Clinical and Applied Thrombosis/Hemostasis
Volume 16 Number 4
July/August 2010 435-439

10.1177/1076029609335518
http://cath.sagepub.com

Inherited Thrombophilia is Associated With Pregnancy Losses That Occur After 12th Gestational Week in Serbian Population

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Recurrent fetal loss (RFL) is a significant clinical problem, occurring in 1% to 5% of reproductive females. Inherited or acquired thrombophilia has been diagnosed in 50% to 65% of women with history of unexplained fetal loss. The objective of our study was to determine the prevalence of thrombophilia in women with unexplained RFL in Serbian population and to find out whether the presence of thrombophilia is associated with pregnancy losses that occur later than 12th gestational week. We have examined 147 women with unexplained RFL or intrauterine fetal death and 128 healthy women with at least 1 uncomplicated pregnancy. The antithrombin (AT), protein C (PC), protein S (PS), activated protein C (APC) resistance, factor V (FV) G1691A, factor II (FII) G20210A, and *MTHFR* C677T were determined. At least 1 inherited thrombophilic defect was found in 54 (36.7%) of 147 women with repeated fetal losses and in 11 (8.59%) of 128 controls ($P < .001$, OR 6.17, 95% CI 3.06-12.48). The most common thrombophilic abnormalities were homozygosity for *MTHFR* 677TT, FV Leiden, and FII G20210A. Deficiency of natural anticoagulants occurred in 10 patients, with protein S deficiency being the most frequent one. Thrombophilia was found in 46 of 94 women with RFL that occurred later than the 12th gestational week and in only 8 of 53 with RPL earlier than 12th week ($P = .001$). Our study has shown the association between the hereditary thrombophilia and RFL that occurred after the 12th gestational week in Serbian population.

Keywords: clinical thrombophilia; gene polymorphisms; recurrent abortion; thrombophilia

Introduction

Recurrent fetal loss (RFL) is a significant clinical problem, occurring in 1% to 5% of reproductive females.[1] The association between acquired thrombophilia caused by the presence of antiphospholipid antibodies or myeloproliferative disorders and RFL is well established.[2,3] During the past decade, the link between inherited thrombophilia and RFL has also been made.[4-7] Inherited or acquired thrombophilia has been diagnosed in 50% to 65% of women with history of unexplained fetal loss.[8,9] Whether thrombophilia is a cause or is just associated with unexplained RFL is still a matter of debate.[10] The

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objective of our study was to determine the prevalence of thrombophilia in women with unexplained RFLs in Serbian population and to find out whether the presence of thrombophilia is associated with pregnancy losses that occur later than 12th gestational week.

Patients and Methods

We have examined 169 women with RFL or intrauterine fetal death of unknown cause, who were referred for evaluation of thrombophilia to the Thrombosis and Hemostasis Unit in two centers in Serbia, the Clinical Centre Novi Sad and the National Blood Transfusion Institute, Belgrade, from January 2002 to June 2008. Prior to thrombophilia investigation, other potential causes of RPL have been excluded (chromosomal abnormalities in both parents, anatomic malformations of the uterus, reproductive hormones dysfunction, diabetes mellitus, thyroid disorders, hyperprolactinemia, autoimmune, and infective disorders). Women were considered to have RFL if they had at least 3 consecutive first trimester pregnancy losses (before 12th gestational week), at least 2 consecutive second trimester losses (between 13th and 24th gestational week), or at least 1 intrauterine fetal death (after 24th gestational week). No women in the study group had a history of previous venous thromboembolism. The study group consisted of 169 women. The control group consisted of 128 women, healthy hospital employees, who had no previous miscarriages or thrombotic events. Of the 128 women, 6 of them had 3, 79 of them had 2, and 43 had 1 delivery. None of the investigated women were pregnant at the time of investigation; none of them were using oral contraceptives. Blood samples have been taken 6 months after the last pregnancy in 161 women, and at least 2 months after the last pregnancy in 8 women.

After venipuncture of antecubital vein, blood samples were collected into 3.2% trisodium citrate tubes and platelet-depleted plasma was obtained after 2 centrifugation steps at 2500 g for 15 minutes. Supernatant plasma aliquots were frozen until assayed. Genomic DNA for polymerase chain reaction (PCR) analyses were extracted from blood leukocytes.

The antithrombin (AT) and protein C (PC) activities in plasma were determined by chromogenic assays using Instrumentation Laboratory kits ([IL], Milan, Italy). Protein S (PS) activity was determined using ProS automated coagulation assay manufactured by IL. Briefly, the ProS assay determines the functional activity of free PS by measuring the degree of prolongation of a prothrombin time in the presence of tissue factor, phospholipids, calcium ions, and activated PC. The PS activity is proportional to the prolongation of clotting time of PS-deficient plasma to which diluted sample of investigated plasma has been added. Activated PC (APC) resistance was determined using IL kits for APC-R detection. If the results were below the normal range, the assays have been repeated from another plasma sample. Deficiency of AT, PC, and PS were defined as plasma levels below 75% for AT, below 69% for PC, and below 63% for PS. Activated PC resistance was defined as APC-R < 2.0. Presence of FV Leiden was screened in samples with APC-R < 2.44.

Factor V (FV) G1691A, factor II (FII) G20210A, and *MTHFR* C677T were detected by PCR, as previously described in 115 patients, and by allelic discrimination in 32 patients.[11-13] Allelic discrimination was performed on the ABI Prism 7000 Sequence Detection System (Applied Biosystems Foster City, California, USA).

The serum anticardiolipin (ACL), immunoglobulin G (IgG) and IgM antibodie' titer, and the presence of the lupus anticoagulant (LA) were determined.[14] The automated coagulometer ACL 9000, manufactured by IL was used in all the coagulation tests.

Presence of ACL of moderate to high titer or/and LA have been found in 22 women. After these 22 women have been excluded, study group consisted of 147 women with unexplained RFL.

The study was approved by the Medical Faculty Ethical committee and signed informed consent was obtained from all participants.

Statistical Analysis

The prevalence of thrombophilia was compared between patients and controls using Yates corrected χ^2 test and univariate odds ratio was estimated separately for each thrombophilia if it was appropriate. $P \leq .05$ was considered statistically significant.

Results

The mean age of the study group was lower than that of the control group (30.1 years, range 20-42 years vs 34.2 years, range 23-55). The study group was slightly

Table 1. Prevalence of Thombophilia in Women With Recurrent Pregnancy Losses and Controls

	RPL, n = 147 (%)	Controls, n = 128 (%)	*P*	Odds Ratio	95% CI
FV Leiden	13 (8.84)	2 (1.56)	.017	6.11	1.35–27.65
FII G20210A	11/147 (7.48)	3 (2.3)	NS	3.24	0.92-12.36
MTHFR	16/ 90 (17.77)	6 (4.69)	.001	5.32	1.87-15.12
AT, PS, PC deficiencies	10/147 (6.8)	0	.007	NA	
Combined	4/147 (2.7)	0	NS	NA	
Total	54/147 (36.7)	11 (8.59)	<.001	6.17	3.06-12.48

NOTES: AT = antithrombin; FII = factor II; FV = factor V; NA = not applicable; NS = not significant; PC = protein C; PS = protein S; RPL = recurrent pregnancy loss.

Table 2. Prevalence of Thombophilia in Women With Pregnancy Losses Before and After 12th Gestational Week

	Before 12th Week, n = 53 (%)	After 12th Week, n = 94 (%)	*P*
FV leiden	2/53 (3.8)	11/94 (11.7)	.10
FII G20210A	2/53 (3.8)	9/94 (9.57)	.19
MTHFR	4/53 (7.5)	12/94 (12.8)	.33
AT, PS, PC deficiencies	0/53	10/94 (10.6)	.014
Combined	0/53	4/94 (4.25)	.13
Total	8/53 (15.1)	46/94 (48.9)	.001

NOTES: AT = antithrombin; FII = factor II; FV = factor V; PC = protein C; PS = protein S.

different regarding ethnicity, 75.5% were Serbian women and 24.5% of other ethnicity, comparing to 81.25% of Serbian and 18.75% of other ethnicity in the control group, but the difference was not statistically significant ($P = .32$).

At least 1 inherited thrombophilic defect was found in 54 of 147 (36.7%) women with RFLs and in 11 of 128 controls ($P < .001$, OR 6.17, 95% CI 3.06-12.48). Prevalence of thrombophilic polymorphisms in study group and in controls is shown in Table 1. The most common thrombophilic abnormalities were homozygosity for *MTHFR* 677TT, FV Leiden, and prothrombin gene mutation *G20210A* and they have been found in 17.77%, 8.84%, and 7.48%, respectively. Deficiency of natural anticoagulants was found in 10 patients (6.8%), with PS deficiency being the most frequent one. Combined thrombophilia was found in 4 women (5.4%), 1 of them was both FV Leiden and FII G20210A heterozygous, 3 of them were homozygous for *MTHFR* 677TT with FV Leiden, FII G20210A, and PS deficiency, respectively.

Thrombophilia was found in 21 of 45 women with intrauterine fetal death; in 26 of 49 with at least 1 miscarriage later than 12th gestational week and in only 8 of 53 with repeated miscarriages earlier than 12th week. The difference in the presence of thrombophilia between the group with RFL before the 12th gestational week and the group with RFL after the 12th gestational week is statistically significant ($P = .001$).

Antithrombin, PC, and PS deficiencies taken together have been found more frequently in women with pregnancy losses after the 12th gestational week ($P = .014$; Table 2).

The total number of previous pregnancy losses was 357: 51 were intrauterine fetal deaths and 306 were RFLs. Fifty-four women with thrombophilia had 122 pregnancy losses, of which 52 occurred in early pregnancy before the 12th gestational week, and 70 occurred after the 12th gestational week (Table 2). Compared to the group of nonthrombophilic women who had 235 pregnancy losses of which 160 occurred within 12 weeks of gestation and 75 after this period, it becomes clear that there is a significant correlation between the presence of thrombophilia and fetal loss that occurs after the12th gestational week ($P = .001$; Table 3).

Discussion

Recurrent fetal losses represent a significant medical and social problem, as many as 5% of women had 2 consecutive fetal losses during their reproductive period and 1% of women had at least 3 consecutive

Table 3. Distribution of Pregnancy Losses (PLs) Before and After 12th Gestational Week in 54 and 93 Women With and Without Thrombophilia, Respectively

	122 PL in 54 Women With Thrombophilia	235 PL in 93 Women Without Thrombophilia	*P*
PL before 12 weeks of gestation	52 (42.60%)	160 (68.10%)	$P = .000$
PL after 12 weeks of gestation	70 (57.40%)	75 (31.90%)	$P = .000$

fetal losses during their reproductive period. In about 50% of the cases, the etiology remains unexplained. Recently, great interest has been developed in determining the connection between RFL and thrombophilia. If it can be determined that the thrombosis of blood vessels in the placenta is responsible for such complications, the possibility of applying thromboprophylaxis arises in the pregnancies to follow, with great chances of favorable pregnancy outcomes.

In our study, inherited thrombophilia was found in 54 of 147 (36%) women. There was significantly increased frequency of thrombophilia among 94 women with pregnancy losses that occurred later than 12th gestational week (49%) in comparison to 53 women with repeated miscarriages earlier than 12th week (15%).

The total frequency of hereditary thrombophilia in our study group was twice as high as the frequency of all thrombophilia cases in the study of Martinelli et al, but lower than the results of Brenner and Sarig, in which studies even after the exclusion of cases of the *MTHFR* C677T homozygous gene mutation, the frequency of the other mutations was 40%.[8,9,15]

In our study, the frequency of FV Leiden mutation was 8.84%, which is similar to data from the studies of Lissalde-Lavigne, Ridker, and Martinelli. In all other investigations a much higher incidence of FV Leiden has been found.[7,15-20]

The prevalence of FII mutation in our study (7.48%) is within the range of results obtained by Brenner and Martinelli.[8,15]

Regarding the frequency of the deficit of natural coagulation inhibitors, our results are very similar to that in the study of Sarig, in which he investigated 145 women with RFL of unknown etiology.[9]

In our study, 4 women (2.7%) with recurrent pregnancy losses had 2 thrombophilic mutations (in 3 cases, *MTHFR* C677T homozygous mutation was present). The high percentage of combined thrombophilia has been described by Brenner and Sarig (8% and 21%), which is probably the result of high frequency of FV Leiden mutation in the Jewish population.[8,9]

The difference between the results obtained through the similar investigations in the world is probably because of different prevalence of thrombophilic mutations and the different criteria of inclusion into the study, the most important being the gestational week at the time of fetal loss.

In our study, patients have been divided into 2 groups, depending on the gestational age at the time of pregnancy loss. One group consisted of women with pregnancy losses that have occurred before the 12th gestational week and the other consisted of women with pregnancy losses that have occurred after this gestation. Because the 10th gestational week is the time when the transfer to placental circulation is completed, the presence of thrombophilia can cause blood vessel thrombosis with a resultant placental infarction after this period.[7] Our study has proved the connection between the presence of hereditary thrombophilia and RFL after the 12th gestational week. We have also found the significant connection between the presence of AT, PC, and PS deficiencies and pregnancy losses that occur after the 12th gestational week. According to our results, it seems justified that women with only 1 pregnancy loss after the 12th gestational week should be screened for thrombophilia, as previously suggested.[21]

Thrombophilia screening among women with unexplained RFL is of special interest in Serbia, because there is negative natality trend in our country, and the diagnosis of thrombophilia and the subsequent application of thromboprophylaxis during following pregnancies may result in favorable pregnancy outcome.

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