Received: April 2, 2022
Accepted: May 5, 2022

Acad Med J 2022;2(1):109-118
UDC: 616.132.2-056.7-053.6(497.7)
616.14-005.6/.7-056.7-053.6(497.7)
DOI: 10.53582/AMJ2221109b
Original article

FREQUENCY OF GENETIC VARIANTS ASSOCIATED WITH CORONARY ARTERY DISEASE AND VENOUS THROMBOEMBOLISM IN YOUNG PATIENTS IN REPUBLIC OF N. MACEDONIA

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Abstract

Introduction: Coronary artery disease - CAD and venous thromboembolism - VTE (deep venous thrombosis - DVT and/or pulmonary embolism - PE) are two major manifestations of cardiovascular diseases. Genetic mutations responsible for the occurrence of CAD and VTE in young population are subject of numerous researches and studies.

Aim: To determine the allelic and genotypic frequencies of the analyzed gene variants and to examine whether there is overlap of the most common genetic mutations between the two groups.

Material and methods: This clinical study analyzed the demographic, clinical, and genetic data of a group of 36 patients up to 50 years of age with CAD and a second group of 32 patients up to 50 years of age with VTE. The selection of subjects was according to previously established inclusion and exclusion criteria. Genetic testing was performed on each patient to determine the presence of certain genetic mutations.

Results: The occurrence of CAD and VTE in young population is significantly more common in men. In terms of risk factors, overweight and obesity were found to be the most common in both groups, while hypertension and smoking being more prevalent in the CAD group. Heterozygous mutations for: MTHFR C677T, eNOS 786 T>C, e NOS 894T and B-fibrinogen were most common in group 1, while in group 2 heterozygous mutations for: eNOS -786 T>C, MTHFR C677T, MTHFR a1298C and B-fibrinogen.

Conclusion: Genetic polymorphisms in MTHFR, B-fibrinogen and eNOS genes should be tested in patients with CAD and VTE under 50 years of age as additional risk factors.

Keywords: coronary artery disease, venous thromboembolism, deep vein thrombosis, pulmonary embolism, genetic mutation

Introduction

Coronary artery disease - CAD and venous thromboembolism (deep vein thrombosis - DVT and pulmonary embolism - PE) are two major manifestations of cardiovascular diseases

(CVD). There are genes involved in endothelial dysfunction, hyperlipidemia, hypertension and inflammation. On the other hand, a combination of other factors including hormone replacement therapy, immobilization, surgery or neoplasms and variations in the genes responsible for the coagulation system, can lead to deep vein thrombosis[1].

Coronary artery disease (CAD) is a complex multifactorial disease leading to ischemic heart disease and myocardial infarction. The hereditary component of CAD has already been proven and recent studies have shown it in about 40-50% of cases[2]. Genetic analysis is advancing with the discovery of genes as biological mechanisms responsible for the occurrence of CAD. Since 2007, genome-related studies have greatly helped us to understand the relationship between genetics and disease[3,4]. They have investigated the association between millions of individual nucleotide polymorphisms and disease, comparing healthy to diseased individuals. Many studies have begun to work on genetics and CAD, and certain genetic polymorphisms are already known to be associated with the occurrence of CAD in the population[5,6]. A study of more than 20,000 twins in Sweden confirmed the claims that the risk of CAD was increased in close relatives, and the analysis estimated a hereditary relationship of about 50% for CAD[7,8]. In the Framingham Heart Study, a family history of cardiovascular disease in a parent or sibling was a strong predictor of the disease[9,10].

Venous thromboembolism (VTE) is a disease involving two entities: deep vein thrombosis (DVT) and pulmonary embolism (PE). Hypercoagulable states can be inherited or acquired. Inherited hypercoagulable states can be caused by a loss of function of natural anticoagulant pathways or a gain of function in procoagulant pathways[11]. Besides the well-known provocative risk factors, VTE has a strong genetic basis, including about 50-60% of the incidence of VTE attributed to genetic mutations. Some genetic variants have been identified that contribute to an increased risk of venous thrombosis, mainly associated with blood clotting and responsible for inherited hypercoagulable conditions. The most common known gene mutations responsible for the occurrence of VTE are: factor V Leiden, prothrombin G20210A and fibrinogen gamma[12, 13].

Factor V Leiden (FV) (1691G>A; R506Q) mutation is a genetic disorder characterized by a poor anticoagulant response to activated Protein C and an increased risk for venous thromboembolism[14]. The prothrombin 20210 G/A mutation is associated with elevated levels of factor II in plasma, and significantly increases the risk of developing venous thrombosis. In fact, this polymorphism is the second most important genetic risk factor for venous thrombosis in Caucasian populations. The role of this procoagulant mutation in arterial vascular disease is, however, unclear[15]. Methylenetetrahydrofolate reductase (MTHFR) is an enzyme involved in the metabolism of homocysteine. Mutations in MTHFR may account for reduced enzyme activity and elevated plasma homocysteine levels. Hyperhomocysteinemia has been identified as an independent risk factor in arterial and venous thrombosis[16]. Beta-fibrinogen (FGB) - 455G>A polymorphisms may be associated with elevated levels of plasma fibrinogen, which are related to an increased frequency of coronary heart disease[17]. Endothelial Nitric Oxide Synthase (eNOS; NOS3) is essential for maintaining vascular homeostasis and plays vital roles in modulating vascular endothelial function. Therefore, if a polymorphism can impact gene expression or protein structure of eNOS, it is likely that this polymorphism may lead to a severe vascular endothelial dysfunction and influence predisposition to CAD[18]. Lymphotoxin alpha is one of the cytokines produced in the early stages of vascular inflammatory processes. LTA has been implicated in the pathogenesis of atherosclerosis and coronary heart disease (CHD). Since LTA is an inflammatory mediator, it is likely that functional variations in the gene encoding this protein confer a high susceptibility to MI by affecting the degree of inflammation at the lesion[19].

Objectives

To determine the allelic and genotypic frequencies of the analyzed gene variants of: Factor V (Leiden and R2), Prothrombin (G20210A), Factor XIII (V34L), B-fibrinogen (-455 G> A), Methylenetetrahydrofolate reductase (MTHFR, C677T and A1298C), Endothelial Nitric Oxide Synthase (eNOS -786 T> C and eNOS G894T), Lymphotoxin Alpha (LTA) in both groups, and to investigate whether there was an overlap of the most common genetic polymorphisms and mutations between the first (CAD) and second (VTE) group.

Material and methods

This clinical study analyzed the demographic, clinical, laboratory and genetic data in a study group of 36 patients up to 50 years of age with proven (documented) CAD and 32 patients up to 50 years of age with proven (documented) VTE. The study was approved by the Ethics Committee of the Faculty of Medicine at the Ss. Cyril and Methodius University in Skopje and was in accordance with the principles of the Declaration of Helsinki. Each participant in this clinical trial signed an informed consent to participate in the study, after the study was previously explained and read by the participant. The selection of the subjects was according to previously established inclusion and exclusion criteria.

The inclusion criteria for the first group with coronary artery disease (CAD) were persons aged 18-50 years of both sexes, as well as previously performed coronary angiography based on a previously set medical indication. A positive coronary angiographic finding (finding at least one significant lesion > 50% of the coronary arteries) categorized the study participant in the first group with CAD [15]. Existing risk factors were also considered in patients, such as: gender, smoking, family history, arterial hypertension (systolic pressure > 140 mmHg, diastolic > 90 mmHg), hypercholesterolemia (LDL cholesterol > 2.6 mmol/L, non-HDL cholesterol > 3.4 mmol/L, or triglycerides > 1.7 mmol/L), type 2 diabetes mellitus (already proven or newly discovered - HgbA1c > 6.5%, fasting glycemia > 7.0 mmol/L), overweight and obesity (body mass index) BMI> 25 kg/m2[20-24].

The inclusion criteria for the second group with VTE - deep vein thrombosis and/or pulmonary embolism were persons aged 18-50 years of both sexes, and previously performed color doppler ultrasound of deep veins to prove DVT, i.e., computed tomography angiography to prove PE. Patients in whom DVT and/or PE was diagnosed with these diagnostic methods were categorized in the second study group. Existing risk factors were also considered in patients, such as: malignancy, surgery, trauma or fracture, immobilization, long-distance travel, hospitalization, catheterization, acute infection, obesity or overweight, use of oral contraceptives or use of hormone therapy, use of corticosteroids, sedentary lifestyle.

Exclusion criteria were: persons under 18 or above 50 years of age, pregnant women, persons with type 1 diabetes mellitus, persons with valvular diseases, persons with cardiomyopathies, persons with congenital heart defects, persons with active malignant disease, persons with acute or chronic inflammatory or infectious conditions/diseases, as well as persons who have a relative or absolute contraindication to perform coronary angiography and/or percutaneous coronary intervention and persons allergic to contrast for angiography.

A sample of 3 ml of venous blood with anticoagulant (ethylenediaminetetraacetic acid - EDTA) was taken from all subjects for routine laboratory tests, as well as blood for genetic testing for the presence of genetic polymorphisms and mutations and they were examined at the Institute of Immunobiology and Human Genetics. Polymorphism analyses were performed by reverse hybridization, using a commercially available kit (CVD, ViennaLab, Vienna, Austria); a multiplex polymerase chain reaction (PCR) was performed to amplify the genes using biotinylated primers. The amplicons were subsequently hybridized by probes fixed on nitrocellulose tape. The positive signals were read by enzymatic reaction and the genotype of the participant was determined. The gene mutations and polymorphisms examined in the study were the following:

Factor V (Leiden and R2), Prothrombin (G20210A), Factor XIII (V34L), B-fibrinogen (-455 G> A), Methylenetetrahydrofolate reductase (MTHFR, C677T and A1298C), Endothelial Nitric Oxide Synthase (eNOS -786 T> C and eNOS G894T), Lymphotoxin Alpha (LTA).

For the purposes of this research descriptive analyses were performed, in order to determine the demographic characteristics and risk factors of patients, type of CAD or VTE, localization of disease, method of treatment, and also, relative frequency of each mutation was obtained in both groups to determine the frequency of the mutations. Comparison between two groups was performed using the statistical Z-test.

Results

A total of 68 patients were included in the study, divided into two groups. The first group with CAD included 36 patients, while the second group with VTE (DVT and/or PE) included 32 patients. In terms of age, patients were equally distributed in both groups with an average of 38 years. From the distribution by gender, it can be noticed that in both groups men were significantly more represented than women. In the first group 83% were males and 17% females, while in the second group 75% were males and 25% females. Analysis of variance showed that both groups were homogeneous in terms of age and gender (Table 1).

Table 1. Demographic characteristics and risk factors

	Group 1 n (%)	**Group 2 n (%)**	**z-test**	**p-value**
Gender (M/F)	30/6 (83.3/16.6%)	24/8 (75/25%)	/	/
Age	38.81±7.16	38.38±9.70	/	/
Diabetes mellitus	2 (5.56%)	0 (0%)	1.353	0.176
Hypertension	12 (33.33%)	3 (9.38%)	2.378	0.017
Smoking	23 (63.89%)	13 (40.63%)	1.918	0.055
BMI Class – Overweight and obesity	30 (83.3%)	23 (71.88%)	1.137	0.255

In terms of risk factors, in both groups the most common risk factor was overweight and obesity, while hypertension ($p<0.05$) and smoking ($p<0.1$) were significantly more common in the group with CAD.

Table 2. Group 1 characteristics

Type of CAD	STEMI (69,44%)
Localization of CAD	Anterior wall (58,33%)
Type of intervention	PCI/stenting LAD (50%)
Single *vs* Multivessel disease	Multivessel disease (61,11%)
Triglycerides (mmol/L)	2.43±1.37
LDL Cholesterol (mmol/L)	3.25±1.10
HgbA1c (%)	6.16±1.75

In the first group with coronary artery disease (CAD), the type of coronary artery disease, its localization and method of treatment were analyzed. From the data obtained from a total of 36 patients, according to the type of CAD, the most common were patients with myocardial infarction with ST segment elevation (STEMI) with 69.44%, followed by patients with myocardial infarction without ST segment elevation (NSTEMI) with almost 23% (Table 2). Regarding the localization of CAD, the most common was the anterior wall in more than half of the patients (58.33%), and inferior wall was found in 28%. Regarding the number of coronary arteries affected with CAD, 61% of patients had multivessel CAD, as opposed to 39% with single vessel CAD. Analyzing the method of treatment, 88% underwent percutaneous coronary intervention (PCI), 6% were treated with coronary artery by-pass graft (CABG), and 6% were treated only with medication. In 50% of patients, percutaneous coronary intervention

(PCI) of the left anterior descending coronary artery was performed while in 31% of patients PCI of the right coronary artery was made, with a lower percentage of PCI on other coronary arteries. The average values of triglycerides and LDL cholesterol were above upper limits, and the average value of HgbA1c was in normal range.

The analysis of the second group showed that, out of 32, same number of patients had isolated PE or DVT, while almost equal number of patients had both conditions. Frequency analysis was performed according to the localization of DVT, and it showed that the most common were DVT of the common femoral veins: left common femoral vein with 36%, right common femoral vein with 25%, left popliteal vein with 25%, and right popliteal vein with 14%.

Table 3. Frequency of the genetic mutations in group 1 and 2 patients

	Group 1 N (f)	**Group 2 N (f)**
F V Leiden		
-Normal	34 (0.94)	21 (0.66)
-Heterozygous	2 (0.06)	11 (0.34)
-Homozygous	0 (0.00)	0 (0.00)
F V (R2)		
-Normal	29 (0.81)	26 (0.81)
-Heterozygous	7 (0.19)	6 (0.19)
-Homozygous	0 (0.00)	0 (0.00)
Prothrombin		
-Normal	35 (0.97)	29 (0.91)
-Heterozygous	1 (0.03)	2 (0.06)
-Homozygous	0 (0.00)	1 (0.03)
MTHFR C677T		
-Normal	12 (0.33)	11 (0.34)
-Heterozygous	18 (0.50)	16 (0.50)
-Homozygous	6 (0.17)	5 (0.16)
MTHFR A1298C		
-Normal	21 (0.58)	18 (0.56)
-Heterozygous	10 (0.28)	14 (0.44)
-Homozygous	5 (0.14)	0 (0.00)
Factor XIII		
-Normal	21 (0.58)	25 (0.78)
-Heterozygous	9 (0.25)	7 (0.22)
-Homozygous	6 (0.17)	0 (0.00)
eNOS -786 T>C		
-Normal	14 (0.39)	10 (0.31)
-Heterozygous	15 (0.42)	16 (0.50)
-Homozygous	7 (0.19)	6 (0.19)
eNOS G894T		
-Normal	21 (0.58)	12 (0.38)
-Heterozygous	14 (0.39)	13 (0.41)
-Homozygous	1 (0.03)	7 (0.22)
LTA		
-Normal	19 (0.53)	22 (0.69)
-Heterozygous	14 (0.39)	8 (0.25)
-Homozygous	3 (0.08)	2 (0.06)
B-fibrinogen		
-Normal	21 (0.58)	17 (0.53)
-Heterozygous	13 (0.36)	14 (0.44)
-Homozygous	2 (0.06)	1 (0.03)

The frequency of mutations of the examined genes in patients by groups was statistically analyzed. We obtained the following results: in group 1 (patients with CAD) the most common were heterozygous mutations for: MTHFR C677T with frequency of 50%, eNOS with 786 T>C 42%, e NOS 894T with 39%, and B-fibrinogen with 36%, while homozygous mutations were rare: eNOS 786 T>C with frequency of 19%, MTHFR C677T and Factor XIII with 17% (Table 3).

In the second group of patients with VTE, the most common were heterozygous mutations for: eNOS -786 T> C and MTHFR C677T with frequency of 50%, followed by MTHFR A1298C and B-fibrinogen with 44%, eNOS G894T with 41%, FV Leiden with 34%. Homozygous mutations were rare, the most common being: eNOS G894T with 22%, eNOS -786 T> C 19% and MTHFR C677T with 16%.

The frequencies of the mutations between the groups were compared. According to the statistical analysis for heterozygous mutations, only the frequency of Factor V Leiden was significantly higher in the second group ($p<0.05$). Concerning the homozygous mutations, the frequencies of MTHFR A1298C and Factor XIII were significantly higher in the first group with CAD, while the homozygous mutation for eNOS G894T was significantly higher in the second group ($p<0.05$). There was a significant overlap of heterozygous mutations for eNOS -786 T> C, MTHFR C677T and B Fibrinogen, as most common mutations in both groups.

Discussion

The number of young patients with CAD has been increasing lately. The incidence of symptomatic CAD in young individuals up to 40 years of age is still low at about 3%, but there is an increasing trend[25,26]. Over the next ten years, we are hopeful that human genetics will prove useful in identifying novel root causes of CAD, guiding drug development efforts in anticipating the safety and efficacy profile of a given therapeutic drug, and providing patients and their providers with genetic data that will aid in CAD prevention and treatment[27]. Environmental factors and genetic factors rarely act in isolation, but in most cases they act synergistically, contributing to an increased risk of a VTE event. Risk factors are not always associated in isolation with VTE, and therefore understanding how the interaction of environment and genetic factors affects the pathophysiology of VTE greatly increases the potential for targeted prevention and treatment in the future, which would significantly prevent and reduce the morbidity and incidence of such events[28].

In our study, patients were divided into two groups: the first group included 36 young patients with CAD, and the second group 32 young patients with VTE. The mean age in both groups was 38 years and the distribution by gender showed that males were significantly more represented than females in both groups. In terms of risk factors, in both groups the most common risk factor were overweight and obesity, while hypertension and smoking were significantly present among patients in the group with CAD. Regarding the statistical analysis of the frequency of examined genetic mutations, we found that in group 1 (patients with CAD) most common were heterozygous mutations for: MTHFR C677T with frequency of 50%, eNOS 786 T>C 42%, e NOS 894T 39% and B-fibrinogen with 36%. Meta-analysis of published case control and cohort studies in PubMed, correlating factor V Leiden, Prothrombin (PT) G20210A, and methylenetetrahydrofolate reductase (MTHFR) C677T (TT genotype) mutations with myocardial infarction and ischemic stroke, have shown that these genetic abnormalities increase the risk of developing the above-mentioned diseases, especially in the younger population[29].

A cross-sectional, case-control study to determine the association of atherogenic and thrombogenic markers of lymphotoxin-alfa gene mutations and the risk of coronary artery disease at a young age was performed in 336 patients with CAD younger than 50 years and 189 healthy individuals. The results suggest that the inclusion of lipoprotein (a) and

lymphotoxin-alfa mutations in the set of conventional risk factors has shown to be an additional factor, with a slight increase in the predictable risk of CAD[30].

In the second group with VTE, the most common were heterozygous mutations for: eNOS -786 T> C and MTHFR C677T with frequency of 50%, followed by MTHFR a1298C and B-fibrinogen with 44%, eNOS G894T with 41%, FV Leiden with 34%. According to these results, overlap of 3 genetic mutations was observed as the most common in both groups: eNOS -786 T> C, MTHFR C677T and B-fibrinogen. The frequencies of the mutations between the groups were compared. According to the statistical analysis for heterozygous mutations, only the frequency of Factor V Leiden was significantly higher in the second group ($p<0.05$). Concerning the homozygous mutations, the frequencies of MTHFR A1298C and Factor XIII were significantly higher in the first group with CAD, while the homozygous mutation for eNOS G894T was significantly higher in the second group ($p<0.05$).

A meta-analysis performed in 2018, to determine the correlation between methylenetetrahydrofolate reductase (MTHFR) gene polymorphism and VTE risk identified 99 studies published in PubMed, Medline, Embase, and Web of Science. The analysis showed that MTHFR polymorphism may serve as a potential biological marker for the occurrence of DVT and VTE[31].

In 2019, a study was conducted to determine the role of risk factors and gene polymorphisms in young patients with acute coronary syndrome (ACS). A total of 299 patients and a control group of 53 healthy volunteers aged 25 to 44 years were included. Genetic polymorphisms for: Prothrombin FII G20210-A, FV Leiden G1691-A and MTHFR C677-T were examined by polymerase chain reaction (PCR). The results showed that in the group with ACS the following risk factors were present in a significant percentage: increased levels of LDL cholesterol, decreased levels of HDL cholesterol, smoking, the presence of MTHFR homozygous polymorphism, FV Leiden homozygosity, smoking in combination with MTHFR[32].

Tomsk National Research Medical Centre in 2019 conducted a study to investigate the distribution of genes from the coagulation system and their impact on serum parameters and hemostasis in 913 patients with acute coronary syndrome. Eight polymorphic variants of genes associated with thrombophilia were analyzed: Prothrombin FII (G20210-A), FV Leiden (G1691-A), FVII (G10976-A), FXIII (G163-T), FI (G455-A), GP Ia-Ila (C807), GP Ilb-IIIa (T1565-C), PAI-I (G6755-G). The results showed that at least 1 polymorphic variant of the above-mentioned genes was found in 97% of patients with ACS[33].

To determine the frequency of recurrent VTE events in patients with factor V Leiden (FVL) or prothrombin G20210A mutation in comparison with patients without mutation of these genes, and to determine the frequency of VTE events in relatives of patients with and without mutations in these genes, a meta-analysis was performed based on relevant studies searched in Medline, EMBASE, the Cochrane Library, the Cumulative Index to Nursing and Allied Health Literature, and PsycInfo. The results showed that heterozygous and homozygous mutations for FVL have predictive value for recurrent VTE event as well as VTE event in close relatives compared to individuals without mutation of this gene. On the other hand, a heterozygous mutation of Prothrombin G20210 is not predictive of recurrent VTE event. The studies on the predictive value of the homozygous mutation of Prothrombin G20210A for the occurrence of recurrent VTE event or VTE event in close relatives are insufficient[34].

In 2014, it was investigated whether there was a link between arterial and venous thrombosis. Several studies revealed that subjects with idiopathic venous thrombosis had an increased risk of cardiovascular events compared to subjects with secondary thrombosis or the control group. On the other hand, atherosclerosis had the potential to cause the development of a thrombogenic disorder in the venous system. The different nature of arterial and venous

thrombogenic disorders remains a challenge. More studies are needed to find and clarify the nature of their association and to evaluate their impact in clinical practice[35].

At the University Clinic for Cardiology in Skopje, in cooperation with the Institute of Immunobiology and Human Genetics, in 2008 a genetic study of Macedonian population was performed to determine the association of methylenetetrahydrofolate reductase (MTHFR-677 and MTHFR-1298) genetic polymorphisms with CAD and DVT. Eighty-three healthy subjects, 76 patients with CAD and 67 patients with DVT were included. The results did not show a significant association between MTHFR-677 and MTHFR-1298 polymorphisms with coronary artery disease and deep vein thrombosis in Macedonians, except for the protective effect of MTHFR/CA:CC diplotype in CAD[36].

In 2006, a retrospective case-control study involved 190 patients with venous thromboembolic disease and 200 healthy individuals, to determine the prevalence of factor V Leiden mutation in patients with venous thromboembolic disease and healthy individuals in the Republic of Macedonia. The prevalence of factor V Leiden mutation among patients with venous thromboembolic disease was 21.1%, compared to 5.5% in healthy individuals[37].

The limitation of this study was that we did not include a control group of healthy individuals, to compare the frequencies of genetic mutations in patients in both groups with the frequencies in healthy population and that is our next objective. Making correlation between patient's groups and healthy population, and logistic regression with the risk factors, can determine whether there is a significant association of certain mutations and the occurrence of disease (i.e., a significant deviation in these two groups). This study included a small number of patients, which may have introduced bias into the study.

Conclusion

There are environmental risk factors (overweight, obesity, hypertension, smoking) that should be considered in the assessment of patients with CAD or VTE under 50 years of age. Our study identified genetic polymorphisms in MTHFR, eNOS and B-fibrinogen genes that are associated with a higher risk of CAD and VTE and should be analyzed in these patients.

Conflict of interest statement. None declared.

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