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Project: Thrombophilia – Hereditary syndrome

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INTRODUCTION

Thrombophilia may be defined as acquired or hereditary tendency to arterial or venous thrombosis. Inherited thrombophilia can be defined as a genetically determined predisposition to develop thromboembolic complications, while acquired thrombophilia can be associated with the presence of antiphospholipid antibodies, acquired hyperhomocysteinemia and several other conditions (*Nicolaides 2005*). In inherited thrombophilia thrombotic complications are usually limited to the venous side of circulation although also arterial thrombosis can be observed. Inherited thrombophilic defects include antithrombin deficiency, protein C and protein S deficiencies, activated protein C (APC) resistance, inherited hyperhomocysteinemia, factor V Leiden, prothrombin G20210A variant, dys- and hyperfibrinogenemia and elevated factor VIII levels *(Francini et Veneri 2005).* Recognition of thrombophilic defects has altered the diagnostic and therapeutic approach to patients with venous thromboembolism (VTE) and has had an important influence on counselling and screening of family members, especially women of childbearing age.

In comparison to VTE, the role of genetic predisposition for the pathogenesis of arterial occlusive disease (atherothrombosis) is unknown, although recent publications suggest a definite link. Certain thrombophilic defects have a definite pathophysiological role in atherothrombosis e.g. inherited or acquired hyperhomocysteinaemia. Other thrombophilic conditions, such as prothrombin gene G20210A polymorphism or factor V Leiden, have been investigated, but current evidence does not unequivocally support the hypothesis of a pathophysiological role in atherothrombosis. Routine screening for thrombophilia in patients with atherothrombosis is therefore not generally recommended on the basis of current evidence, but there is a role for selective screening (*Bohm et Al-Khaffaf 2003*).

Since some thrombophilic defects show geographical distribution (*Rees et al 1995, Rosendaal et al 1998)*, the aim of the present study was to establish prevalence of thrombophilic defects in patients with venous and arterial trombosis in Macedonia.

MATERIALS AND METHODS

Subjects

Altogether 150 patients with a history of VTE, VTE during pregnancy (VTE + P), thrombosis of the central retinal vein (TVCR), acute myocardial infarction (AMI) or ischaemic cerebrovascular infarction (CVI) from Macedonia were investigated. The patients were 21 to more than 70 years old.

As a control group 22 apparently healthy volunteers from Macedonia were recruited among acquaintances and co-workers. They had no history of thromboembolism and were not on anticoagulant therapy at the time of the study.

Neither patients nor controls were related to each other. They participated in the study after they had given their full informed consent.

Blood sampling and preparation of plasma in Macedonia

Blood was sampled from an antecubital vein between 7 and 9 a.m. after an overnight fast and a 20-minute rest. Both in patients and controls blood was sampled on a single occasion.

For coagulation assays 18 ml of blood was collected in two vacuum tubes each containing 1 ml of 0.11 mol/L sodium citrate (Beckton Dickinson, Vacutainer System Europe), thoroughly mixed with the anticoagulant, placed immediately in ice water and centrifuged within 2 hours of venepuncture at 4 °C and 2000 x g for 30 min. Aliquots of platelet-poor plasma were then frozen in liquid nitrogen and stored at –70 ^{o}C until analysis.

For DNA analysis 8 ml of blood was collected in K_3-EDTA vacuum tubes (Beckton Dickinson, Vacutainer System Europe), thoroughly mixed and stored at –20 ^{o}C.

Isolation of DNA in Slovenia

DNA was isolated from peripheral blood leukocytes by standard methods.

Laboratory methods performed in Slovenia

Antithrombin activity (Berichrom AT III, Dade Behring), protein C activity (Berichrom Protein C, Dade Behring), and resistance to activated protein C (Coatest APC Resistance V-S, Chromogenix) were measured on an automated coagulation analyser (Behring Coagulation Timer, Dade Behring) according to the instructions of the manufacturers. According to our reference values antithrombin and protein C activities below 0.70 and 0.72 relative to normal plasma, respectively, were regarded as deficiency. A ratio of APTT with activated protein C versus APTT without activated protein C below 1.93 indicated APC resistance. The method was 100% sensitive for factor V Leiden (*Božič et al 2000*). Free protein S was determined with enzyme-linked immunosorbent assay in supernatant after precipitation of protein-bound protein S with poly-ethylene glycol (*Espana et al 1991*). Free protein S below 0.55 relative to normal plasma was considered as protein S deficiency.

Factor V Leiden and prothrombin G20210A polymorphism were detected by the real time polymerase chain reaction utilizing a factor V Leiden and a prothrombin (G20210A) polymorphism detection kits (both Applied Biosystems) on a ABI Prism 7000 equipment (Applied Biosystems).

Laboratory methods performed in Macedonia

Antithrombin activity (Berichrom AT III, Dade Behring), protein C activity (Berichrom Protein C, Dade Behring), protein S (Protein S, Dade Behring), and resistance to activated protein C (ProC® Ac R, Dade Behring) were measured on an automated coagulation analyser (Behring Coagulation Timer, Dade Behring) according to the instructions of the manufacturer. According to our reference values antithrombin activity below 0.75, protein C activity below 0.72 and free protein S below 0.50 relative to normal plasma, respectively, were regarded as deficiency. A ratio of activated protein C below 2.22 indicated APC resistance.

Statistical analysis

For statistical analysis, Statistica 6.0 software (Stat Soft Inc.2001, USA) was utilised. Variables are shown as means and standard deviations and differences between groups tested with Student's *t* test or Kruscall Wallis analysis of variance (ANOVA). Differences in numbers of patients were tested by χ^2 test. A p level of 0.05 or less was considered statistically significant.

RESULTS

Age distribution of different subgroups of patients is shown in Table 1.

Table.1 Age distribution of patients with different diagnosis: venous thromboembolism (VTE), venous thromboembolism during pregnancy (VTE + P), thrombosis of the central retinal vein (TVCR), acute myocardial infarction (AMI) or ischaemic cerebrovascular infarction (CVI).

Age (years)	21-30	31-40	41-50	51-60	61-70	>70
VTE (n=58)	0 (0 %)	14 (24 %)	16 (27 %)	18 (31 %)	8 (14 %)	2 (3 %)
VTE + P (n=9)	5 (56 %)	4 (44 %)	-	-	-	-
TVCR (n=24)	1 (4 %)	1 (4 %)	4 (17 %)	9 (37 %)	7 (29 %)	2 (8 %)
AMI (n=19)	2 (10 %)	1 (5 %)	3 (16 %)	5 (26 %)	7 (37 %)	1 (5 %)
CVI (n =38)	2 (5 %)	1 (3 %)	10 (26 %)	24 (63 %)	-	1 (3 %)

In Table 2, presence of several risk factors for thromboembolism and presence of positive family history for thromboembolism is shown.

Table.2 Number of risk factors of patients with different diagnosis: venous thromboembolism (VTE), venous thromboembolism during pregnancy (VTE + P), thrombosis of the central retinal vein (TVCR), acute myocardial infarction (AMI) or ischaemic cerebrovascular infarction (CVI).

	Diabetes mellitus	Hypertension	Increased cholesterol	Smoking	Positive family history
VTE (n=58)	1 (2 %)	16 (27 %)	7 (12 %)	11 (19 %)	40 (69 %)
VTE + P (n=9)	-	-	2 (22 %)	1 (11 %)	6 (67 %)
TVCR (n=24)	6 (25 %)	8 (33 %)	3 (12.5 %)	4 (17 %)	12 (50 %)
AMI (n=19)	6 (31 %)	5 (26 %)	9 (47 %)	10 (53 %)	8 (42 %)
CVI (n =38)	1 (3 %)	18 (47 %)	10 (26 %)	14 (37 %)	22 (58 %)

Mean values of resistance to activated protein C, antitrombin and protein C activity and concentration of free protein antigen are shown in Table 3. Results obtained in Slovenia are presented. Subgroups of patients did not differ significantly in APC resistance and antitrombin, while significant differences were observed for protein C and free protein S.

Table 3. Mean values with standard deviations of APC resistance, antithrombin, protein C and protein S in patients with venous thromboembolism (VTE), venous thromboembolism during pregnancy (VTE + P), thrombosis of the central retinal vein ((TVCR), acute myocardial infarction (AMI), ischaemic cerebrovascular infarction (CVI) and apparently healthy controls (Controls).

	APC ratio (rel)	Antithrombin (rel)	Protein C (rel)	Protein S (rel)
VTE (n=58)	2.29 ± 0.32	0.97 ± 0.14	0.95 ± 0.30	0.91 ± 0.29
VTE + P (n=9)	2.32 ± 0.33	0.97 ± 0.10	1.42 ± 0.27	1.00 ± 0.73
TVCR (n=24)	2.30 ± 0.28	0.99 ± 0.16	1.18 ± 0.41	1.02 ± 0.47
AMI (n=19)	2.45 ± 0.34	0.97 ± 0.16	1.14 ± 0.32	1.09 ± 0.56
CVI (n =38)	2.27 ± 0.26	1.00 ± 0.14	1.21 ± 0.28	0.98 ± 0.50
Controls (n=22)	2.48 ± 0.18	0.91 ± 0.11	1.09 ± 0.22	1.25 ± 0.27
ANOVA (p)	0.058	0.061	0.005	0.002

Percentages of thrombophilic defects in subgroups of subjects investigated are shown in Table 4.

Table 4. Percentages of thrombophilic defects in patients with venous thromboembolism (VTE), venous thromboembolism during pregnancy (VTE + P), thrombosis of the central retinal vein (TVCR), acute myocardial infarction (AMI), ischaemic cerebrovascular infarction (CVI) and apparently healthy controls (Controls).

	APC resistance	Antithrombin deficiency	Protein C deficiency	Protein S deficiency
VTE (n=58)	14.3	2.0	16.1	14.3
VTE + P (n=9)	9.1	0	0	30.0
TVCR (n=24)	8.7	0	4.4	18.8
AMI (n=19)	11.1	0	16.7	10.0
CVI (n =38)	10.0	0	2.5	14.3
Controls (n=22)	0	0	4.6	0

Table 5. indicates distribution of factor V Leiden and prothrombin G20210A genotypes in subgroups of patients studied. Among all patients studied prevalence of factor V Leiden was 12.3 % and prevalence of prothrombin G20210A polymorphism 6.3%. Prevalence of factor V Leiden was highest among CVI patients, while prevalence of prothrombin G20210A was the highest in VTE patients. The differences in prevalences between subgroups of patients and between patients and controls did not reach statistical significance.

Table 5. Distribution of factor V Leiden genotypes and prothrombin G20210A genotypes in subgroups of patients with venous thromboembolism (VTE), venous thromboembolism during pregnancy (VTE + P), thrombosis of the central retinal vein (TVCR), acute myocardial infarction (AMI), ischaemic cerebrovascular infarction (CVI) and apparently healthy controls (Controls). Numbers of patients with percentages are given.

	Factor V Leiden				Prothrombin G20210A			
genotype	n	GG	AG	AA	n	GG	AG	AA
VTE	51	44 (86.2 %)	6 (11.8 %)	1 (2.0 %)	51	45 (88.2 %)	6 (11.8 %)	0 (0 %)
VTE + P	11	10 (90.9 %)	1 (9.1 %)	0 (0 %)	10	9 (90.0 %)	1 (10.0 %)	0 (0 %)
TVCR	23	21 (91.3 %)	2 (8.7 %)	0 (0 %)	23	23 (100.0 %)	0 (0 %)	0 (0 %)
AMI	18	16 (88.9 %)	2 (11.1 %)	0 (0 %)	18	16 (88.9 %)	2 (11.1 %)	0 (0 %)
CVI	43	37 (86.0 %)	6 (14.0 %)	0 (0 %)	42	42 (100.0 %)	0 (0 %)	0 (0 %)
All patients	146	128 (87.7 %)	17 (11.6 %)	1 (0.7 %)	144	135 (93.8 %)	9 (6.3 %)	0 (0 %)
Controls	20	20 (100.0 %)	0 (0 %)	0 (0 %)	20	19 (95.0 %)	1 (5.0 %)	0 (0 %)

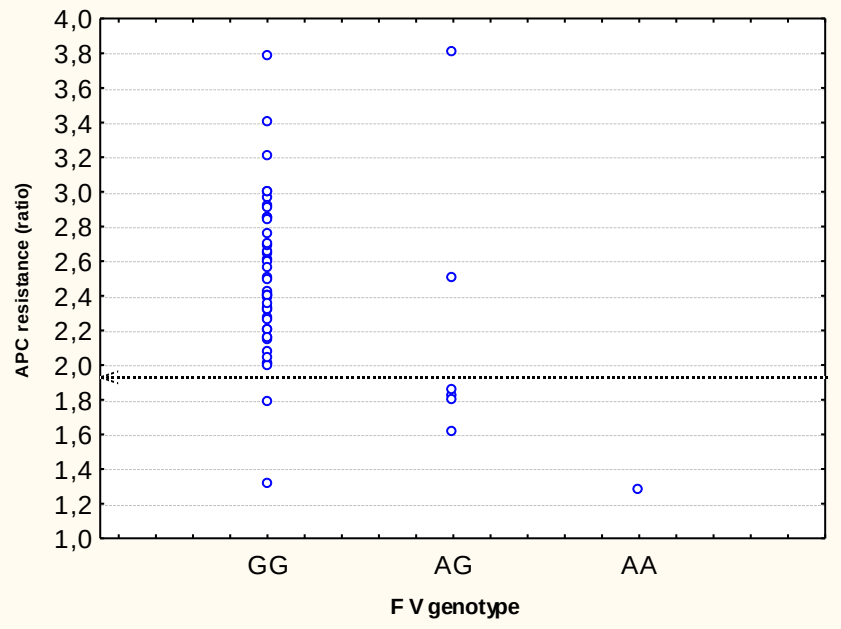
In 52 patients results of APC resistance, antithrombin, protein C and free protein S were obtained in both laboratories. No significant differences were observed (Table 6)

Table 6. Mean levels of APC resistance, antithrombin, protein C and free protein S obtained in Macedonia and Slovenia in 52 patients.

	Macedonia	Slovenia	t-value	p
APC resistance (ratio)	2.44	2.30	1.67	0.097
Antithrombin (rel)	0.98	0.99	-0.29	0.767
Protein C (rel)	0.87	0.94	-1.09	0.278
Protein S (rel)	0.84	0.77	1.24	0.217

According to the results of APC resistance obtained in Slovenia, only one (1.9 %) false positive result in a patient with a GG genotype was obtained and no false negative results were observed. According to the results obtained in Macedonia, 11 (21.1 %) false positive results and two false negative results were obtained in patients with APC resistance above 2.22. If the cut-off for APC resistance was decreased from 2.22 to 1.95, better agreement between APC resistance and factor V Leiden was observed (Fig. 1).

Figure 1. (Dys)Agreement between APC resistance and genotyping for factor V Leiden (Results from Macedonia).



COMMENTS AND CONCLUSIONS

For VTE the most common genetic predisposition in patients of European origin is activated protein C (APC) resistance (Factor V Leiden). Others include: antithrombin, protein C or protein S deficiencies, hyperhomocysteinemia, prothrombin G20210A gene polymorphism or combined thrombophilias. (*Nicolaides 2005*). Not all hereditary thrombophilias are associated with the same thrombotic risk. The highest incidence is found in combined defects and in antithrombin deficiency and the lowest in factor V Leiden (*Vossen et al 2004*). In the European Prospective Cohort on Thrombophilia (EPCOT) study, 575 asymptomatic carriers of antithrombin, protein C or protein S deficiency or factor V Leiden, and 1118 controls were included and followed for 5.7 years on average. The incidence of a first event was 0.8% per year in carriers and 0.1% in controls (*Vossen et al 2004).* The risk of thrombosis when homozygous factor V Leiden or prothrombin G20210A defects are present is considered to be extremely high, 50-80 fold compared with the normal population. The risk is also very high when heterozygous defects in both polymorphisms are present (*Samama et al 2003*).

Our study showed that in all subgroups of patients studied the most prevalent defects were protein S and protein C deficiencies, followed by APC resistance (or factor V Leiden), prothrombin G20210A gene polymorphism and antithrombin deficiency, which was observed only in one patient with VTE. In patients with VTE the same order of deficiencies was observed. Combined defects were observed 17 (11.6 %) of all patients investigated.

Presence of thrombophilic defects associated with pregnancy complications has gained much attention, although data on this topic are conflicting. Besides an established association between antiphospholipid antibodies and pregnancy loss, available data suggest additional associations for antithrombin deficiency, hyperhomocysteinemia and also for factor V Leiden, prothrombin G20210A polymorphism, and protein S deficiency. However, a limited number of prospective studies have failed to reveal an increased risk of pregnancy complications in unselected women with thrombosis risk factors *(Pabinger et Vormittag 2005).* In our study only 11 patients with VTE during pregnancy were included, so this group is too small to draw any conclusions.

Hereditary deficiencies of protein C, protein S, or antithrombin are not believed to cause atherosclerosis and are rare among patients with arterial thrombosis, even in young patients without atherosclerosis. Protein C and antithrombin levels were not associated with myocardial infarction or cardiac death in a prospective study (*Folsom et al 1997*). Conversely, there is no clear evidence that the incidence of arterial thrombosis is increased among patients with protein C, protein S, or antithrombin deficiencies (*Van Cott et al 2002*). Factor V Leiden and prothrombin G20210A may be more prevalent among patients with myocardial infarction who do not have atherosclerosis and/or who have certain other risk factors (smoking, hypertension, obesity, high cholesterol, or diabetes), when compared with control groups (*Van Cott et al 2002*). Our study showed that in patients with AMI the prevalence of APC resistance, antithrombin, protein C and S deficiency was similar or lower than in patients with VTE. Also the prevalence of factor V Leiden and prothrombin G20210A polymorphism was similar among patients of these subgroups.

Of the inherited thrombophilias, factor V Leiden and the prothrombin G20210A polymorphism have been associated with stroke, but this association is significant only in children and adults under age 40. The risk of stroke in persons with these polymorphisms is substantially increased by concomitant exposure to oral contraceptives. Of the acquired thrombophilias hyperhomocystinemia is a major risk factor for stroke and the antiphospholipid syndrome is strongly associated with transient ischemic attacks and cerebral infarction. The diagnosis of thrombophilia should be considered in stroke patients who are young, have a family history of thrombosis, suffer venous dural sinus thrombosis, or have recurrent strokes (*Green 2003*). In the presents study, the most prevalent defect in CVI patients was free protein S deficiency, followed by factor V Leiden, APC resistance and protein C deficiency. No patients with antithrombin deficiency or prothrombin G20210A polymorphism were observed in this subgroup.

To conclude, high prevalence of protein S and protein C deficiencies, followed by APC resistance (factor V Leiden) and prothrombin G20210A polymorphism in patients with venous or arterial thrombosis from Macedonia suggest that these patients populations might be genetically different from other European populations. However, the present study is limited by very small numbers of patients, therefore these results require validation in studies with larger numbers of patients.

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