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Thrombophilia genetic testing in Romanian young women with acute thrombotic events: role of Factor V Leiden, Prothrombin G20210A, MTHFR C677T and A1298C polymorphisms

Evaluarea genetică a trombofiliilor la femei tinere din România cu evenimente acute trombotice: rolul Factorului V Leiden, Protrombinei G20210A, polimorfismelor MTHFR C677T și A1298C

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Abstract

Objective: *The present case-control study aimed at evaluating the contribution of thrombophilic polymorphisms to acute venous (VTE) as well as arterial thrombotic events (ATE) in a population of young women with few traditional thrombotic factors (CVRF).*

Methods: *We consecutively enrolled patients under 45 years of age, with less than 3 CVRF, evaluated for VTE or ATE, women and men as a comparator. The control group consisted of healthy young women. A thrombophilia panel and genetic testing for Factor V Leiden (FVL), G20210A Prothrombin and MTHFR polimorphisms were done.*

Results: *A total of 323 persons were enrolled: 71 women and 121 men with thromboembolic events, and 131 healthy female as controls. Hyperhomocysteinemia was more frequent in ATE (30.4%) than VTE female patients (6.25%), p<0.01. Genetic testing was available in 45 women and 84 men with acute thrombotic events and in all controls. Homozygous FVL was associated with VTE in young women (10.3% vs 0% controls, p<0.01). Prothrombin G20210A polymorphism had the lowest prevalence - 5.4% and only heterozygosity was found. MTHFR C677T heterozygosity showed no significant difference between women patients and controls (62.2 % vs 43.5% respectively, p=0.1). The homozygous status, less frequent (6.6%), was not associated with ATE or VTE. Homozygous MTHFR A1298C was associated with VTE in women (17.2% patients vs 4.5% controls, OR 4.34, p 0.02, CI 1.22-15.3).*

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Conclusion: *In young women with few CVRF, mild hyperhomocysteinemia, homozygosity for FVL and for MTHFR A1298C polymorphisms increase the risk for VTE but not ATE. MTHFR polymorphisms are found with increased frequency in both healthy persons and patients therefore, their significance as an important thrombotic risk modifier remains unclear.*

Keywords: *Thrombophilia;Factor V Leiden;Prothrombin G20210A;MTHFR C677T and A1298C.*

Rezumat

Scop: *Acest studiu caz-control a dorit evaluarea contribuţiei polimorfismelor trombofilice la apariţia evenimentelor acute trombotice venoase (VTE) şi arteriale (ATE) la femei tinere cu puţini factori de risc trombotici tradiţionali (FRCV).*

Material şi metodă: *Am inclus consecutiv pacienţi sub 45 ani, cu mai puţin de 3 FRCV, evaluaţi pentru VTE şi ATE, femei şi bărbaţi ca şi comparator. Grupul control a constat în femei tinere sănătoase. S-a efectuat un panel de trombofilie şi testare genetică pentru Factor V Leiden (FVL), Protrombina G20210A şi polimorfismele MTHFR.*

Rezultate: *Am inclus 323 de persoane: 71 femei şi 121 bărbaţi cu evenimente tromboembolice şi 131 femei sănătoase ca şi control. Hiperhomocisteinemia a fost mai frecventă la femeile cu ATE (30.4%) comparativ cu cele cu VTE (6.25%), p<0.01. Testele genetice au fost disonibile la 45 femei si 84 barbati cu evenimente trombotice acute, si la tot lotul control. FVL homozigot a fost asociat cu VTE la femei (10.3% vs 0% control, p<0.01). Protrombina G20210A a fost găsită cu cea mai joasă prevalenţă - 5.4% şi doar forma heterozigotă. Prevalenţa MTHFR C677T heterozigot nu a fost semnificativ diferită între femeile pacient şi control (62.2 % vs 43.5%, p=0.1). Statusul homozigot, mai rar (6.6%), nu a fost asociat cu ATE sau VTE. MTHFR A1298C homozigot a fost asociat cu VTE la femei (17.2% vs 4.5% control, OR 4.34, p 0.02).*

Concluzii: *La femeile tinere cu puţini FRCV, hiperhomocisteinemia uşoară, statusul homozigot pentru FVL şi MTHFR A1298C cresc riscul de VTE dar nu şi de ATE. Polimorfismele MTHFR au frecvenţă crescută atât la pacienţi cât şi la persoanele sănătoase, semnificaţia lor ca factori de risc trombotic rămânând neclară.*

Cuvinte cheie: *Trombofilie;Factor V Leiden;Protrombina G20210A;MTHFR C677T si A1298C.*

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INTRODUCTION

Venous thromboembolism (VTE) is a common condition, affecting an estimated 1-2 person per 1000 annually (1). Substantial morbidity and mortality result from the development of VTE in both young and elderly individuals. Furthermore, arterial thrombosis events (ATE), consisting of acute coronary syndromes or ischemic stroke, bring significant socio-economic impact especially when they affect the young population (2).

Young patients with acute venous or arterial thrombotic events usually have a different profile of risk factors than older people. In these patients other factors, such as inherited thrombophilia, may play a significant role (3). In recent decades several hereditary coagulation abnormalities have been identified as additional risk factors for thrombotic events and the most frequently found genetic thrombophilic factors in the European Caucasian population were Factor V Leiden (FVL) (1-14% in the healthy population and 9-50% in VTE) and factor II – prothrombin G20210 A polymorphisms (0-5% in the healthy population and 3.2-17.2% in VTE) (4, 5). Although the predominant clinical manifestation of inherited thrombophilia is venous thrombosis, its contribution to arterial thrombosis still remains debated (6).

Venous and arterial thrombotic events used to be regarded as two separate entities, however, this hypothesis has changed in recent years. On the one hand, there are conventional cardiovascular risk factors (CVRF) common to both dis-

orders, such as smoking, obesity, older age. On the other hand, there are studies showing an increased risk of arterial thrombotic complication in patients with VTE (7). The role of inherited thrombophilia in ATE is less clear but hyperhomocysteinemia, inherited or acquired, has been demonstrated to be an independent risk factor for atherothrombosis (8). Two polymorphisms of the methylenetetrahydrofolate reductase (*MTHFR*) gene - C677T and A1298C - are the most common inherited causes of hyperhomocysteinemia. Homozygosity for these alleles usually leads to mild to moderate elevations of serum homocysteine and a risk for arterial thrombosis (9).

One special population in which thromboembolic events and identification of genetic thrombophilias have a big socio-economic and psychological impact is represented by young women. Due to the different hormonal and risk factor profile, young women are generally considered at lower risk for arterial or venous thrombotic events than men. However, oral contraception and hormone replacement therapy are recognized risk factors for thrombosis and in the presence of an inherited thrombophilia, this risk is further increased (10). Therefore, identification of a thrombophilic mutation in young women poses several management problems and even though acute venous or arterial thrombosis in these persons represents a rare event, it can have a high mortality and a big impact on the further management. Unfortunately, the current knowledge and guidelines are still controversial related to thrombophilia screening in young women with an unprovoked VTE and even less data are available for ATE (11, 12).

The present case-control study aimed at evaluating the contribution of thrombophilic polymorphisms to acute venous as well as arterial thrombotic events in a population of young women with few traditional thrombotic factors, comparing prevalence of genetic factors with both young men with thromboembolic events and a healthy population of young women.

MATERIAL AND METHODS

Patient population

Between 2010 and 2013 we studied consecutive young patients, irrespective of sex, under 45 years of age, admitted to or referred for consultation in our hospital for acute thrombotic events, either for VTE (deep vein thrombosis or pulmonary thromboembolism) or ATE (acute coronary syndromes, stroke, peripheral arterial ischemic events). Exclusion criteria were the presence of more than 3 traditional cardiovascular risk factors (CVRF) (such as smoking, arterial hypertension, dyslipidemia, obesity) or diabetes mellitus, pregnancy, and the presence of comorbidities known to predispose to thrombotic diseases, such as cancer (including hematological malignancies), fracture of lower limb or major trauma, hip or knee replacement, chemotherapy, congestive heart or respiratory failure, infections, post-partum period, prolonged immobilization.

Diagnosis of VTE or ATE was done according to the current guidelines of the European Society of Cardiology. Venous thromboembolism was confirmed by either venous Duplex Ultrasound or CT scan. In the case of ATE, electrocardiograms, cardiac biomarkers, echocardiography and angiography were employed to establish the diagnosis. Patients were treated and followed according to the standard clinical practice protocol.

In order to appreciate the real contribution of thrombophilic polymorphisms in the women population, the study was designed as an observational case-control study; therefore, we also enrolled and tested a control group of 131 healthy young women randomly selected from

the Romanian general population. The healthy population was defined by the absence of a venous or arterial thrombotic history, or spontaneous abortions.

Informed consent was obtained from all patients and controls and their participation was voluntary. The study was approved by the Ethics Committee of the hospital.

Laboratory tests

In male and female cases blood samples were taken in order to test for a thrombophilia panel – genotype analysis for FVL, Factor II G20210A and MTHFR mutations, as well as protein C (PC), protein S (PS), antithrombin III (AT III), antiphospholipid syndrome testing (lupus anticoagulant, anticardiolipin antibodies and Beta 2 glycoprotein 1(GP1)) and serum levels of homocysteine.

For the genotype analysis, 5 ml samples of peripheral blood were collected in K2-EDTA tubes and stored at -20°C until DNA isolation. The FVL and Factor II G20210A were genotyped by real time polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphism (RFLP) assays, while MTHFR C677T and A1298C polymorphisms were genotyped by PCR-RFLP and allele-specific PCR assays, as previously described, with minor modifications (13-16).

The Factor V Leiden was genotyped either by a real-time TaqMan assay or by a PCR-RFLP assay, as previously described [13]. Briefly, a 267-bp fragment from the Factor V gene was obtained by PCR, using the primers previously described [13]. The PCR amplification consisted in an initial denaturation at 95°C for 10 min, followed by 40 cycles, each comprising denaturation at 95°C for 40s, annealing at 55°C for 40s and extension at 72°C for 2 min. A final extension at 72°C for 7 min was also performed. Ten microliters of the amplicon were digested overnight at 37°C with 2U of the restriction enzyme MnlI (Fermentas MBI, Vilnius, Lithuania). The wild-type allele was cut in three fragments of 163, 67 and 37 bp, while the Factor V Leiden allele abolishes one of the restriction sites, yielding two fragments, of 200 and 67 bp.

The Prothrombin G20210A was genotyped by a real-time TaqMan assay or by a PCR-RFLP assay, as previously described [14]. Briefly, a 195-bp fragment from the prothrombingene was obtained by PCR, using the primers previously described [14]. The PCR amplification consisted in an initial denaturation at 94°C for 5 min, followed by 30 cycles, each comprising denaturation at 94°C for 20s, annealing at 52°C for 30s and extension at 72°C for 30s. A final extension at 72°C for 5 min was also performed. Ten microliters of the amplicon were digested overnight at 37°C with 2U of the restriction enzyme HindIII (Fermentas MBI, Vilnius, Lithuania). The wild-type allele remained uncut, while the prothrombin G20210A allele creates a restriction site, yielding two fragments of 176 and 19 bp.

The MTHFR C677T polymorphism was genotyped by a PCR-RFLP assay, as previously described, with minor modifications [15]. Briefly, a 265-bp fragment from the MTHFR gene was obtained by PCR, using the primers previously described [15]. The PCR amplification consisted in an initial denaturation at 94°C for 5 min, followed by 35 cycles, each comprising denaturation at 94°C for 30s, annealing at 57°C for 30s and extension at 72°C for 30s. A final extension at 72°C for 5 min was also performed. Ten microliters of the amplicon were digested overnight at 37°C with 2U of the restriction enzyme HinfI (Fermentas MBI, Vilnius, Lithuania). The wild-type allele remained uncut, while the MTHFR C677T allele creates a restriction site, yielding two fragments of 171 and 94 bp.

The MTHFR A1298C polymorphism was genotyped by an AS-PCR assay, as previously

described, with minor modifications [16]. The common reverse primer was combined with the corresponding forward primer (wild-type and polymorphic specific). The PCR amplification consisted of an initial denaturation at 94°C for 5 min, followed by 30 cycles, each comprising denaturation at 94°C for 30s, annealing at 58°C for 20s and extension at 72°C for 20s. A final extension at 72°C for 5 min was also performed. The size of the amplicon was 296-bp in case of both alleles (wild-type and polymorphic).

In the case of PCR-RFLP assays for Factor V Leiden and Prothrombin G20210A, the DNA fragments were resolved in 3% high resolution agarose gel electrophoresis (MetaPhor, Lonza, Rockland, ME, USA), while the DNA fragments obtained by the assays for MTHFR polymorphisms were resolved in 2% agarose gel electrophoresis (Seakem, Lonza, Rockland, ME, USA), all stained with ethidium bromide.

The coagulation tests were performed in a central experienced laboratory of hemostasis, in the absence of current anticoagulant therapy. Determination of PC was done by the chromogenic method (Stachrom® Protein C) (normally 70-140%), determination of free PS was done by immunological assays (Liatest® Free Protein S): (normally 54-123% female), determination of AT III was done by the chromogenic method (Stachrom ATIII®) (normal 80-120%), a simplified dilute Russell's venom test for detection of lupus anticoagulant was used (Siemens LA1 and 2 reagent®) (normal negative), and for anti-cardiolipin and anti-Beta 2 GP1, Ig G, and IgM antibodies an ELISA based method (ORGENTEC Diagnostika GmbH®) was used (normal 0-15 EU/Ml for anticardiolipine antibodies and 0-20 EU/mL for anti-beta 2 GP1 antibodies). The laboratory uses its own age and sex matched controls for these coagulation tests.

Fasting serum levels of homocysteine were determined by an enzymatic assay using EDTA plasma. Hyperhomocysteinemia was defined as values above 12 μmol/l.

Statistical analysis

The categorical variables were presented as counts and percentages while continuous variables were presented as mean values ± standard deviation. The frequency of the genotypes in the groups was compared using two-by-two contingency table and analyzed with chi-square $\chi 2$ with Yates correction test. The Fischer exact test was used for those groups in which relative frequencies were low. The strength of the association between genotype and thrombotic risk was evaluated by odds ratio (OR) with 95% confidence intervals (CI). Continuous variables were compared using Student T test. A p value < 0.05 was considered statistically significant. Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS) 16.0 for Windows.

RESULTS

General characteristics

This study enrolled a total of 323 persons of which 192 young patients (71 women, 36.9%) with thromboembolic events and 131 young female controls. Demographic characteristics are summarized in **Table 1**. Females with ATE had a family history of ATE at young age more frequently than men. In turn, men, although young, under 45 years of age, had already a mean of 1.9 CVRF in the ATE group, significantly more than in the corresponding female group. Moreover, as shown in **Table 2**, both men and women with ATE were older than VTE cases.

Thrombophilia panels

In the study population, genetic testing was available in 45 female (29 VTE, 16 ATE patients) and 84 male (18 VTE, 66 ATE patients) patients.

Table 1. Demographic description and medical history data in the study groups.

Parameter	71 FEMALE PTS		CONTROLS (C)	p value	p value	121 MALE PTS		P value	P value
	VTE	ATE		VTE vs C	ATE vs C	VTE	ATE	VTE vs F - VTE	ATE vs F - ATE
No. of patients	48	23	131	-	-	37	84	< 0.01	< 0.01
AGE (years, mean ± SD)	30.1 ± 8.2	41.4 ± 9.6	37.4 ± 7.6	< 0.01	0.07	32.8 ± 13.2	39.2 ± 7.7	0.3	0.3
History of recurrent thrombotic events	15 (31.2%)	5 (21.7%)	0 (0%)	-	-	18 (48.6%)	15 (17.8%)	0.1	0.7
Family history of CV events - Arterial	3 (6.2%)	9 (39.1%)	-	-	-	4 (10.8%)	16 (19.0%)	0.6	0.05
Family history of CV events - Venous	7 (14.5%)	0 (0%)	-	-	-	6 (16.2%)	1 (1.1%)	1	1
History of spontaneous abortion	8 (16.6%)	4 (17.3%)	0 (0%)	-	-	-	-	-	-
No. of patients with recurrent spontaneous abortion	1 (0.2%)	0 (0%)	0 (0%)	-	-	-	-	-	-
Mean no. CVRF no ± SD	0.86 ± 0.8	1.5 ± 1.3	-	-	-	1.1 ± 1	1.9 ± 1.1	0.01	< 0.01

ATE – arterial thromboembolic event; VTE – venous thromboembolic event; F-VTE – female VTE; F-ATE – Female ATE; CVRF – cardiovascular risk factors; SD – standard deviation.

Table 2. Non-genetic thrombophilia panel – frequency and comparison between ATE (arterial thromboembolic events) and VTE (venous thromboembolic events) groups of thrombophilic factors in young.

	FEMALE			MALE		
Thrombophilia test	VTE	ATE	P value	VTE	ATE	P value
No. of patients	48	23	-	37	84	-
AGE (years, mean ± SD)	30.1 ± 8.2	41.4 ± 9.6	< 0.01	32.8 ± 13.2	39.2 ± 7.7	0.01
Protein C Deficiency (< 70%)	6 (12.5%)	3 (13.0%)	0.94	6 (16.2%)	5 (5.9%)	0.08
Protein S Deficiency (< 60%)	17 (35.4%)	6 (26.0%)	0.43	7 (18.9%)	18 (21.4%)	0.8
Antithrombin III Deficiency (< 80%)	1 (2.0%)	1 (4.3%)	0.29	2 (5.4%)	4 (4.7%)	1
Lupus anticoagulant	8 (16.6%)	5 (21.7%)	0.60	3 (8.1%)	21 (24%)	0.04
Anticardiolipin antibodies	1 (2.0%)	0 (0%)	0.48	1 (2.7%)	0 (0%)	0.3
Beta 2 GP 1 (> 21 U/mL)	10 (20.8%)	7 (30.4%)	0.78	13 (35.1%)	42 (50%)	0.1
APS	1 (2.08)	0 (0%)	0.48	1 (2.7%)	0 (0%)	0.3
Hyperhomocysteinemia (> 12 µmol/L)	3 (6.25%)	7 (30.4%)	< 0.01	9 (24.3%)	30 (35.7%)	0.2

SD – standard deviation; APS – antiphospholipidic syndrome.

Factor V Leiden was present in 20 patients (15.5%; 11.1% women and 17.8% men, p NS) with acute thrombotic events, 17.0% in VTE and 14.6% in ATE patients. The prevalence of FVL was 8.3% in the control group. In women with VTE, homozygosity for FVL was significantly more frequent than in controls (**Table 3**).

Prothrombin G20210A polymorphism presented the lowest prevalence – 5.4% and only the heterozygote type was found. However, it was also found in control women with a frequency of 2.2% and had no significant association with thrombotic events (**Table 3**).

Only females with ATE had hyperhomocysteinemia more frequently, with no other differences between ATE and VTE group regarding the non-genetic thrombophilia panel. The prevalence of protein C and S, as well as ATIII deficiency were similar in all groups, and while lupus anticoagulant appeared as more prevalent in male patients with ATE, it was not associated with positivity of other antiphospholipid syndrome specific tests.

MTHFR C677T heterozigosity was found in 48.0 % of patients, 55.3% in the VTE and 43.9% in the ATE group with no difference between sexes. The prevalence of this mutation was very high in the female control group (43.5% for the MTHFR C677T heterozygous state, 38.1% for MTHFR A1298C heterozygous state), without any significant difference compared with women patients, showing no association with thrombotic events in this young group. In turn, the homozygous state was found with a lower frequency of

Table 3. Genetic mutation frequency in young women with acute thrombotic events compared with young healthy women.

Mutation	HETEROZYGOSITY			VTE vs. CONTROL				ATE vs. CONTROL			
	VTE (29)	ATE (16)	CONTROL (131)	P	OR	95% CI LL	95% CI UL	P	OR	95% CI LL	95% CI UL
Factor V Leiden (G1691A) (n, %)	2 (6.8%)	0 (0%)	11 (8.3%)	0.56	0.8	0.16	3.85	0.5	-	-	-
Prothrombin Mutation (G20210A) (n, %)	2 (6.8%)	1 (6.2%)	3 (2.2%)	0.22	3.1	0.5	19.8	0.37	2.84	0.27	29.1
MTHFR C677T(n, %)	18 (62.0%)	10 (62.5%)	57 (43.5%)	0.10	2.12	0.93	4.85	0.24	2.16	0.74	6.30
MTHFR A1298C (n, %)	11 (37.9%)	5 (31.2%)	50 (38.1%)	0.84	0.99	0.43	2.26	0.79	0.73	0.24	2.24
Mutation	**HOMOZYGOSITY**			**VTE vs. CONTROL**				**ATE vs. CONTROL**			
	VTE (29)	ATE (16)	CONTROL (131)	P	OR	95% CI LL	95% CI UL	P	OR	95% CI LL	95% CI UL
Factor V Leiden (G1691A) (n, %)	3 (10.3%)	0 (0%)	0 (0%)	< 0.01	-	-	-	-	-	-	-
Prothrombin Mutation (G20210A) (n, %)	0 (0%)	0 (0%)	0 (0%)	-	-	-	-	-	-	-	-
MTHFR C677T (n, %)	1 (3.4 %)	2 (12.5%)	11 (8.3%)	0.47	0.38	0.04	3.14	0.63	1.55	0.31	7.75
MTHFR A1298C (n, %)	5 (17.2%)	1 (6.2%)	6 (4.5%)	0.02	4.34	1.22	15.37	1	1.38	0.15	12.33

ATE – arterial thromboembolic event; VTE – venous thromboembolic event; MTHFR - methylenetetrahydrofolate reductase; CI – confidence interval; LL – lower limit, UL – upper limit; OR – Odds ratio.

13.9%, 2.1% in the VTE and 20.7% in the ATE group (p value VTE vs ATE 0.00, OR 0.08, CI 0.01-0.64).

MTHFR A1298C heterozygosity was found in 44.1 % of patients, 48.9% in the VTE and 41.4% in the ATE group with the prevalence being higher in the TVP male group than in the TVP women group, however, without reaching statistical significance (66.6% vs 37.9%, p 0.1). The prevalence of this mutation was also very high in the female control group, without any significant difference compared with women patients (**Table 3**). The homozygous status was found with a frequency of 10.0%, 12.7% in the VTE and 8.5% in the ATE group (p value VTE vs ATE 0.2, OR 0.27, CI 0.03 - 2.3). However,

the frequency of the homozygous status was found with a higher prevalence in women with VTE than controls with an OR 4.34 (p 0.02, CI 1.22-15.3).

In women, 93.1% of the VTE (p 0.07 against control, OR 3.66; 95% CI 0.82 – 16.37) and 14 (87.5%) from the ATE (p 0.40 against control, OR 1.9, 95% CI 0.4 – 8.87) group had at least one genetic mutation with no differences between these two groups (p 0.79). In turn, 78.6% had at least one genetic mutation in the control group. Moreover, complex mutations (one MTHFR polymorphism associated with one of the G20210 A or FVL) was significantly more frequent (24.1%) in the VTE group compared with controls (p 0.02, OR 3.1; 95% CI 1.1 –8.9). In turn, 6.25% from the ATE (p 0.69, OR 0.66, 95% CI 0.08– 5.45) group and 9.1 % in the control group had a complex polymorphism. There were no differences between ATE and VTE group regarding the combined polymorphism prevalence.

The MTHFR polymorphisms are found with an increased frequency and as much as 89.6% from the VTE (p 0.13 vs. control, OR 2.57; 95% CI 0.72 –9.09) and 87.5% from the ATE (p 0.34 vs. control, OR 2.07, 95% CI 0.44 – 9.66) group had at least one MTHFR polymorphism. In turn, 101 (77.09 %) had a compound polymorphism.

Table 4. Gender differences in the frequency of thrombophilic mutations in patients with venous thromboembolic events (VTE). MTHFR - methylenetetrahydrofolate reductase.

Mutation	VTE					
	HETEROZYGOSITY		P Value	HOMOZYGOSITY		P Value
	FEMALES	MALES		FEMALES	MALES	
Factor V Leiden (G1691A) (%)	6.8%	11.1%	0.58	10.3%	5.5%	0.58
Prothrombin Mutation (G20210A) (%)	6.8%	5.5%	0.87	0 %	0%	-
MTHFR C677T(%)	62%	44.4%	0.29	3.4%	0%	0.43
MTHFR A1298C(%)	37.9%	66.6%	0.1	17.2%	5.5%	0.24

Table 5. Gender differences in the frequency of thrombophilic mutations in patients with arterial thromboembolic events (ATE). MTHFR - methylenetetrahydrofolate reductase.

Mutation	ATE					
	HETEROZYGOSITY		P Value	HOMOZYGOSITY		P Value
	FEMALES	MALES		FEMALES	MALES	
Factor V Leiden (G1691A) (n, %)	0%	18.1%	0.11	0%	0%	-
Prothrombin Mutation (G20210A) (n, %)	6.8%	4.5%	0.77	0%	0%	-
MTHFR C677T (n, %)	62.5%	39.3%	0.16	12.5%	22.7%	0.5
MTHFR A1298C (n, %)	31.2%	43.9%	0.52	6.25%	9.0%	1

DISCUSSIONS

The present study examined the contribution of four genetic thrombophilic polymorphisms to acute venous or arterial thrombotic events in a population of young women with few CVRF, and led to the following findings: young women seem to be more prone to VTE compared with young men; women with ATE had mild hyperhomocysteinemia more frequently than those with VTE; homozygosity for Factor V Leiden and MTHFR A1298C polymorphisms increase the risk for venous but not arterial thromboembolic events, with compound genotypes (one MTHFR polymorphism associated with one of the G20210 A or FVL) significantly more frequent in the VTE group compared with controls.

Acute thrombotic events in young patients are associated with important morbidity and a high socio-economic impact (2). These patients usually have a different profile of CVRF than older patients and thrombophilia is increasingly recognized as an additional causative factor in this particular population (17). Moreover, in the United States, despite an overall reduction in the death rate due to cardiovascular disease over the last several decades, the rate of decline is smaller for women than men(18).

Arterial and venous thrombotic events have been recently looked at as a spectrum of the same disease sharing similar risk factors (19). In our young patients, ATE occurred at an older age than VTE both in women and males. However, women had a familial history of acute thrombotic events at young age more frequently compared to males while the latter had cumulated more CVRF. This shows once again the multifactorial etiology of the disease involving both genetic as well as environmental risk factors for thrombotic events (20). Age is one of the factors modifying both the frequency of thrombotic events as well as the causative factors. The influence of inherited risk factors decreases with age whereas acquired factors begin to cumulate (21).

In our study, young women seem to be more prone to VTE while men present more frequently with ATE, in agreement with previously reported data in which, at a younger age, there is an overwhelming male majority in patients with acute myocardial infarction (MI) (22). Moreover, premenopausal women have a much lower incidence of heart disease compared to men of the same age explained mostly by the hormonal profile (23).

In our data, women with ATE had mild hyperhomocysteinemia more frequently than those with VTE. There are studies pointing at hyperhomocysteinemia as a risk factor mostly for ATE with a lower effect on VTE. However, this differentiation was not found in our study in males. Probably, females who develop CV disease 7 to 10 years later than men need additional risk factors to trigger the arterial thrombotic event (24). Interestingly, even though hyperhomocysteinemia was more frequent in ATE women, we found no differences in the prevalence of MTHFR polymorphisms between these two groups. In fact, current data from the literature suggests that only MTHFR C677T polymorphism is associated with increased homocysteine plasma levels while A1298C is not (25). Furthermore, several dietary factors such as folate or vitamin B12 intake can influence plasma homocysteine and could account for the discrepancies between genetic testing and homocysteine levels in our study group (26).

In our cohort, the prevalence of protein C deficiency was 10.4%, for protein S deficiency 25%, and for ATIII 4.1%, similar to the literature, and without differences between women and men.

One of the established heritable causes of VTE is FVL, a factor V resistant to activated protein C inactivation resulted from a single nucleotide substitution with a R506Q missense

mutation (27). In our study population, the prevalence of FVL was 17% in the VTE group, lower than that reported for Caucasians in South-East Europe (Serbia 29.9%, Croatia 21-28.2%, Hungary 44%) while in the control female group the prevalence was 8.3%, similar with other surrounding countries (5.8% Serbia, 6.9% Hungary) (28-30). Only the homozygous FVL was associated with VTE in our female population. Probably an additional prothrombotic factor is needed to prompt an acute event when only heterozygous FVL is present and indeed, previous studies have shown a 7-fold increase in the first VTE event in this latter case, whereas homozygosity confers an 80-fold higher risk of venous thrombosis by itself (27). FVL involvement in ATE is still controversial (31, 32). A case control study done in young Caucasian women showed a higher prevalence of MI in patients with FVL; however, while among non-smokers the FVL mutation had little effect, in the smokers it was associated with a 32-fold increased risk of MI (32). We found no FVL in the female ATE group and we could not conclude on this association.

This was also the case for prothrombin G20210A polymorphism, which was found only in the heterozygous form with a prevalence similar to that reported for European Caucasians of 3-17% in the VTE and 0.7-8% in the healthy population (5). The homozygous G20210A type is very rare and was not found in several VTE studies (33, 34). There was no association between heterozygous Factor II mutation and VTE in accordance with data from a large cohort showing that G20210A prothrombin gene variant is not associated with MI or stroke and the risk associated with VTE and VTE recurrence is small (35).

Hyperhomocysteinemia has been associated with ATE as well as with VTE, therefore MTHFR polymorphisms (C677T and A1298C) have been studied as causative factors in thrombotic events, more so in young patients. The results are still controversial. We found a high prevalence of both MTHFR polymorphisms in VTE and ATE patients as well as control females. Heterozigosity of MTHFR C677T and A1298C in the young population with acute thrombotic events was found with a frequency around the one reported in countries like Russia (52.6% for C677T) and Croatia (54% for C677T), being somewhat higher than the one reported previously in Romanian patients with deep vein thrombosis (32.2% for C677T and 31% for A1298C) (33, 34, 36, 37). In the young female group there was no association between heterozigosity of MTHFR polymorphisms and acute thrombotic events and the prevalence of heterozigozity for C677T and A1298C in the control/ healthy women was as high as 43.5% and 38.1%, respectively. In turn, homozygosity of both MTHFR mutation had a lower prevalence than reported in other studies (33, 34, 36, 37). However, contrary to other studies, our data suggests an association between homozygous A1298C MTHFR mutation and VTE in young women with a 4.35 increase in risk (38). MTHFR polymorphisms have been linked mostly to arterial thrombotic events and C677T was the most frequent variant studied. There are small studies and several meta analyses suggesting an influence of MTHFR C677T mutation on VTE even though the MEGA study (Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis) gathering data from 4375 patients with VTE found no significant association (39). The A1298C MTHFR mutation has been less studied in relation to VTE. A previous study done on patients with idiopathic VTE has shown an association with thrombotic events only for homozygous C677T and not for A1298C. However, the study group was small, not comparable to the magnitude of the MEGA study. Nonetheless, our association is reported for young women with VTE and gender differences could account for the results of the current study.

The four gene mutations discussed above are the most frequently studied in relation to acute thrombotic events with the aim of establishing a subgroup of population in which genetic thrombophilia testing would influence management and improve prognosis. To date, the indications for thrombophilia screening have been controversial. In fact, some guidelines suggest testing only in young patients with a family history of thrombosis as the duration of anticoagulation will not be influenced otherwise (11). In our study, 78.6% of women had at least one thrombophilic mutation. Therefore, when screening young patients with acute thrombotic events, there is a high probability of finding one thrombophilic mutation but the management would be influenced only in the presence of associated risk factors. Data from the current literature suggest a significant increase in thrombotic risk in combined polymorphisms such as FVL with prothrombin G20210A mutation or prothrombin G20210A mutation with MTHFR polymorphism (40). We found that in young females, the presence of MHTFR polymorphism along with either prothrombin G20210A mutation or FVL increases the risk of VTE 3.1-folds compared to healthy young women. However, the association of two MTHFR polymorphisms had no influence on thrombotic risk in women. While it appears clear that multiple risk factor concur to the development of acute thrombotic events, in the case of genetic mutation association it remains to be established which would significantly increase the risk so as to modify the patient's future management.

Limits of the study

Our study included a relatively small sample size, the study population being composed of young patients, under 45 years of age, with very few CVRF in which acute thrombotic events are relatively rare. Therefore, a type II statistical error cannot be excluded, and further studies on a larger population are necessary to confirm our findings. Focusing on acute thrombotic disease in young women, we included only a female control group, but we could compare findings between men and women with the same pathology showing that genetic background is similar while the associated CVRF are the one altering the phonotypical manifestation.

We did not have access to more laboratory data in the control group (like protein C, S, antithrombin III or homocysteine levels), but the focus of our study was to understand the importance of thrombophilia genetic factors in relation to acute thrombosis in the young. Regarding the antiphospholipid syndrome tests, a limit of our study is related to the absence of complete data for APS parameters at more than 12 weeks from the baseline test, based on the current criteria for definition.

CONCLUSION

In young women with acute thrombotic events and few cardiovascular risk factors, mild hyperhomocysteinemia and homozygosity for Factor V Leiden and MTHFR A1298C polymorphisms increase the risk for venous but not arterial thromboembolic events, with compound genotypes (one MTHFR polymorphism associated with one of the G20210 A or FVL) significantly more frequent in the VTE group compared with controls. In this latter case, family history of CV events appears as a risk factor. MTHFR polymorphisms are found with increased frequency in both healthy females and patients with thromboembolic pathology; therefore, their significance as an important thrombotic risk modifier in women remains unclear.

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