analysis of 628 patients with transient ischemic attacks (TIAs) and stroke found that 57% underwent thrombophilia testing, with 14% yielding positive results [18]. The findings of our study may provide preliminary evidence to support the consideration of long-term anticoagulant therapy in patients with CI who also have hereditary thrombophilia. Such an approach could reduce the risk of recurrent cerebral ischemia and other thrombophilia-related complications. Furthermore, it is advisable that family members of patients with confirmed inherited thrombophilia undergo testing, ensuring that those diagnosed with a coagulation disorder receive appropriate prophylactic treatment.

## Conclusion

Based on the findings of this study, we recommend conducting similar research on a larger cohort of patients with reported cerebral ischemia to determine whether a significant association between inherited thrombophilia and cerebral ischemia exists. Some of the results from our study deviate from the majority of comparable research, emphasizing the need for more extensive studies. Such research will provide more definitive results, allowing for the validation or refinement of current clinical recommendations.

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