Original Article

SND rs1799889(-) IN THE PROMOTOR OF THE PLASMINOGEN ACTIVATOR INHIBITOR-1 GENE CONTRIBUTES TO THE RISK OF DVT IN WOMEN

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Summary

The incidence of deep venous thrombosis (DVT) depends on the specific genotype, inheritance of prothrombotic polymorphisms and the influence of environmental risk factors. Rs1799889(-) polymorphism in the promotor of PAI-1 gene has been described as a risk factor for hypercoagulable state. Objective: To evaluate the contribution of thrombophilic rs1799889 (-) in the promotor of PAI-1 gene on the incidence of DVT in women and men in groups below and above 45 years of age. There was significantly higher rs1799889 (-) polymorphism carriage among female patients with DVT vs controls (Chi squared =5.506, OR=2.170, p=0.021) but not in male patients (Chi squared =0.090 OR=1.147, p=0.825). A significant contribution of rs1799889 (-) polymorphism to early onset of the disease was found in female patients aged 45+ and carriers of the polymorphism (Chi squared =7.476, p=0.006), but not in young women.
Key words: deep venous thrombosis, thrombophilia, Rs1799889(-)s, PAI-1

Introduction

Deep venous thrombosis is a relatively common but preventable cause of morbidity and mortality worldwide, leading to complications such as pulmonary embolism (PE), post-phlebitic syndrome and death. According to a population based study, deep venous thrombosis (DVT) has an estimate of annual incidence of about 0.67 per 1000 among general populations. The presence of multiple risk factors is a prerequisite for venous thromboembolism (VTE) development with synergistic gene-gene and gene environment interactions, often increasing the risk above the sum of individual risk factors [1]. The most common risk factors associated with VTE are as follows: non-modifiable factors as age and inheritance [2]; modifiable factors of lifestyle (tobacco smoking, obesity, and oral contraceptives), clinical parameters (multiple trauma, major surgery, pregnancy, and central venous catheters) and medical conditions (nephrotic syndrome, myocardial infarction, stroke,

active cancer, etc.) [3].

Impaired balance between fibrinolysis and coagulation might significantly contribute to prothrombotic state. Raised plasma concentration of serpine1 – plasminogen activator inhibitor type 1 (PAI-1), has been associated with a risk of stroke, cardiovascular diseases and VTE, which could be possibly due to suppressed fibrinolysis. Fibrin clots are dissolved by plasmin produced from plasminogen under activation by urokinase plasminogen activator (u-PA), or tissue plasminogen activator (t-PA). This activation, regulated by PAI-1 [4], was postulated to play an important role in the pathogenesis of disorders associated with thrombosis.

Overexpression of PAI-1 may compromise normal fibrin clearance mechanisms and promote fibrin deposition and, as a consequence, thrombotic events [5]. PAI-1 is synthesized in various tissues and cell types including the liver, spleen, adipocytes, and endothelial cells. It is also found in platelets. The human PAI-1 gene is located on chromosome 7q22. The promoter region of the PAI-1 gene contains a single guanine nucleotide deletion SND 675 (4G) polymorphism, which influences transcription of the gene, thus leading to loss of and additional repressor binding site in 5G allele carriers. The 4G allele cells (rs1799889(-) produce up to 6 times more mRNAin vitro. In vivo, it is associated with higher plasma PAI-1 level in blood. High levels of PAI-1 were found to produce hypofibrinolytic state. Apart from that, PAI-1 seems to influence the processes of smooth muscle cell proliferation and matrix remodelling in the direction of promoting thrombosis. Numerous studies have concluded that inheritance of the 4G/4G genotype confers an increased risk of stroke, coronary heart disease and influences venous thrombosis.

However, it is not clear whether the elevated PAI-1 level contribute to VTE in a similar way in male and female patients, or there are factors contributing to different expression of PAI-1 in women, since its concentration is influenced by a variety of hormones and cytokines. Reports on gender-related difference in VTE episodes are controversial and these differences still remain unexplained [6].The DVT incidence is distributed extremely unequally in lifespan, which makes thrombosis extremely uncommon in young subjects, while being a leading cause of death in the elderly.

Investigations of the association of SND rs1799889(-) (a single nucleotide deletion) in the promotor of the PAI-1 gene with the risk of DVT in male and female patients could contribute to better understanding of the underlying pathology and better diagnosis of DVT. Although current knowledge of the DVT diagnosis is well established there are still unresolved options on the risk of DVT onset and recurrent incidents.

Material and Methods

Selection of patients and study protocol

This prospective cohort study evaluated the consecutive incident cases ofDVT and PE in patients older than 18, admitted to the Clinic of Surgery, University Hospital – Pleven Bulgaria from May 2012 to November 2014.

A total of 210 unrelated patients with DVT were investigated (106 men and 104 women). The selection criterion was a history of one or more incidents of DVT with or without consequent PE. Deep venous thrombosis was diagnosed by compression ultrasonography, and Doppler ultrasonography and D-dimer measurements according to criteria set by Evidence-Based Clinical Practice Guidelines [7].

Diagnostic algorithms: D-dimer test and Imaging tests. The diagnostic strategy was based on clinical prediction rules [8], D-dimer and imaging tests in a stepwise approach. At the first step, a validated clinical prediction rule to determine pre-test probability was used. In cases the prediction rule suggested a low, moderate, or unlikely probability of VTE, a negative D-dimer test was used. All other patients and those positive on D-Dimer test underwent an imaging test -compression ultrasonography with whole-leg ultrasound, in which a negative result ruled out DVT, or by compression ultrasonography limited to the proximal venous system.

Data Collection

A specific questionnaire was used for the personal interview of patients with thrombotic incidence. The survey listed number and type of vascular incidents; age at onset of the first episode; number of recurrent incidents; family history; previous and present anticoagulation therapy; presence of risk factors such as surgery, trauma, immobilization, obesity, tobacco smoking, and oral contraceptive use, pregnancy or postpartum period within the last 3 months for

females. There were no patients with malignant or myeloproliferative diseases or systemic infections in the study group.

The control group included 202 unrelated healthy individuals (82 men and 120 women) with no family history of venous thrombosis and/or embolism.

Both patients and control groups were of similar demographic data and from the same geographical area. The demographic and baseline characteristics and common risk factors, obtained by reviewing medical records are presented in Table 1.

The study protocol was approved by the ethics commission of Medical University-Pleven. All investigated subjects gave written informed consent for the investigation.

Sample collection and DNA analysis

Venous blood was collected in vacutainers with 0.084 ml 15% EDTA (Becton, Dickinson and Company). DNA was isolated following the procedure of GFTTM Genomic Blood DNA Purification Kit (Amersham Pharmacy Biotech Inc) and quantified using the agarose gel procedure.

The gene for PAI-1 is located in the region of 7q-21.22. Allelic polymorphism of the gene is expressed in an internal promoter region 4G / 5G polymorphism of 675b.p "upstreams" from the transcription start site, in the promoter of the gene.

The highly sensitive method of allele-specific PCR / AS - PCR / was used for amplification.

The promoter genotype was established for each individual patient by PCR of genomic DNA with allele specific primers. Amplification was performed in two separate series, with the use of two constitutive primers and two primers with high specificity (respectively primer sequence GGGG 4G and 5G primer sequence GGGGG). Constitutive primers limit region of about 270 bp and the inner primer lead to amplification product of 170 bp. Polymerase Chain Reaction (PCR) was performed in a total volume of 20µl, containing the following: 1 µl (100 ng/µl) genomic DNA, 0.4 µl (20 pmol/µl) of the respective forward and reverse primers, 1.8 µl (5mmol/µl) deoxynucleotide triphosphates, MgCl 25 mmol/µl, 2.0 µl, 2.0 µl Buffer for Taq polymerase and Taq polymerase 1 U per sample (AB gene) [9].

The amplification was carried out by thermocycler (Techne, version 11.04).

The primers used have the following nucleotide sequence:

Pr F 5 'AAG CTT TTA CCA TGG TAA CCC CTG GT 3'

Pr R 5 'TGC AGC CAG CCA CGT GAT TGT CTA G 3'

Pr 4G 5 'GTC TGG ACA CGT GGG G 3'

Pr 5G 5 'GTC TGG ACA CGT GGG GG 3'

PCR products were fractionated by electrophoresis through 2.5% agarose (AppliChem) and visualized in UV light by ethidium bromide staining (10mg/ml, 10 µl).

Guidelines to avoid PCR contamination were strictly followed. Sample collection, DNA isolation and quantitation were performed in the laboratory physically separated from the laboratory used for sample amplification and analysis [10].

Statistical analysis

Statistical analyses were performed using SPSS 21.0 for Windows (SPSS Inc.,Chicago, IL, USA). Continuous variables were expressed as means ±standard deviation or standard error. Categorical variables were expressed as percentages. Differences between continuous variables were analyzed using the Student t test, while those between categorical variables were analyzed using the 2 test. All quantitative data was tested for normality distribution with Shapiro-Wilk $p>0.05$.

The association between genotype PAI -1 4G/4G and total DVT risk was calculated using descriptive analysis method of cross tabulation. Prevalence was taken from cross tabs, Chi-Square (2) estimates were calculated as Pearson 2 and Fisher's Exact Test, Risk estimates were measured as odds ratio (OR), and 95% confidence intervals (CIs) and asymptotic significance (2-sided) for the whole group of patients as compared to controls (with adjustment for sex and age) and stratified by gender. The Mentel-Haensel test of conditional independence and odds ratio estimate test were used to compare the statistical data. Cumulative incidence of first and recurrent VTE events was estimated by the Kaplan-Meier survival method, censoring at the time of thrombotic event, event of recurrence, the end of the follow-up.

The Kaplan–Meier analysis was used to measure the fraction of patients carriers of prothrombotic mutation as compared to non-carrier for the “survival”time in anticipation of first VTE event, as well as for “survival”-time to the recurrent episode for estimating the survival function to the repeated event. The time interval

was taken from medical records and from follow-up data. The time interval for the patients, who did not develop the recurrent episode was taken from follow-up history. Cox proportional hazard model was used to estimate the risk/probability of VTE event at younger age associated with potential risk factors.

Results

A thrombophilic genetic variant of rs1799889 (-) polymorphism was significantly pronounced in the women (OR =2.170, p=0.021), but not in the men (OR=1.147, p=0.825) or in the whole group of patients (OR=1.689, p=0.079). Age-dependent Kaplan Meier probability/likelihood of "survival" before DVT event was higher in young male patients non-carriers of rs1799889(-) polymorphism, as compared to carriers of rs1799889(-) (Chi squared 3.872, p=0.049) but not in the elder patients. The opposite was seen inn female patients: the likelihood of "survival" before DVT event was higher in older female patients non-carriers of rs1799889(-) (Chi squared 7.476, p=0.006).

The clinical parameters and life style factors according to gender (male and female) are presented in Table1.

Women included in the study were older than men (48.40 years vs. 45.84) and had had the first embolic incident later in life (median age at first incident 42.6 years for females, and 39.8 years for males).

The episodes of pulmonary embolism were fewer in women as compared to men (25.49 % versus 30.18%, p=0.3), but the number of recurrent incidents (observed and taken from medical history records) was higher in women than in men - 40.8 % versus 34.9%, respectively.

Table 1. The Clinical parameters and life style factors according to gender. (Values are numbers and percentages in the brackets)

Characteristics	Total	Male patients	Female patients
Patients (n,%)	210	106 (53.9%)	104 (46.1%)
Age (mean±SD, years)	46.87 ±16.82	45.83±16.67	47.93±16.98
Range (mean, years)	17-79		
Age of the first thrombotic incidence (mean±SD, years)	44.5 ± 17.01	43.61± 17.49	44.88 ±16.37
Diagnosis of DVT (n %)	178 (84.36%)	87 (82%)	95 (91%)
Thrombophlebitis/superficial thrombosis (n %)	33 (15.7%)	22 (20.8%)	11 (10.6%)
Pulmonary embolism (n %)	57 (26.6%)	31 (29.52%)	26 (23.2%)
Previous pulmonary embolism/deep vein thrombosis (n %)	58 (27.62 %)	32 (30.2%)	26 (25.5%)
Surgery (n %)	28 (13.45 %)	15 (14.06%)	13 (12.81%)
Trauma (n %)	32 (15.44%)	25 (23.18%)	8 (7.46%)
Immobilisation (n %)	27 (13.07%)	24 (22.72%)	5 (5.1%)
Obesity (n %)	45 (21.25%)	21 (19.67%)	24 (22.72%)
Tobacco smoking (n %)	81 (38.46%)	50 (47.61%)	29 (28.03%)
Hormonal therapy/oral contraceptive (n %)			8 (7.69%)
Pregnancy-related incident (n %)			25 (24.27%)
Total modifiable risk factors (presented in the table) (n %)		62 (58.94%)	58 (55.96%)

The clinical presentation of the VTE depended on the underlying cause. Women had an additional risk such as recent pregnancy or pregnancy-related incidents (24.27%) and were using oral contraceptives or being on hormonal therapy (7.69%). Altogether, the contribution of acquired/modifiable risk factors was 55.34% and 50.48% in male and female patients, respectively.

The results of DNA testing for carriage of SND rs1799889(-) in the promotor of PAI-1 gene polymorphism in the 206 patients and 210 controls we investigated are presented in Table 2 .The carriage of rs1799889(-) gene polymorphism was higher, though insignificantly, in the patients than in the controls (29.34% versus 20.87% in controls, p=0.080).

Table 2. Prevalence, Pearson Chi-Square, Odds Ratio, Fisher's Exact Test, and 95% CI of SND rs1799889(-) in the promotor of PAI-1 gene in male and female patients as compared to controls

Statistical values rs1799889(-) in the PAI-1 gene	Prevalence in patients %	Prevalence in controls %	Pearson Chi-Squared	Fisher's Exact Test	Odds Ratio	95% Confidence Interval
Total Patients	29.34	20.87	3.072	0.080	1.689	1.004-2.841
Male	24.8	21.95	0.090	0.825	1.147	0.469-2.807
Female	34.1	19.6	5.506	0.021	2.170	1.128-4.173

The carriage of rs1799889 (-) polymorphism in female patients only was significantly higher than in female controls (34.10% versus 19.6%, p=0.021), while carriage of rs1799889 (-) PAI -1polymorphism was only slightly higher in the male group of patients (24.80% vs. 21.95%, p>0.05). Kaplan-Meier method was used to compare a probability of survival before DVT event between different groups.

By applying this approach for statistical calculations, we found out that this polymorphism influenced the thrombotic risk

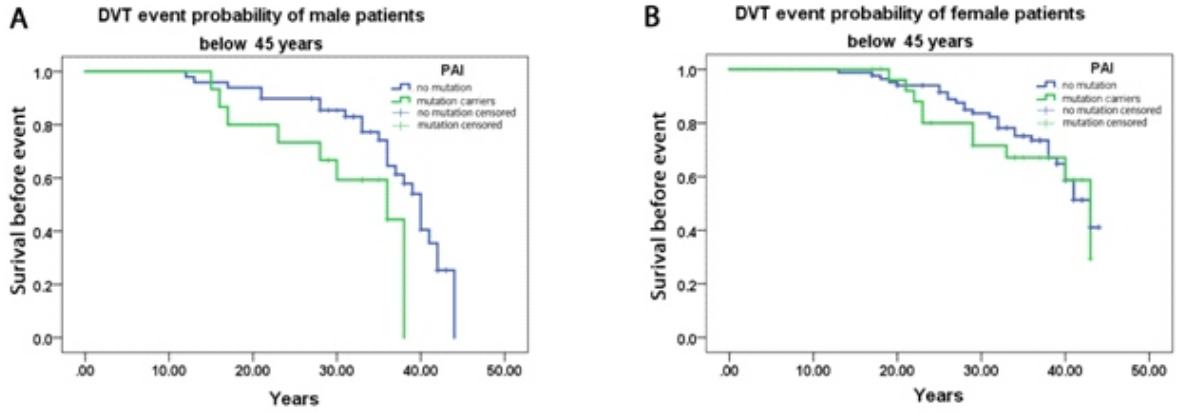

Figure 1. Survival Time probability before DVT event for patients with DVT below 45 years, carriers and non-carriers of SND rs1799889(-) in the promotor of PAI-1 gene: (A) male patients; (B) female patients

A significant contribution of rs1799889(-) polymorphism to the early onset of the disease was found in male patients below 45 years, (Chi-Square 3.782. p=0.049). The contribution was not significantly higher in female patients below 45 years. The opposite relationship was found in the patients above 45 years: the female patients above 45 years and carriers of rs1799889(-) polymorphism had a higher probability for earlier onset of a DVT event (Chi-Square 7.476. p=0.006).

Table 3. Means of survival time probability before DVT event for patients with DVT, below 45 years, carriers and non-carriers of SND rs1799889(-) in the promotor of PAI-1 gene

Gender	Carriage of rs1799889 (-)	Mean			
		Estimate	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
Male	non-carriers	37.008	1.248	34.561	39.454
	carriers	31.044	2.414	26.312	35.777
Female	non-carriers	38.443	.898	36.684	40.203
	carriers	36.842	1.858	33.199	40.485
Overall	Overall	37.344	.675	36.020	38.668

Table 4. Overall comparisonsof survival time probability before DVT event for patients with DVT below 45 years, carriers and non-carriers of SND rs1799889(-) in the promotor of PAI-1 gene

Gender		Chi -Square	p
Male	Log Rank (Mantel -Cox)	3.872	0.049
Female	Log Rank (Mantel -Cox)	0.144	0.705
Pooled [b]	Log Rank (Mantel -Cox)	2.101	0.147

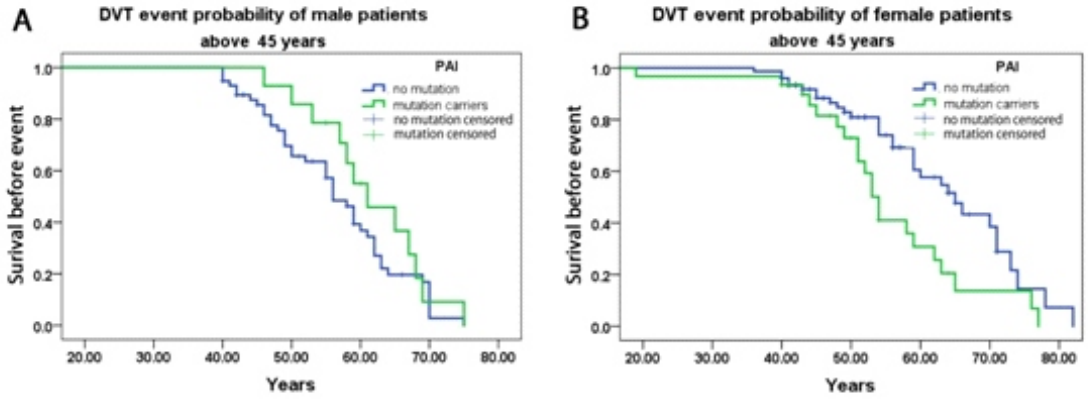

Figure 2. Survival time probability before DVT event for patients with DVT above 45 years, carriers and non-carriers of SND rs1799889(-) in the promotor of PAI-1 gene: A Male patients. B Female patients

Table 5. Means of survival time probability before DVT event for patients with DVT above 45 years, carriers and non-carriers of SND rs1799889(-) in the promotor of PAI-1 gene

Gender	Carriage of rs1799889 (-)	Mean			
		Estimate	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
Male	non-carriers	58.729	1.300	56.181	61.277
	carriers	61.439	2.315	56.902	65.976
Female	non-carriers	64.907	1.832	61.317	68.498
	carriers	56.169	2.770	50.740	61.599
Overall	Overall	60.652	0.970	58.751	62.553

Similar mean and median values confirm the uniform distribution of data. Taking into account age difference in the survival probability before DVT events, further analysis of mutation carriage was performed in the subgroups of the patients below 45 years and above 45 years of age.

Table 6. Overall comparisons for survival time probability before DVT event for patients with DVT above 45 years, carriers and non-carriers of SND rs1799889(-) in the promotor of PAI-1 gene

Gender		Chi - Square	Sig.
Male	Log Rank (Mantel - Cox)	0.323	0.570
Female	Log Rank (Mantel - Cox)	7.476	0.006
Pooled	Log Rank (Mantel - Cox)	2.262	0.133

The results from analysis of the subgroup of the patients below and above 45 years are presented in Table 7. Female patients above 45 years of age presented with a significant difference in the carriage of SND rs1799889(-) in the promotor of PAI-1 (OR 4.926, p=0.006) as compared to the age adjusted controls. This confirms a contribution of rs1799889(-) polymorphism to the risk of DVT development in women above 45 years of age.

Table 7. Prevalence, Pearson Chi-Square, Odds Ratio, Fisher's Exact Test, and 95% CI of SND rs1799889(-) in the promotor of PAI-1 gene in male and female patients as compared to controls

Statistical values rs1799889(-) in the PAI-1 gene	Prevalence in patients %	Prevalence in controls %	Pearson Chi-Squared	Fisher's Exact Test	Odds Ratio	95% Confidence Interval
Male below 45 years	25.0	21.9	0.090	0.761*	1.190	0.374 - 3.793
Female below 45 years	25.6	22.6	0.560	0.821	1.116	0.453-2.478
Male above 45 years	24.0	22.0	0,130	0.908*	1.105	0.202-6.051
Female above 45 years	41.3	17.4	7.531	0.006	4.926	1.482-16.138

Discussion

Plasminogen activator inhibitor (PAI-1) is an important component of the fibrinolytic system. SND 4G/4G polymorphism (rs1799889(-) has been correlated with higher levels of plasma PAI-1, associated with an increase in the risk for intravascular thrombosis.

No significant relationship was found between the carriage of rs1799889(-) polymorphism in PAI gene and the development of VTE in the whole group of unselected patients, as it was presentedin the meta-analysis[11] and in most of the studies quoted on the association between the PAI-1 4G/5G polymorphism and venous thrombosis, as well as in other publications and our preliminary study [12].

When the patients in our study were analysed in the groups matched by gender and subgroups matched by age, a significantly higher carriage of rs1799889(-) polymorphism in PAI gene was found among DVT female patients than in the female controls. No difference was found between the male groups. When analysed by age, a significant difference in polymorphism carriage was found in older female patients only.

Age- and gender-dependent thrombotic event development has been found and discussed in the literature, but so far it is still not fully understood. The results regarding carriage of rs1799889(-) polymorphism in PAI gene were registered in older female patient with a history of stroke [13].

Recently, a broad spectrum of findings in thrombotic events has been reported. A meta-analysis of nine studies has shown that the 4G/4G genotype is associated with a 20% increase of the risk for myocardial infarction [14]. Several studies also addressed the association of the 4G/5G polymorphism with stroke [15], and some of them found a particular association in women [16] at young [17] or older age [18]. It was suggested that this polymorphism could contribute to the risk for VTE in patients with other inherited prothrombotic risk factors. The severity and phenotypic manifestation of thrombophilic state is affected by a genetic background of thrombophilia, since thrombosis is a multigenic disorder [19, 20]. However, it could be affected by other factors like age and gender as well.

The contribution of age as factor in our study was gender-related and was manifested in a different way in male and female patients. Age-dependent Kaplan Meier probability/likelihood of "survival" before DVT event was higher in young male patients non-carriers of rs1799889(-) polymorphism, as compared to carriers of rs1799889(-), (Chi squared 3.872, p=0.049) but not in the elder patients. The opposite was true for the female patients: likelihood of "survival" before DVT event was higher in older female patients non-carriers of rs1799889(-) (Chi

squared 7.476, p=0.006). As was mentioned above, only in females older than 45 years the risk of DVT was significantly higher.

Thus, it seems that rs1799889(-) polymorphism is probably a mild risk factor, which in itself conveys little risk, as it does not contribute to the DVT risk in all the patients, but only in older female patients. The clinical manifestations become more evident in combination with other possible risk factors. We could suggest an important contribution – that of changes in hormonal status in women after 45 years. Through decreasing PAI-1 concentration in blood [21], estrogen levels could prevent DVT development in young females carriers of rs1799889(-) polymorphism. Estrogens could also be the reason why young females do not experience earlier onset of the DVT events, as compared to young males.

PAI-1 regulation is possibly an adaptive mechanism of blood coagulation duringpregnancy and delivery. PAI-1 plays a multi-functional role as a fast-acting inhibitor of plasminogen activators; urokinase-plasminogen activator and tissue type plasminogen activator in regulating blood clotting, cell proliferation, adhesion, migration, and signal transduction pathways. It's important function was found to play a role in inflammation and pregnancy.Its attenuation by estrogen hormones make us assume that the level of PAI is regulated in a much more complicated way in women. It depends on estrogen concentration fluctuation. This gender-related difference in the risk of VTE events remained an enigmatic. The main hypothesis could be that PAI-1 blood levels in women are related to estrogenand estrogen contributes to eliminate the effect of higher PAI-1 concentration in young menstruating women.

From our data, we could support the idea of Tsantes [[1]1] that the presence of the 4G allele might significantly increase the thrombotic risk in patients with inherited or aqired thrombophilic factors and, to a lesser degree, in patients without known risk factors.

Conclusion

Significantly higher rs1799889(-) polymorphism carriage among DVT the female patients was found, as compared to female controls. No such significant difference was found in males and the whole group of patients.

A significant contribution of rs1799889(-) polymorphism to the early onset of the disease was found in female patients above 45 years, but not in those below 45 years. The probability to survive to a DVT event in female patients above 45 years was higher in non-carriers of PAI polymorphism, as compared to mutations carriers.

In older women rs1799889(-) polymorphism significantly contributed to a higher risk for DVT development. In young women, the effect of this polymorphism was not be seen.

A significant contribution of rs1799889(-) polymorphism to the early onset of the disease was found in male patients below 45 years. The probability to survive to a DVT event in male patients below 45 years was higher in patients non-carriers of PAI polymorphism, as compared to mutations carriers.

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