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Genetic Variants and Biochemical Parameters Associated with the Risk for Venous Thromboembolism in a Romanian Cohort

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Abstract: Thrombophilia can be defined as a predisposition to form clots inappropriately. We studied the carrier status for thrombophilia-related genetic variants in a cohort of 237 Romanian patients who were referred to Synevo Romania between January 2021 and April 2021. Two groups of patients consisting of 117 subjects were evaluated for the underlying causes of a VTE (venous thromboembolism) - group A and group B, consisting of 120 patients with no thromboembolic events. All patients were screened for PC, PC, AT-III, FVL, FII, and MTHFR. The presence of thrombophilia was compared between groups. Out of the 117 patients in group A, 113 (96.58 %), revealed at least one of the analyzed mutations, while just 4 (3.41 %) there were no identified mutations; in comparison, the mutation carrier/non-carrier ratio in group B was 104 patients (86.67%) and 16 (13.33%), respectively. The prevalence of FVL and FII mutation in group A, 21/117 (17.94%) heterozygous and 17/117 (14.53%) heterozygous, respectively, was notably higher when compared to 10/120 (8.33%) FVL heterozygous and 6/120 (5%) FII heterozygous in group B (p=0.034 and p=0.0156). The prevalence of inherited natural anticoagulants was comparable between groups with no statistically significant difference (p=0.6592, p= 0.0992, p= 0.6809).

Keywords: thrombophilia; prothrombin G20210A mutation; MTHFR (C677T/ A1298C) polymorphisms; factor V Leiden; protein C; protein S; antithrombin III.

1. Introduction

Thrombophilia can be defined as a tendency to develop thrombosis due to predisposing hereditary and/or acquired causative factors. The prevalence of this condition in the general population is 5-8% [1-3]. Although thrombosis may occur in both veins and arteries, the term thrombophilia is usually considered in the context of venous thromboembolism (VTE), since most of the well-defined thrombophilic risk factors are commonly associated with thrombosis

in venous blood vessels. Today, VTE is a serious health problem that affects approximately 1–2/ 1000 individuals in the general population of Western countries each year [4, 5].

When evaluating subjects with a VTE event, hereditary thrombophilia may be suspected in the following situations: young patients below 50 years old with VTE associated or not with minor risk factors (such as oral contraceptives), recurrent VTE episodes occurring at a young age, heredocollateral history of VTE and VTE developed in unusual anatomic locations, such as cerebral / splanchnic veins [6-9]

Venous thromboembolism (VTE) is a complex disease (thrombophilia) that is caused by a number of genetic and acquired/environmental risk factors [10, 11].

Hereditary thrombophilia that is largely recognized as a strong independent risk factor for VTE development includes the rare inherited deficiencies of natural anticoagulants protein C, protein S, and antithrombin, caused by loss-of-function mutations, and the more common gain-of-function prothrombotic mutations in F5 and F2 genes (factor V Leiden - FVL- and prothrombin G20210A mutation) [9, 12, 13].

Hereditary thrombophilia, which is widely recognized as a strong independent risk factor for VTE development, includes the rare inherited deficiencies of anticoagulants such as protein S, protein C, and antithrombin, which can be induced by loss-of-function mutations and the more frequent gain-of-function prothrombotic mutations in Factor V and Factor II genes (FVL and prothrombin G20210A mutation) [9, 12, 13]. There are also other genetic risk factors whose contribution to VTE is debatable, such as the MTHFR (methylenetetrahydrofolate reductase) C677T and A1298C polymorphisms of the MTHFR gene [14].

Other genetic factors causing thrombosis and pulmonary embolism have been identified in recent studies, including the 4G/4G polymorphism of the PAI-1 gene [15].

The increasing risk for VTE incidence, especially in the elderly, requires new biomarkers for disease diagnosis and prognosis [16]. Recently, high levels of factors VIII (FVIII) [17] and von Willebrand (vWF) [18] were related to a higher risk of both arterial and venous thrombotic events.

The prevalence of hereditary thrombophilia in the general population varies from 0.2%-0.4% for protein C deficiency, 0.2% for protein S deficiency, 0.02% for AT III deficiency, and 4-5% for FVL [19, 20].

Almost 70% of the patients diagnosed with thrombophilia show at least one major genetic defect of the procoagulant (Factor V & Factor II) or/and anticoagulant (protein C, S, and antithrombin) factors. It was found that mutations in the Factor V Leiden gene and the G20210A mutation in Factor II cause more than 2/3 of all cases of hereditary thrombophilia and that genetic defects in protein C, S, and antithrombin occur in 15 - 20% of patients with venous thrombosis [21-23].

Genetic testing for inherited thrombophilia is becoming easier as a result of the latest technological progress and automation. Even though there is a low number of published validated test guidelines, screening for hereditary hypercoagulable disorders is frequently requested in specialized clinics. [24].

The aim of our work was to evaluate the prevalence and distribution of 6 known inherited risk factors - protein C, protein S, antithrombin deficiency and Factor V Leiden, Factor II, methylenetetrahydrofolate reductase (MTHFR) mutations, as a cause of thrombophilia in a Romanian cohort, referred to a private laboratory in Bucharest.

2. Materials and Methods

2.1. Patient and samples.

A retrospective study has been performed on a cohort of Romanian patients that were analyzed from January-April 2021 at Synevo Central Laboratory for a complete hereditary thrombophilia screening. Two groups of patients have been selected, consisting of 117 subjects evaluated for the underlying causes of a VTE and a group consisting of 120 patients with no personal history of thromboembolic events.

All subjects included in this study were tested for protein C (PC), protein S (PS), and antithrombin III (AT-III) deficiency, mutations in the F5 (factor V Leiden), F2 (prothrombin G20210A mutation), and MTHFR (the c.677C>T, c.1298A>C mutations) genes.

All patients included in this study signed the informed patient consent.

Patients on anticoagulant therapy were excluded from the study.

All whole blood samples were collected in two vacutainers – one for DNA extraction (on EDTA - ethylenediaminetetraacetic acid) and one for the quantitative detection of PS, PC, and AT-III (on sodium citrate).

2.2. Real-Time PCR (polymerase chain reaction) genotyping for thrombophilic variants.

DNA (genomic DNA) was isolated from 200 µl peripheral blood using the MagNA pure LC DNA Isolation kit and the automated extractor based on the magnetic-bead technology (MagNA pure LC Instrument), according to the manufactures's instructions. The extracted and purified DNA was analyzed by Real-Time PCR using the following commercial kits (Roche Diagnostics): Factor V Leiden, Factor II G20210A, MTHFR C677T LightMix, MTHFR A1298C LightMix, on Cobas Z 480 instrument. The genotyping assays are based on FRET (Fluorescence Resonance Energy Transfer)-allowing distinction between the three genotypes: homozygous wild type, homozygous mutant, or heterozygous.

2.3. Quantitative detection of protein S, C, and Antithrombin III.

Quantitative assays were also conducted for protein S, C, and antithrombin III (through the chromogenic method). Blood samples were drawn using vacutainer tubes with sodium citrate for the PS, PC, and AT-III determination. The blood was drawn a jèune, each sample being prepared immediately using a centrifuge or stored at -20°C until work. STA-R Evolution was used to determine the activity level of protein C chromogenic (normal range 70-130%), antithrombin III (normal range >80%), and protein S coagulometric activity (normal range for men 66-143%; 57-131% for women) using specific kits, i.e., protein C-STAGO, STA ATIII-STAGO, and protein S-STAGO, respectively. Antithrombin activity was analyzed immediately. A modified APTT (activated partial thromboplastin time) test was used to establish the activity level of protein S.

2.4. Statistical analysis.

For statistical analysis, we used GraphPad Prism 6.01 for Windows. Gender-based differences and the incidence of the analyzed genetic variants were determined for both groups and compared by a two-tailed Fisher's exact test. A *P* value below 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Patient characteristics.

Out of the 117 individuals in group A, 54 were female (46.15%), and 63 were male (53.84%). 79 of the 120 individuals in group B were women (65.83%), while 41 were men (34.16%).

The average age for the patients in group A was 44 years old (15/ 76, ± 12.42**)**. The average age for the patients in group B was 39 years old (18/ 65, ± 10.54).

The average age in women was 43 in group A and 35 in group B. In men, the average age was 45 in group A and 44 in group B.

3.1.1. Cohort distribution by age groups.

Thirty-four of the VTE patients (15 males, 19 females) and 83 of the 120 controls (29 males, 54 females) had ages ranging between 18 and 40 years old. One hundred nineteen of all tested individuals were 40 years older or older, 83 in group A (48 males, 35 females) and 37 in group B (12 males, 25 females). Only one patient in the analyzed cohort was aged <18 years old.

Group B consisted of patients with different clinical indications, including miscarriage and other obstetrical problems, female infertility, pre-conceptional screening or screening during pregnancy, hematological recommendations, a.o.

3.2. Carrier frequencies and genotypes` distribution of thrombophilic variants.

The FVL gene mutation was detected in a heterozygous status in 17.94% of the patients in group A, while the F2 G20210A gene mutation in 14.53%. In group B, the FVL gene mutation was found in 8.33% and 5 % F2, both in heterozygous status.

Statistically significant differences, between groups, in F5 and F2 genes were found (p=0.034 and p=0.0156).

Pathogenic homozygous genotypes were not found in the F5 and F2 genes.

The MTHFR c677C>T, and c1298A>C mutations in group A were detected as follows: 57.26% (71.64% heterozygous, 28.36 homozygous) and 54.7 % (73.43 % heterozygous, 26.57% homozygous).

In group A, 57.26% of all individuals revealed the c677C>T mutation. Forty-eight patients (71.64%) of the c677C>T positive patients were heterozygous for the mutation, and 73.43% of the c1298A>C positive individuals (47 patients) also revealed a heterozygous genotype.

A homozygous genotype was detected in 19 of the individuals carrying the c677C>T variant (28.36%) and 17 of the c1298A>C positive patients (26.57%).

In group B, 46.67% of all analyzed individuals revealed the c677C>T mutation, while 40% (48 patients) carry the c1298A>C variant.

57.14% of the c677C>T positive group were heterozygous for the mutation, 42.86% (24 individuals) being homozygous, while 30 patients of the c1298A>C group (62.5%) showed the heterozygous genotype and 37.5% were homozygous (18 individuals).

We had statistically significant differences when comparing the frequency of the heterozygotes between groups A and B: p= 0.095 (c677C>T) and p= 0.0136 (c1298A>C)

(Fisher exact test). No statistical difference was identified when comparing the homozygotes frequencies between the two cohorts (p=1 and p=0.568, respectively).

5.98% (7 patients) of the patients in group A were identified with protein C deficiency and 10.25% (12 patients) with a lack of protein S, while AT-III deficiency was found in 3 patients (2.56 %). In group B, the following deficiencies have been detected: 8.33 % (10 patients) for protein S, 1.66 % (2 patients) for protein C and 1.66% (2 patients) for AT-III.

Between groups no statistically significant difference was found (p=0.6592, p= 0.0992, p= 0.6809, Table 1).

Table 1. Inherited thrombophilia in the study population.

Genetic variant genotypes/ PS, PC, AT- III deficiency	**A group n=117**	**B group n=120**	***p* value**
FVL			
Homozygous WT	96 (82.06 %)	110 (91.66 %)	
Heterozygous	21 (17.94 %)	10 (8.33 %)	**0.034**
FII			
Homozygous WT	100 (85.47 %)	114 (95 %)	
Heterozygous	17 (14.53%)	6 (5 %)	**0.0156**
***MTHFR* C677T**			
Homozygous WT	50 (42.74 %)	64 (53.33 %)	
Heterozygous	48 (41.03 %)	32 (26.67 %)	**0.0295**
Homozygous mutant	19 (16.23 %)	24 (20 %)	1
***MTHFR* A1298C**			
Homozygous WT	53 (45.3 %)	72 (60 %)	
Heterozygous	47 (40.18 %)	30 (25 %)	**0.0136**
Homozygous mutant	17 (14.52 %)	18 (15 %)	0.5658
Compound heterozygous MTHFR C677T/ A1298C	27 (23.07 %)	13 (10.83 %)	**0.0401**
protein S (activity)			
Deficiency	12 (10.25 %)	10 (8.33 %)	0.6592
protein C (activity)			
Deficiency	7 (5.98 %)	2 (1.66 %)	0.0992
Antihrombin III (activity)			
Deficiency	3 (2.56 %)	2 (1.66 %)	0.6809
All thrombophilias (no. of mutant alleles)	227	176	

p <0.0001**** (Fisher's exact test, group A vs. group B; (homozygous and heterozygous genotypes of thrombophilia variants versus homozygous wild-type genotype)
Values given in bold show statistical significance ($p < 0.05$)

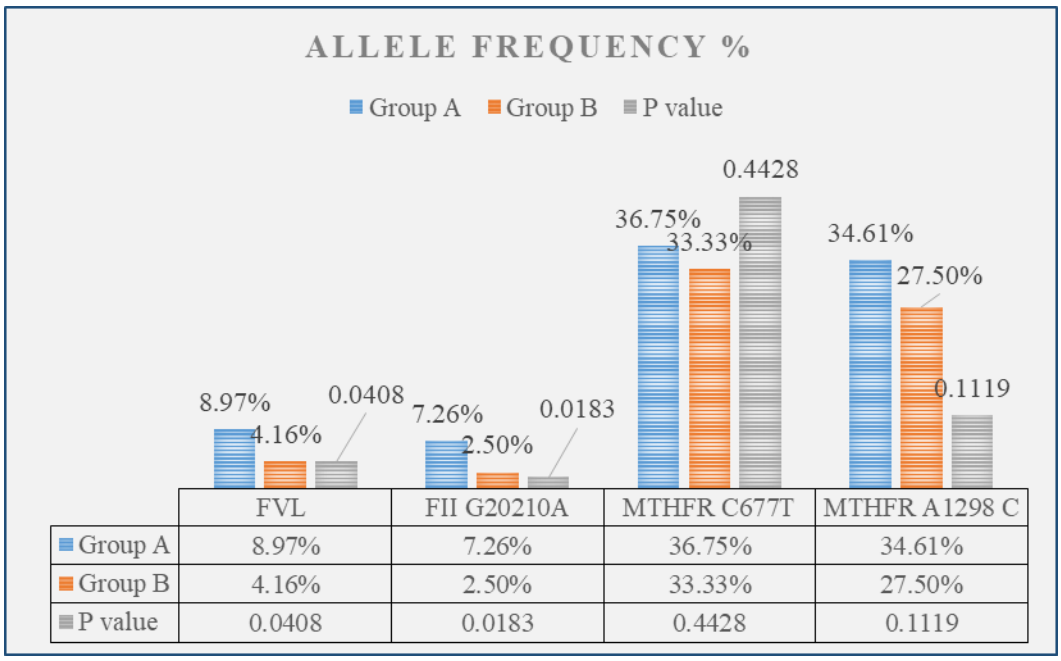
The most frequently detected mutations in all the 237 analyzed individuals were the two tested MTHFR mutations, c677C>T (123 patients/ 51.89%) and c1298A>C (112 patients/ 47.25%). The factor V Leiden heterozygous mutation was found in 13.08% of the entire cohort (31 patients), the heterozygous prothrombotic mutation in 9.7% (23 patients), followed by deficiencies in protein S (22 patients/ 9.28 %), protein C (9 patients/ 3.79 %) and AT-III (5 patients/ 2.1%).

In the entire, A group, the number of identified risk alleles was 227 (out of which 36 were in a homozygous state). Therefore, more than one thrombophilic defect was identified for a single patient with a maximum number of 3 genetic variants per patient.

The prevalence of thrombophilia was statistically significantly different between groups (p=0.0319).

One hundred thirteen patients in group A (96.58%) carried at least one pathogenic genetic variant, while just 4 (3.41%) had none. In comparison, 104 patients in the B group (86.67%) had at least one mutation, while 16 (13.33%) had none.

Deficiencies of proteins S and C and AT-III in group A were correlated with the presence of at least one mutation. In group A, protein S, protein C and AT-III deficiencies were correlated with thrombophilic mutations in F5 and F2 genes in eight of the 22 patients (36.36%).



	FVL	FII G20210A	MTHFR C677T	MTHFR A1298 C
Group A	8.97%	7.26%	36.75%	34.61%
Group B	4.16%	2.50%	33.33%	27.50%
P value	0.0408	0.0183	0.4428	0.1119

Figure 1. Distribution of allele frequency.

The allele frequency and genotype distribution for the FVL (G1691A) and FII (G20210A) mutations were significantly higher in group A in comparison to group B: 8.97% and 7.26% in group A compared to 4.16% (p=0.0484) and 2.5% (p=0.0183) in group B.

Between groups, no statistically significant difference was found among the allele frequencies of the MTHFR c677C>T and MTHFR c1298A>T, between the two groups (p=0.4428; p=0.1119). The results are displayed in Figure 1.

The majority of patients in the VTE group were male (53.84% male vs. 46.15% female), similar to literature data by Maria Khan *et al*. [25], in contrast with group B, in which the majority of patients were female (65.83% female vs. 34.16 % male p= 0.0026).

A considerably higher number of female and male patients were above the age of 40 in group A compared to the patients in group B. (p <0.0001, Fisher's exact test) [26].

Of the total number of patients tested for a complete panel of thrombophilia genetic variants, containing both prothrombotic mutations and the common MTHFR variants, which are known to have a controversial involvement in the hypercoagulable state, a significant rate of positive results was observed.

Our findings identified F5, F2 and/or MTHFR mutations and/or antithrombin III, protein S, protein C, and low levels in 191 of the 117 included patients in group A and 134 of the 120 group B individuals.

The most frequent ones being the c.677C>T (123 patients/ 51.89%) and c.1298A>C (112 patients/ 47.25%) MTHFR mutations, for the all patients in the analyzed cohort.

The FVL, prothrombin mutations and protein S deficiency were detected in approximate 10% of all patients (31 patients/ 13.08%; 23 patients/ 9.7%; 22 patients/ 9.28% respectively). The lowest frequencies were observed for protein C and AT-III deficiencies (9 patients/ 3.79 %; 5 patients/ 2.1%, respectively).

3.2.1. Factor V Leiden.

FVL and prothrombin G20210A mutation is the most common inherited factors associated with thrombosis [27-29]. FVL mutation is an autosomal dominant condition detected in 40% to 60% of patients with hereditary thromboembolism [30]. The incidence of FVL relies on the race. Varies from 5.27-11% in Caucasians to about 1% in Africans [31]. In unselected VTE patients, the FVL prevalence was approximately 20% [32], heterozygous mutations of FVL being observed in 12–20% of patients with incidental VTE and 40–50% of patients with the familial predisposition. Homozygous FVL mutations are less frequent, with an incidence rate of 0.02 % [33]. The risk of thrombosis is a -fold increase in heterozygotes and a 50-fold in homozygotes [34]. In the study by Kreidy *et al*., 9.9% of the analyzed Lebanese patients with VTE were heterozygous for the FVL mutation, while in the study of Jackson *et al*., 17.7% had FVL mutation [35, 36]. In a study conducted by Khalid *et al*., the FVL mutation was detected in 14.2% of all patients with VTE. [37]. Also, Michel C.A. *et al.* found that among the patients with thrombotic events history, 21.5% were carriers for FVL mutation in heterozygosity [38]. In the current study, the carrier frequencies for FVL (17.94%) mutations detected in cohort A are comparable with the results reported in the aforementioned studies. The frequencies of the FVL (8.33%) detected in cohort B, are comparable with the results reported in the general population.

3.2.2. Prothrombin G20210A mutation.

The prothrombin G20210A mutation has an incidence that varies from 1% to 6% in general Caucasians and from 5% to 19% in patients with VTE [39-41]. The prothrombin gene mutation was very rare in individuals from Asia and African countries [42]. Homozygosity for this variant is rarer than homozygosity for the g.1691G>A variant. However, the risk for VTE is high and has been reported to be 30 times increased [34].

Its incidence rate was reported to be 2.6% in a healthy Turkish population. In a study conducted by Ekim *et al*., the heterozygotes FII was detected in 5.5% of a healthy Turkish population [43]. In different studies, its incidence rate has been reported to range from 6.3–7.7% in patients with pulmonary embolism and 6–16% in patients with VTE [33]. In the current study, the carrier frequencies for prothrombin G20210A mutations detected in cohort A (14.53%) are comparable with the results reported in the aforementioned studies. The frequencies of the prothrombin G20210A (5%) mutations detected in cohort B are comparable with the results reported in the general population.

3.2.3. MTHFR C677T and A1298C mutations.

In conformity with data from the 1000 Genomes project, approximately 36% of the European population carries the T allele for the C677T variant, and the estimated frequency of the homozygous TT genotype was 13.5%. The frequencies of the A1298C variant and the CC genotype in Europeans are 31% and 11%, respectively [44]. Although point mutations in the MTHFR gene were associated with a higher risk for arterial and venous thrombosis, a recent study found no relationship between these mutations and an increased VTE risk. The MTHFR C677T gene mutation incidence rate in the Turkish population was reported to range between 20.0–34.9% [33].

In our selected cohorts, the MTHFR c.677C>T and c.1298A>C variants were detected in 57.26% and 54.7%, respectively, in group A and 46.67% and 40%, in group B. The results

are greater than those mentioned in the general population from Europe because of a higher heterozygotes frequency.

Both MTHFR variants were found more frequently in subjects with thromboembolic events than in those from group B.

A statistically significant difference was found for the compound heterozygous status MTHFR C677T/ A1298C between the two groups, 27 patients (23.07 %) in the VTE group were carrying both MTHFR variants in a heterozygous state. In comparison, 13 patients in group B (10.83%) were compound heterozygotes carrying both mutations (p=0.0401). Heterozygous or homozygous and specially compound heterozygous MTHFR variants are related to a higher risk of VTE. VTE occurred in 50% of patients with the compound mutation. [45] This result is consistent with our study. Yet, Coriu and colleagues, in 2014, retrospectively performed genetic analyses of 151 pregnant women. No association was found between the presence of thrombosis and the compound heterozygous MTHFR C677T/A1298C status (OR, 0.63; 95% CI, 0.23-1.7; p=0.36) [46].

The results of the current study detected a higher carrier rate for any of the two tested MTHFR mutations than reported for the Caucasian population, our findings suggesting at the same time a statistical correlation with VTE that is consistent with the literature (p=0.0295; p=0.0136) [45, 47, 48].

Some studies confirm the results obtained in our research, all of them presenting a high prevalence of the MTHFR heterozygous variants, but were unable to associate these results with VTE [10, 35, 36, 49, 50, 51], thus making it difficult to come to a definite conclusion.

3.2.4. Protein C, Protein S, Antithrombin.

Apart from FVL and prothrombin mutations, three major genes have been identified as having significant relevance in thrombophilia expression: protein C, protein S, and AT-III. These factors are routinely screened in the investigation for the cause of VTE. Protein C and protein S are vitamin K-dependent plasma proteins and are a component of the anticoagulant system.

Measuring levels while using anticoagulants, warfarin, heparin, and direct oral anticoagulants (DOAC) can cause significantly different values resulting in an erroneous diagnosis [52-56].

Patients with hereditary deficiency of PS, PC, or AT-III have a higher risk of developing VTE, which ranges from 2 to 11, compared to those without this deficiency. In a comprehensive study by Khalid *et al*. 2004, performed in Pakistan, Protein C deficiency was detected in 2.3% and protein S deficiency in 1.4% of all analyzed individuals [37].

In a meta-analysis of CVA patients, the frequency of PC & PS deficiency has been reported as 14%, 19%, 23% in patients younger than 45 years old, and 6% in patients younger than 60 years old [49].

It is estimated that 50% of people with AT deficiency will present one or more episodes of thrombosis throughout their lifetime. Almost 40% of these events happen without a triggering factor being specifically identified [57, 58].

Antithrombin III deficiency was observed in 2.56% of patients with thrombophilia. In a study conducted by Khalid *et al*., the frequency was 1.5%, and other international studies reported frequencies of 5.5% -7.5%, however [35-37]. Antithrombin III activity is affected by heparin therapy if not considered prior to the assay. In patients on heparin therapy, false-positive results are expected.

Deficiencies of proteins C and S due to gene mutations lead to moderate to severe thrombosis, causing disabilities and critically affecting life quality [59, 60].

In our study, we have normal values of proteins C and S. The prevalence of inherited natural anticoagulants was comparable between groups.

Protein C, S, and AT-III deficiencies in group A with thrombosis were not statistically significant differences in comparison with group B (p=0.6592, p= 0.0992, p= 0.6809). This is comparable with the results of Ufuk *et al*. in 2020 [61].

In one cohort family study, arterial events were diagnosed in 8% of the 144 subjects with protein C or S deficiencies and 1% out of the 94 subjects with antithrombin deficiency [58].

Deficiencies of proteins S and C and AT-III in group A, were correlated with the presence of at least one mutation. In group A, protein S, protein C and AT-III deficiencies were correlated with thrombophilic mutations in FVL and prothrombin G20210A mutation in eight of the 22 patients (36.36%). Heterozygous factor V Leiden and factor II, correlated with inherited lack of natural anticoagulants (protein C, protein S, and antithrombin III) are considered risk factors for thrombotic events [62].

Our observation that at least one inherited risk factor was present in most VTE patients is consistent with previous studies. We observed that the presence of at least one inherited risk factor in an individual patient increased the risk of VTE, in accordance with earlier studies [63, 64].

Knowing that the genetic background increases the risk of thromboembolic events, thrombophilic anomalies have to be analyzed: in patients under 50 years of age; in recurrent and suspicious situations;- during pregnancy and after delivery; -in women under oral anticoagulant treatment or post-menopause hormonal substitution; -in individuals with family history (first-degree relatives) of thrombosis below 50 years of age; - in patients with thrombosis of the cerebral, mesenteric, portal or hepatic veins; - in the case of abortions after ten weeks of pregnancy [65].

Thrombophilia is a pathological disorder caused by many risk factors and associated with hypercoagulability and a tendency towards thrombosis [66].

By determining the hereditary risk factors in patients with a previously described VTE episode, family members with a predisposition to thrombosis, family genetic testing is recommended for relatives at risk for carrying the pathogenic mutation, which is allowed to prevent thromboembolic attacks by avoiding the acquired risk factors [61].

4. Conclusions

In conclusion, this study emphasizes the high prevalence of heterozygotes FVL and FII in patients with VTE. In these two groups of patients, the frequency of hereditary thrombophilia was significantly different between those with and without thromboembolic pathology.

Hereditary thrombophilias are observed more frequently in older age groups and are less common in patients under 40 years of age.

Our study demonstrated that heterozygosity for the FVL, FII, and MTHFR C677T/A1298C variants is more frequently observed among patients with thromboembolic events compared with the non-VTE group and that it can increase the risk of blood clots occurrence.

These results raise the chance that these variants increase venous thromboembolism susceptibility. No statistical correlation with VTE was detected for antithrombin - III, protein C, and protein S deficiency.

Even though solid, the result of the current study needs further confirmation through wider research projects.

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Conflicts of Interest

The authors declare no conflict of interest.

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