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The evaluation of inherited thrombophilic conditions in patients with bleeding in the first trimester of pregnancy

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Abstract. Introduction: Prothrombotic condition induced by inherited thrombophilia is involved in vascularization disorders of the placenta bed. The aim of our study was to investigate the relationship between the presence of specific genetic mutations and the occurrence of complications in the first trimester of pregnancy. Material and method Thirty-six patients in the studied group, who presented bleeding in the first trimester of pregnancy, and 39 patients in the control group were tested for the mutation of factor V Leiden (FVL) (G1691A), prothrombin (G20210A) and methylenetetrahydrofolate reductase (MTHFR A1298C and C677T). Results: Twenty-seven patients in the studied group (75%) and 27 patients in the control group (69.2%) presented genetic thrombophilic mutations (p=0.5). Genetic polymorphism was described in similar percentages in the studied group (22.2%) as compared with the control group (23%) (p=0.9). The prevalence of the FVL mutation was higher in the control group (10.2%) as compared with the studied group (8.3%). The MTHFR A1298C mutation was more frequently isolated in the studied group (52.7%) as compared with the control group (46.1%)(p=0.5). MTHFR C677T occurred more frequently in the control group (38.4%) than in the studied group (36.1%) (p=0.8). The G20210A mutation was not isolated in any of the groups. The MTHFR A1298C mutation as well as homozygotism associated with MTHFR (A1298C and C677T) correlated with a higher risk of complications in the first trimester of pregnancy OR 1.30 95%CI [0.48-3.58] and OR 1.31 95%CI [0.37-4.68] respectively. Conclusion: The thrombophilic mutations determined in our clinical context had a similar distribution in the two groups. Therefore, genetic screening in such patients is only validated by the presence of suggestive patient history.
Keywords: thrombophilia, inherited, pregnancy.

Rezumat. Introducere: Statusul protrombotic indus de trombofilia ereditară a fost implicat în tulburări ale vascularizaţiei în patul placentar. Scopul studiului nostru este de a investiga relaţia între prezenţa mutaţiilor genetice specifice şi apariţia complicaţiilor în primul trimestru de sarcină. Material şi metodă: Un număr de 36 paciente în lotul studiat cu sângerare în primul trimestru de sarcină şi 39 paciente în lotul martor au fost testate pentru mutaţia factorului V, Leiden (FVL), protrombinei (G20210A) şi metilentetrahidrofolatreductazei (MTHFR A1298C şi C677T). Rezultate: Din lotul studiat, 27 de paciente (75%) şi 27 paciente din lotul martor (69.2%) au prezentat mutaţii genetice trombofilice (p=0.5). Polimorfismul genetic a fost descris în proporţii similare în lotul studiat (22.2%), comparativ cu lotul martor (23 %) (p=0.9). Prevalenţa mutaţiei FVL a fost mai mare în lotul martor (10.2%), comparativ cu lotul studiat (8.3%). Mutaţia MTHFR A1298C s-a izolat mai frecvent în lotul studiat 52.7%, comparativ cu lotul martor (46.1%)(p=0.5). MTHFR C677T s-a întâlnit mai frecvent în lotul martor (38.4%), decât în lotul studiat (36.1%) (p=0.8). Mutaţia G20210A nu a fost izolată în nici unul din loturi. Mutaţia pentru MTHFR A1298C, precum şi homozigotismul asociat MTHFR (A1298C si C677T) s-au corelat cu un risc mai crescut al complicaţiilor în primul trimestru de sarcină OR 1.30 95%CI [0.48-3.58] şi respectiv OR 1.31 95%CI [0.37-4.68]. Concluzie: În contextul clinic ales mutaţiile trombofilice determinate au avut o distribuţie similară în cele două loturi. În aceste condiţii, screening-ul genetic la aceste paciente nu este justificat decât în prezenţa unor antecedente sugestive.
Cuvinte cheie: trombofilie, ereditar, sarcină.

Introduction. Inherited thrombophilia is a genetic condition that carries an important prothrombotic risk factor (Walker et al 2001; Martinelli et al 2002). The G1691A mutation, which determines the appearance of a modified factor V, named factor V Leiden (FVL), the G20210A (prothrombin gene) mutation (PGM), antithrombin III deficiency, protein C deficiency and protein S deficiency are some thrombophilic mutations associated with an increased the risk of thrombosis. The methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C mutations are also prothrombotic determinants, especially when they are associated with folate deficiency (Wramsby et al 2000; Greer 2000; Robertson et al 2005). There is an association between inherited thrombophilic defects and fetal loss and late pregnancy complications, with a presumed mechanism being thrombosis of uteroplacental circulation.

The thrombogenic potential is increased by the existence of the procoagulant condition, which is characteristic of pregnancy. Consequently, the prognostic of the fetus and mother is affected. Pregnancy-associated venous thromboembolism increases maternal morbidity and constitutes one of the main causes of maternal mortality (Friederich et al 1996; Mc Cool et al 1997; Grandone et al 1998; Gerhardt et al 2000). Thrombosis in the intervillous space and the concomitant involvement of the placental perfusion not only increases the thromboembolic risk in pregnancy, but may also influences the obstetrical prognosis due to the risk of miscarriage, spontaneous abortion, fetal death in utero, delayed intrauterine growth or even preeclampsia (Sheppard & Bonnar 1999; Robertson et al 2005). Meta-analyses on the correlation between thrombophilic mutations and the pathology of the first trimester of pregnancy indicate contradictory results. However, there are statistically significant risks when anomalies of procoagulant factors, especially FVL and prothrombin, occur. The thrombotic risk rises for MTHFR, antithrombin III, protein C or S deficiency, but the increase is not statistically significant (Robertson et al 2005).

Material and Method

Patient selection. Thirty-six patients, who reported at the 1st Gynecology Clinic for bleeding in the first trimester of pregnancy, were included in the study between January and August 2006. The control group included 39 patients who were in the first trimester of pregnancy. They had similar descriptive characteristics as the studied group, carried at least one normal pregnancy to full term and gave birth without any maternal or fetal complications. Local ethics committee approval was granted. All individuals gave signed informed consent for the participation in study. From the obstetrical history of the patients included we followed data about previous pregnancies, misscariagies and thromboembolic episodes.

The patients included in the studied group underwent clinical examination to exclude vaginal or cervical pathologies involved in the etiology of bleeding. The ultrasound examination was carried out using a Voluson 730 Expert ultrasound machine. The diagnosis of viable intrauterine pregnancy was confirmed by transvaginal ultrasonography and determination of β-HCG serum level. Thus suspected ectopic pregnancies were excluded and the pregnancies were subsequently monitored according to the protocol of the clinic. In the event of decidual hematoma or massive bleeding, the patients were admitted to hospital and serial ultrasound evaluations were carried out. Blood was collected from the patients in both groups in order to identify the presence of specific mutations in FVL G1691A, prothrombin G20210A, MTHFR A1298C and C677T. The exclusion criteria were antiphospholipidic syndrome patients, thrombocytopenia or other hematological disorders with increased hemorrhage risk.

Laboratory protocol. Genomic DNA extraction was carried out using samples of 300 µl whole blood and the Wizard Genomic DNA Purification kit (Promega®, USA). The amplification was carried out for all the four mutations in the following reaction mix (25 µl): 12.5 µl 2x PCR Mastermix (Fermentas MBI®, Lithuania) containing Taq recombinant polymerase 0.05 U/µl in Taq-polimerase buffer, MgCl2 4mM, dATP, dGTP, dCTP, dTTP at a concentration of 0.4 mM each, 1 µl BSA solution bovine serum albumin 2 mg/ml, 1 µl primer Fw and 1 µl primer Rev (concentration of 1.5 pmols/µl each) (Eurogentec,

Belgium) (see sequences in Table 2), 2 µl genomic DNA 200 ng/µl and H20 nuclease free (qsp up to 25 µl). The PCR reaction was performed in a Mastercycler gradient thermocycler (Eppendorf®, Germany) using the amplification programs mentioned in Table 1. The obtained amplicons underwent enzymatic digestion using the corresponding restriction enzyme, 4 U in 4 µl buffer enzyme, using 10 µl amplicon for digestion, for 12 hours at 37 C. The fragments obtained after digestion were identified using horizontal electrophoresis in 2% agarosis in TBE 1x and stainig gels with ethidium bromide. The gels were read with a Vilber-Lourmat®, France gel documentation system (Bertina et al 1994; Poort et al 1996; Friedman et al 1999; Sharma et al 2006; Zhou-Chun et al 2007).

Table 1

Amplification conditions, enzymes used for PCR-RFLP and fragments obtained after digestion

	PCR conditions	*Restriction enzyme*	*Fragments length*
FactorV G1691A	- Initial denaturation 94 C – 5 min. - 36 cycles (91 C – 40 sec, 55 - 40 sec., 71 C - 2 min.) - Final elongation 72 C - 7 min.	Mnl I	- Wild type: 163 bp, 67 bp, 37 bp - Heterozygous: 200 bp, 163 bp, 67 bp, 37 bp - Homozygous mutant: 200 bp, 67 bp
Prothrombin G20210A	Initial denaturation 94 C – 5 min. - 36 cycles (91 C – 40 sec, 55 - 40 sec., 71 C - 2 min.) - Final elongation 72 C - 7 min.	HindIII	- Wild-type: 345bp - Heterozygous: 345 bp , 322 bp, 23 bp - Homozygous mutant: 322 bp, 23 bp
MTHFR C677T	Initial denaturation 94 C – 5 min. - 35 cycles (94 C – 30 sec, 57 - 30 sec., 72 C - 30sec.) - Final elongation 72 C - 5 min.	HinfI	- Wild-type: 265bp - Heterozygous: 265 bp , 171 bp, 94 bp - Homozygous mutant: 171 bp, 94 bp
MTHFR A1298C	95 C – 5 min., 55 C - 2min, 72 C-2 min. - 35 cycles (95 C – 75 sec, 55 - 75 sec., 72 C - 90 sec.) - Final elongation 72 C - 6 min.	MboII	- Wild-type: 56 bp, 31bp, 30 bp, 28 bp, 18 bp - Heterozygous: 84 bp, 56 bp, 31 bp, 30 bp, 28 bp,18 bp - Homozygous mutant: 84 bp, 31bp, 30 bp,18 bp

Table 2

Sequences of primers used to detect FVL mutation, prothrombin G20210A, MTHFR A1298C, MTHFR C677T

	Sequence of primers
Factor V 1691 G>A	Fw 5'-TGC CCA GTG CTT AAC AAG ACC A-3'
	Rev 5'-TGT TAT CAC ACT GGT GCT AA-3'
Prothrombin	Fw 5'-TCT AGA AAC AGT TGC CTG GC-3'
	Rev 5'-ATA GCA CTG GGA GCA TTG AAG C-3'
MTHFR C677T	Fw 5'- CAT CCC TAT TGG CAG GTT AC -3'
	Rev 5'- GAC GGT GCG GTG AGA GTG -3'
MTHFR A1298C	Fw 5'- CTT TGG GGA GCT GAA GGA CTA CTA C-3'
	Rev 5'- CAC TTT GTG ACC ATT CCG GTT TG-3'

Statistical analysis. The results were processed with the Epi Info Program version 3.4.1. Univariate analysis was used and the average values were expressed together with the standard deviation. The results were expressed as odd ratios (OR) with a 95% confidence interval (CI). The Pearson correlation coefficient was calculated and the level of statistical significance was $p < 0.05$.

Results. The average age in the studied group ($\pm$ standard deviation) was of 31.1$\pm$3.4 years (between 24 and 40 years) and of 29.8$\pm$3.9 years (between 24 and 40 years) in the control group. Table 3 includes the description of the two groups with regard to the patient's obstetrical and thromboembolic history.

Table 3

Obstetrical and thromboembolic history in the patients included in the study

	Studied Group, n=36	*Control Group, n=39*
No obstetrical history	7 (19.4%)	31(79.4%)
Recurrent miscarriage	6(16.6%)	0 (0%)
Early pregnancy loss	22(61.3%)	8(20.6%)
Late pregnancy loss	1(2.7%)	0 (0%)
History of thromboembolism (deep venous thrombosis)	2(5.5%)	0(0%)

Twenty seven (75%) patients in the studied group and twenty seven patients (69.2%) in the control group presented thrombophilic genetic mutations (p=0.5). Genic polymorphism was described in similar percentages: eight cases (22.2%) in the studied group, as compared with nine cases (23%) in the control group (p=0.9). The prevalence of the investigated thrombophilic mutations in the homozygous or heterozygous genotype in both groups was presented in Table 4. Their distribution did not differ significantly between the two groups. The prevalence of FVL heterozygous mutation was higher in the control group (10.2%), as compared with the studied group (8.4%) (p=0.6). The homozygous FVL mutation was not isolated in any of the groups. The MTHFR C677T mutation also occurred more frequently in the control group (38.4%) as compared with the studied group (36.1%). The MTHFR A1298C mutation was more frequently identified in the studied group as compared with the control group (p=0.5), which is statistically insignificant. The mutation for the prothrombin gene (G20210A) was not identified in any of the groups.

Table 4

Prevalence of thrombophilic mutations in the two groups

Mutation	*Normal genotype*		*Homozygous genotype*		*Heterozygous genotype*	
	Studied group N=36	Control group N=39	Studied group N=36	Control group N=39	Studied group N=36	Control group N=39
FVL	33 (91.6%)	35 (89.8%)	0 (0%)	0 (0%)	3 (8.4%)	4 (10.2%)
PGM	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
MTHFR C677T	23 (63.8%)	24 (61.5%)	7 (19.4%)	7 (17.9%)	6 (16.8%)	8 (20.6%)
MTHFR A1298C	17 (47.2%)	21 (53.9%)	1 (2.7%)	0 (0%)	18 (50.1%)	18 (46.1%)

The presence of the MTHFR A1298C mutation as well as for associated homozygotism (MTHFR A1298C and C677T) correlated with the risk of bleeding in the first trimester of pregnancy. However, the risk obtained was reduced and statistically insignificant (Table 5).

Table 5

Risk associated with isolated thrombophilic mutations in the patients included in the study

Presence of the mutation	*Studied group N=36*	*Control group N=39*	*OR 95% CI*
FVL heterozygous	3	4	0.8 [0.13-4.67]
MTHFR C677T	13	15	0.9 [0.32-2.56]
MTHFR A1298C	19	18	1.30 [0.48-3.58]
MTHFR A1298C +C677T (homozygous genotype)	8	7	1.31 [0.37-4.68]
FVL+ MTHFR	2	4	0.51 [0.06-3.60]

The association between the FVL mutation and the presence of one or both MTHFR mutations was the genetic polymorphism identified in the studied cases. Nevertheless, a higher risk associated with this type of genotype was not observed.

Twelve patients in the studied group (33.3%) presented decidual hematoma at the first ultrasound examination as compared with three cases in the control group (7.7%) (p=0.005).

One patient presented genic polymorphism (FVL mutation and heterozygous MTHFR A1298C); two patients had a normal genotype while one type of thromophilic mutation was identified in the other nine patients (Table 6).

None of the decidual hematoma cases included in the control group presented thrombophilic mutations.

Table 6

Thrombophilic mutations identified in the patients with decidual hematoma

Presence of the mutation	*No. of cases*
FVL heterozygous	1
MTHFR A1298C heterozygous	3
MTHFR A1298C homozygous	1
MTHFR C677T heterozygous	2
MTHFR C677T homozygous	2
FVL + MTHFR A1298C	1
Normal genotype	2

The presence of inherited thrombophilic condition, represented by the MTHFR A1298C heterozygous mutation, was identified in four out of the six patients with abortive disease in the studied group.

Four patients (11.1%) in the studied group had spontaneous abortions in the first trimester of pregnancy. Genetic polymorphism (the FVL mutation, MTHFR A1298C and C677T) was identified in only one of the four cases.

Thrombophilic mutations were not isolated in two cases while a homozygous genotype for MTHFR A1298C was identified in one case.

Discussions. Numerous studies reported an association between inherited thrombophilia and poor obstetrical prognosis; however the absolute risk is low, statistically insignificant and varies according to study (Robertson et al 2005).

The physiopathogenic mechanisms involved correlated with the occurrence of thrombosis in the spiral arteries, chorial vessels and intervillous space.

Thrombotic processes, which affect the chorial vessels, and impaired trophoblastic invasion, which are probably responsible for the impaired uteroplacental circulation, were noted in fetuses (Preston et al 1996; Rey et al 2003).

A relative correlation was demonstrated between early pregnancy loss and the presence of the FVL mutation in homozygous or heterozygous form as well as the heterozygous genotype in the prothrombin gene. The significance of the correlation increases, if this complication type occurred in previous pregnancies in the patient's history (recurrent abortion, pregnancy stopped in evolution) (Foka et al 2000; Finan et al 2002).

In our study the presence of the heterozygous genotype in FVL could not be correlated with the occurrence of bleeding in the first trimester of pregnancy. The influence of G20210A on the evolution of the first trimester of pregnancy could not be analyzed in our study since it was not identified in the patients included in the 2 groups.

The thermolabile form of MTHFR was associated with the occurrence of obstetrical complications especially in the second trimester of pregnancy. The studies regarding its influence on pregnancies in the first trimester offer contradictory results (Fatini et al 2000).

The risk, derived from the presence of A1298C mutation and MTHFR A1298C-C677T homozygotism in our study, was small and statistically insignificant and it is correlated with literature data

The presence of hyperhomocystinemia associated with the MTHFR homozygous genotype may increase the thrombogenic potential (Morelli et al 2002). The fact that homocysteine levels were not determined and correlated with the presence of MTHFR mutations represents one of the drawbacks of our research.

Acid folic supplementation in the first trimester of pregnancy may influence homocysteine plasma levels, which increases especially in the case of the MTHFR homozygous genotype. The results of our study were not influenced by this aspect; all

patients included in the study receiving a reduced dose of folic acid in order to prevent neural tube malformations (400µg daily).

Genetic polymorphism associated with thrombophilia may favor thrombosis at the maternal-placental interface. This was often associated with significantly increased thombotic risk as compared with carriers of only one mutation (Roque et al 2004).

Such a correlation could not be identified in our study probably due to the small number of patients included in both groups.

Besides genetic polymorphism with thrombophilic potential, numerous anatomical or endocrine factors are held responsible for the occurrence of bleeding in the first trimester of pregnancy. Such factors may influence the results of research in the selected clinical context. In this respect the results of our study are limited.

Furthermore, a correlation between the presence of decidual hematoma and the existence of a certain type of thrombophilic mutation could not be established.

The research was carried out on a limited number of patients. Therefore a real correlation between poor obstetrical prognosis due to spontaneous abortion or pregnancy stopped in evolution and the presence of one of the identified thrombophilic mutations could not be obtained.

Conclusions. In our study the prevalence of thrombophilic mutations in FVL, prothrombin, MTHFR A1298C, MTHFR C677T was similar in both groups and could not be correlated with the occurrence of bleeding in the first trimester of pregnancy.

Routine screening for inherited thrombophilia is not recommended in an unselected population in the clinical context analyzed. Such screening should only be considered after careful anamnesis in patients with an obstetrical history or previous thromboembolism.

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