

**THE PREVALENCE OF PAI-1 4G/5G POLYMORPHISM IN WOMEN WITH FETAL LOSS – FIRST DATA FOR A SERBIAN POPULATION**

## UČESTALOST POLIMORFIZMA PAI-1 4G/5G KOD ŽENA SA SPONTANIM POBAČAJEM – PRVI PODACI ZA SRPSKU POPULACIJU

Valentina Đorđević<sup>1</sup>, Maja Gvozdenov<sup>1</sup>, Iva Pruner<sup>1</sup>, Mirjana Kovač<sup>2,3</sup>,  
Branko Tomić<sup>1</sup>, Marija Stanković<sup>1</sup>, Dragica Radojković<sup>1</sup>

<sup>1</sup>Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Belgrade, Serbia

<sup>2</sup>Faculty of Medicine, University of Belgrade, Serbia

<sup>3</sup>Blood Transfusion Institute of Serbia, Hemostasis Department, Belgrade, Serbia

**Summary**

**Background:** Plasminogen activator inhibitor 1 (PAI-1) is an inhibitor of fibrinolysis. The PAI-1 4G/5G polymorphism is associated with elevated plasma levels of PAI-1. Over-expression of PAI-1 and impaired fibrinolysis in homozygous carriers of the 4G/4G PAI polymorphism may lead to abnormal placental formation and increased risk of fetal loss (FL). The aim of our study was to determine the frequency of this polymorphism in patients with FL in a Serbian population.

**Methods:** The study was carried out in a group of 203 women (91 controls and 112 women with FL). The presence of PAI-1 4G/5G polymorphism was detected by PCR-RFLP analysis.

**Results:** Slightly increased frequency of the PAI-1 4G/4G genotype was observed in the study group compared to the controls (32.1% vs. 30.8%). The frequency of PAI-1 was highest in women experiencing FL in the second trimester of pregnancy (50%), but this difference was not statistically significant.

**Conclusions:** Our findings suggest that PAI-1 4G/4G might be a risk factor for FL occurring in the second trimester of pregnancy. Further studies are required in order to determine the role of PAI-1 4G/5G polymorphism in the etiology of FL.

**Keywords:** PAI-1 4G/5G polymorphism, plasminogen activator inhibitor-1, fetal loss

**Kratak sadržaj**

**Uvod:** Inhibitor aktivatora plazminogena 1 (PAI-1) igra značajnu ulogu u procesu inhibicije fibrinolize. Pokazano je da je PAI-1 4G/5G polimorfizam povezan sa povišenim nivoom PAI-1 proteina u plazmi. Povećana ekspresija PAI-1 i smanjena fibrinoliza kod homozigotnih nosilaca PAI-1 4G/5G polimorfizma može dovesti do poremećaja tokom formiranja placente i povećanja rizika za spontane pobačaje (SP). U okviru ove studije analizirali smo učestalost PAI-1 4G/5G polimorfizma kod pacijentkinja sa spontanim pobačajima.

**Metode:** Studija je obuhvatila grupu od 203 žene (91 u kontrolnoj grupi i 112 žena sa SP). Prisustvo PAI-1 4G/5G polimorfizama je detektovano PCR-RFLP analizom.

**Rezultati:** Detektovana učestalost homozigotnih nosilaca PAI-1 4G/5G polimorfizma je bila nešto viša u grupi pacijentkinja u odnosu na kontrolnu grupu (32,1% vs. 30,8%). Najviša učestalost je detektovana kod žena koje su imale SP u drugom trimestru trudnoće (50%), ali ova razlika nije bila statistički značajna.

**Zaključak:** Rezultati naše studije ukazuju da PAI-1 4G/4G može biti faktor rizika za pojavu SP u drugom trimestru trudnoće. Potrebna su dalja ispitivanja u cilju određivanja uloge PAI-1 4G/4G polimorfizma u etiologiji spontanih pobačaja.

**Ključne reči:** PAI-1 4G/5G polimorfizam, inhibitor aktivatora plazminogena, spontani pobačaj

Address for correspondence:

Maja Gvozdenov  
Institute of Molecular Genetics and Genetic Engineering  
Vojvode Stepe 444A, P.O.Box 23  
11010 Belgrade, Serbia  
Tel: +381 11 3976658  
Fax: +381 11 3975808  
e-mail: majagvozdenov@yahoo.com

List of non-standard abbreviations: FL, fetal loss; PAI-1, plasminogen activator inhibitor-1; tPA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; bp, base pairs; DVT, deep vein thrombosis.

## Introduction

Thrombophilia refers to a group of inherited or acquired coagulation disorders leading to venous and/or arterial thrombosis as well as reproductive disorders such as fetal loss (FL) (1–3). The most frequent genetic risk factors associated with thrombophilia include factor V Leiden (G1691A), prothrombin G20210A and PAI-1 4G/5G gene variants (1).

Plasminogen activator inhibitor-1 (SERPINE1, also known as PAI-1) is a 55-kd glycoprotein synthesized by endothelial cells, hepatocytes and megakaryocytes (4). PAI-1 acts as an inhibitor of endogenous fibrinolytic activity due to its ability to inhibit the activity of tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (u-PA) (5–7). Gene coding for PAI-1 is located on chromosome 7 (7q21.3-q22.1) and contains eight introns and nine exons (4).

The 4G/5G polymorphisms located at position –675 bp in the PAI-1 gene have alleles with four or five guanosines due to a single guanosine insertion/deletion in the promoter region (4, 7). A 5G allele binds both a transcriptional activator and a repressor protein at the overlapping binding site, which leads to normal PAI-1 expression. In contrast, a 4G allele binds only a transcriptional activator, resulting in an increased expression of PAI-1 (4). High levels of PAI-1 due to the presence of 4G/4G may contribute to the risk for deep vein thrombosis (DVT) and myocardial infarction (4, 8). The PAI-1 overexpression and impaired fibrinolysis may also cause compromised and insufficient trophoblast invasion, leading to abnormal placental formation and increased risk of FL (9–11).

Fetal loss is a common health problem affecting 1–5% of women of reproductive age (12). Although several etiologies, such as anatomic defects of the uterus, immune disorders, endocrinological abnormalities and chromosomal translocations and inversions, have been recognized as causes of FL, the pathophysiology still remains unexplained (9, 12, 13). It has been suggested that acquired or inherited thrombophilia is associated with FL (9, 14).

Some studies reported significantly higher frequency of the PAI-1 4G/4G polymorphism in patients with FL (9, 15, 16), while other studies demonstrated no difference in the PAI-1 4G gene variant prevalence among patients with FL compared with controls (17–19).

The aim of this study was to determine the frequency of PAI-1 4G/5G polymorphism in women with FL and healthy controls. This is the first study in which the prevalence of this polymorphism was investigated in a Serbian population.

## Materials and Methods

### Patients

Our study included 203 women divided into a study group and a control group. The study group included 112 women ( $35 \pm 5.42$  years) with at least one FL, referred to the Institute of Molecular Genetics and Genetic Engineering for thrombophilia testing in the period from 2002 to 2012. Women with a history of infective, gynecological and endocrinological disorders, chronic hypertension, diabetes, malignancy disorders and chromosomal abnormalities were not included in the study, as well as patients with protein C, S and antithrombin deficiencies.

Out of 112 patients, 78 patients with available anamnestic data on the period of FL occurrence were divided in 4 groups. First, second and third group included women who had FL only in one trimester (first, second and third trimester, respectively). The 4th group (combined) included women who had miscarriages in two different or in all three trimesters.

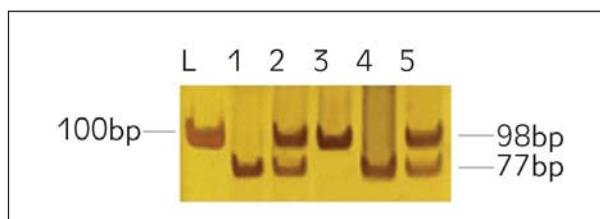
Control group consisted of 91 healthy women ( $40 \pm 12$  years) with no history of miscarriages or thrombosis. The study was approved by the local research ethics committee.

### Laboratory Methods

Peripheral blood was taken on 3.8% sodium citrate as anticoagulant. Genomic DNA was purified from 200  $\mu$ L of human whole blood using the QIAamp DNA blood mini kit (QIAGEN, Germany) according to manufacturer's protocol.

All women were tested for FV Leiden and FII G20210A mutations using PCR-RFLP analysis as previously described (20, 21).

The fragment of the PAI-1 gene that included position –675 in the promoter region was amplified by PCR using previously described primers, followed by digestion with BslI restriction enzyme (Biolabs, New England) (22). Normal (77 and 21 bp) and mutated (98 bp) alleles were distinguished by the size of the restriction fragments, using electrophoresis on 10% polyacrylamide gels and visualised by silver staining (23) (Figure 1).



**Figure 1** Polyacrylamide gel electrophoresis of the PAI-1 4G/5G polymorphism.

L – Ladder size marker (100bp) 2,5- PAI – 1 4G/5G genotype  
1,4 – PAI-1 5G/5G genotype 3- PAI – 1 4G/4G genotype

*Statistical Analysis*

Statistical analysis was performed using Statistical Package for Social Sciences 13.0 for Windows (SPSS Inc., Chicago, Illinois, USA). Deviations of genotype distributions from the Hardy-Weinberg equilibrium were assessed by  $\chi^2$ -test. The prevalence of PAI-1 gene variants was compared between patients and controls with the use of Fisher’s exact test. Odds ratio (OR) and 95% confidence interval (CI) were calculated. P value less than 0.05 was considered as statistically significant.

**Results**

In this study, we investigated 112 women with FL and 91 controls. There were more than 292 miscarriages with only 11 successful deliveries in the study group, while in the controls 82 successful pregnancies and no fetal loss were recorded. Family history of thrombotic disorders was more frequent in the patient group (38.4% vs. 2.2%).

In the study group, 40.2% of women were heterozygous and 31.2% were homozygous carriers of

the PAI-1 4G/5G gene variant, which was similar to the frequency in controls (49.5% heterozygous and 30.8% homozygous) (Table I). Control group was in the Hardy-Weinberg equilibrium, while the study group was not ( $\chi^2=4.25$ ) due to a slight excess of homozygotes for the 4G allele, which was probably the result of small sample size.

The four groups of patients, divided according to FL occurrence period, included: 1st trimester – 52 women, 2nd trimester – 10 women, 3rd trimester – 6 women and a combined group – 10 women. Characteristics of these groups are shown in Table II. No statistically significant differences in the PAI-1 4G/5G frequency were found between the control group and groups of patients divided according to trimesters. The highest frequency of the PAI-1 4G/4G genotype was found in women who have had miscarriages in the second trimester. In this group, the risk of FL was 2.25-fold higher than in controls (Table III).

In order to determine whether the PAI-1 gene variant may increase the risk of FL in combination with other thrombotic mutations, we evaluated the number of PAI-1 4G/4G carriers heterozygous for FV

**Table I** Genotype frequencies of PAI-1 4G/5G polymorphism in women with fetal loss and control group.

Genotypes	Controls n=91 (%)	Patients n=112 (%)	P OR (95% CI)
PAI-1 5G/5G	18 (19.8%)	31 (27.7%)	0.19 1.55 0.8–3.01
PAI-1 4G/5G	45 (49.4%)	45 (40.2%)	0.18 0.69 0.39–1.20
PAI-1 4G/4G	28 (30.8%)	36 (32.1%)	0.8 1.06 0.59–1.93

**Table II** Characteristics of the study group according to trimesters.

	1st trim. (n=52)	2nd trim. (n=10)	3rd trim. (n=6)	combined (n=10)
Age (years±SD)	36±3.9	34±4.4	32±3.4	34±4.2
Number of successful pregnancies	3	2	2	None
Number of FL	131	20	10	35
Family history of thrombotic disorders (%)	48.1	40	50	40

**Table III** Genotype frequencies of PAI-1 4G/5G polymorphism in women with fetal loss according to trimesters.

Genotypes	Controls n=91 (%)	1st trim. n=52 (%)	2nd trim. n=10 (%)	3rd trim. n=6 (%)	combined n=10 (%)	Pa OR <sup>a</sup> (95% CI <sup>a</sup> )	Pb OR <sup>b</sup> (95% CI <sup>b</sup> )	Pc OR <sup>c</sup> (95% CI <sup>c</sup> )	Pd OR <sup>d</sup> (95% CI <sup>d</sup> )
PAI-1 5G/5G	18 (19.8%)	12 (23.1)	2 (20)	2 (33.3)	2 (20)	0.64 1.22 0.53–2.78	0.98 1.01 0.20–5.20	0.43 2.03 0.34–2.91	0.98 1.01 0.20–5.20
PAI-1 4G/5G	45 (49.4%)	18 (34.6)	3 (30)	3 (50)	6 (60)	0.09 0.54 0.27–1.09	0.25 0.44 0.11–1.80	0.97 1.02 0.2–5.33	0.53 1.53 0.41–5.80
PAI-1 4G/4G	28 (30.8%)	22 (42.3)	5; (50)	1 (16.8)	2 (20)	0.16 1.65 0.81–3.35	0.23 2.25 0.60–8.40	0.47 0.45 0.05–4.03	0.48 0.56 0.11–2.82

<sup>a</sup> 1st trim. patient group vs. control group were tested  
<sup>b</sup> 2nd trim. patient group vs. control group were tested

<sup>c</sup> 3rd trim. patient group vs. control group were tested  
<sup>d</sup> combined patient group vs. control group were tested

Leiden and FII G20210A. In the study group, 1.8% of women with FL were homozygous carriers of the PAI-1 polymorphism and heterozygous for FV Leiden mutation, while in controls the frequency of these mutations was 1.1%. In the study group, 2.7% of women homozygous for PAI-1 were heterozygous carriers of FII G20210A mutation, but none of the women in the control group had both of these two mutations.

## Discussion

In our study, we investigated the prevalence of the PAI-1 4G/5G polymorphism in women with FL and healthy women who had no history of miscarriages. Our results show that the PAI-1 4G/4G genotype was slightly more frequent in the study group (32.14%) compared to controls (30.77%), but the difference was not statistically significant. These results are in concordance with the study of Buchholz et al. (18) which reported that the PAI-1 4G/4G polymorphism frequency was higher in women with FL (39.1%) compared to controls (32.3%), but the difference did not reach statistical significance ( $P=0.22$ ). Similar data was reported in a study by Goodman et al. (19). Conversely, Jeddi-Tehrani et al. (9) and Coulam et al. (2) have shown that the frequency of homozygous PAI-1 4G allele carriers was significantly higher in women with FL compared to controls.

In our study, the frequency of PAI-1 4G/4G carriers in patients who have had FL in the first trimester (42.3%) was slightly higher compared to controls (30.8%). Dossenbach-Glaninger et al. (24) showed that homozygosity for the PAI-1 4G polymorphism might contribute to increased risk of early pregnancy loss by disrupting fibrin cross-linking and fibrinolysis (OR 2.4; 95% CI 1.1–5.5).

Our study shows that the frequency of homozygous carriers of the PAI-1 gene variant was highest in patients suffering from FL in the second trimester (50%). This group of patients had 2.25-fold increased risk of FL. The difference was not statistically significant compared to controls ( $P=0.23$ ), but it indicates that the PAI-1 4G/4G polymorphism might play a role in the pathogenesis of FL in the second trimester of pregnancy. This result is consistent with the study of Glueck et al. (25) who reported that in a group of women homozygous for the PAI-1 4G allele, heritable hypofibrinolysis was the major cause of prematurity ( $P=0.001$ ) and second- and third-trimester fetal death ( $P=0.025$ ). They also suggested that the 4G/4G genotype is associated with complications of pregnancy, probably by acting through thrombotic induction of placental insufficiency (25).

In our study, only six women experienced FL in the third trimester. In this group, the frequency of PAI-

1 4G/4G genotype was lower (16.8%) compared to controls, which indicates that PAI-1 4G/5G polymorphisms might not represent risk factors for late FL. However, further larger studies are needed to confirm our findings.

It has been reported that the prevalence of multiple thrombophilic mutations was significantly higher in patients with FL compared to controls (26). We have investigated whether PAI-1 4G/4G might increase risk for FL in FV Leiden and FII G20210A mutation carriers. Martinelli et al. (27) showed that the presence of FV Leiden and FII G20210A mutations alone are risk factors for FL. Recent studies elucidate the relevance of PAI-1 4G/5G polymorphism as a potential risk factor contributing to FL in FV Leiden and FII G20120A carriers (28). Glueck et al. (29) showed that the hypofibrinolytic 4G/4G PAI-1 genotype is associated with FV Leiden mutation in women with severe preeclampsia, *abruptio placentae*, fetal growth restriction and stillbirth. Our data show that patients homozygous for the PAI-1 4G allele and heterozygous for FV Leiden (1.8%) are not at increased risk for FL occurrence compared to controls (1.1%). These data might be the result of small sample size and larger further studies are needed to confirm our results.

The concomitant presence of PAI-1 4G/4G genotype with the FII G20210A mutation in patients with venous thromboembolism was related to an increased risk for venous thrombosis (30). In our study, heterozygosity for FII G20210A and homozygosity for the PAI-1 4G allele were more common in patients with FL (2.7%) compared to controls, where none of the women had a combination of these two mutations. This suggests that heterozygous carriers of the FII G20210A mutation might be at increased risk for FL in combination with the PAI-1 4G/4G polymorphism.

In conclusion, this is the first study in which the prevalence of the PAI-1 4G/5G polymorphism was determined in a Serbian population. Our findings suggest that women carrying the PAI-1 4G/4G genotype might be at increased risk of FL, and indicate that during pregnancy, the second trimester might be the most critical for FL to occur. The limitation of our study was a small sample size, but our results highlight the possible role of PAI-1 4G/5G polymorphism in FL, so further larger studies are needed to establish the significance of the PAI-1 4G/5G polymorphism in the etiology of FL and validate our findings.

**Acknowledgements.** This work was supported by grant No 173008 from the Ministry of Science and Technological Development of the Republic of Serbia.

## Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

## References

1. McNamee K, Dawood F, Farquharson RG. Thrombophilia and early pregnancy loss. *Pract Res Clin Obstet Gynaecol* 2012; 26: 91–102.
2. Coulam CB, Wallis D, Weinstein J, et al. Comparison of thrombophilic gene mutations among patients experiencing recurrent miscarriage and deep vein thrombosis. *Am J Reprod Immunol* 2008; 60: 426–31.
3. Kupfermanc MJ. Thrombophilia and pregnancy. *Curr Pharm Des* 2005; 11: 735–48.
4. Akhter MS, Biswas A, Ranjan R, et al. Plasminogen activator inhibitor-1 (PAI-1) gene 4G/5G promoter polymorphism is seen in higher frequency in the Indian patients with deep vein thrombosis. *Clin Appl Thromb Hemost* 2010; 16: 184–8.
5. Shammaa DM, Sabbagh AS, Taher AT, et al. Plasminogen Activator Inhibitor-1 (PAI-1) gene 4G/5G alleles frequency distribution in the Lebanese population. *Mol Biol Rep* 2008; 35: 453–7.
6. Hoekstra T, Geleijnse JM, Kluit C, et al. 4G/4G genotype of PAI-1 gene is associated with reduced risk of stroke in elderly. *Stroke; a journal of cerebral circulation* 2003; 34: 2822–8.
7. Ringelstein M, Jung A, Berger K, et al. Promotor polymorphisms of plasminogen activator inhibitor-1 and other thrombophilic genotypes in cerebral venous thrombosis: a case-control study in adults. *J Neurol* 2012; 259: 2287–92.
8. Gong LL, Peng JH, Han FF, et al. Association of tissue plasminogen activator and plasminogen activator inhibitor polymorphism with myocardial infarction: A meta-analysis. *Thromb Res* 2012; 130: e43–51.
9. Jeddi-Tehrani M, Torabi R, Zarnani AH, et al. Analysis of plasminogen activator inhibitor-1, integrin beta3, beta fibrinogen, and methylenetetrahydrofolate reductase polymorphisms in Iranian women with recurrent pregnancy loss. *Am J Reprod Immunol* 2011; 66: 149–56.
10. Vora S, Shetty S, Khare M, et al. Placental histomorphology in unexplained foetal loss with thrombophilia. *Indian J Med Res* 2009; 129: 144–9.
11. Dossenbach-Glaninger A, van Trotsenburg M, Schneider B, et al. ACE I/D polymorphism and recurrent first trimester pregnancy loss: interaction with SERPINE1 4G/5G and F13 Val34Leu polymorphisms. *Br J Haematol* 2008; 141: 269–71.
12. Pruner I, Đorđević V, Miljić P, et al. +1040 C/T polymorphism in coding region of thrombin-activatable fibrinolysis inhibitor gene and the risk of idiopathic recurrent fetal loss. *Blood Coagul Fibrinolysis* 2010; 21: 679–82.
13. Brenner B. Thrombophilia and pregnancy loss. *Thromb Res* 2002; 108: 197–202.
14. De Santis M, Cavaliere AF, Straface G, et al. Inherited and acquired thrombophilia: pregnancy outcome and treatment. *Reprod Toxicol* 2006; 22: 227–33.
15. Sun L, Lv H, Wei W, et al. Angiotensin-converting enzyme D/I and plasminogen activator inhibitor-1 4G/5G gene polymorphisms are associated with increased risk of spontaneous abortions in polycystic ovarian syndrome. *J Endocrinol Invest* 2010; 33: 77–82.
16. Ciacci C, Tortora R, Scudiero O, et al. Early pregnancy loss in celiac women: The role of genetic markers of thrombophilia. *Dig Liver Dis* 2009; 41: 717–20.
17. Wolf CE, Haubelt H, Pauer HU, et al. Recurrent pregnancy loss and its relation to FV Leiden, FII G20210A and polymorphisms of plasminogen activator and plasminogen activator inhibitor. *Pathophysiol Haemost Thromb* 2003; 33: 134–7.
18. Buchholz T, Lohse P, Rogenhofer N, et al. Polymorphisms in the ACE and PAI-1 genes are associated with recurrent spontaneous miscarriages. *Hum Reprod* 2003; 18: 2473–7.
19. Goodman C, Hur J, Goodman CS, et al. Are polymorphisms in the ACE and PAI-1 genes associated with recurrent spontaneous miscarriages? *Am J Reprod Immunol* 2009; 62: 365–70.
20. Đorđević V, Rakičević L, Spasić M, et al. Factor V Leiden, FII G20210A, MTHFR C677T mutations as risk factors for venous thrombosis during pregnancy and puerperium. *Vojnosanit Pregl* 2005; 62: 201–5.
21. Nestorov J, Matic G, Elaković I, Tanić N. Gene expression studies: how to obtain accurate and reliable data by quantitative real-time RT-PCR. *J Med Biochem* 2013; 32: 325–38.
22. Brown NJ, Murphey LJ, Srikuma N, et al. Interactive effect of PAI-1 4G/5G genotype and salt intake on PAI-1 antigen. *Arterioscler Thromb Vasc Biol* 2001; 21: 1071–7.
23. Radojković D, Kusić J. Silver staining of denaturing gradient gel electrophoresis gels. *Clin Chem* 2000; 46: 883–4.
24. Dossenbach-Glaninger A, van Trotsenburg M, Dossenbach M, et al. Plasminogen activator inhibitor 1 4G/5G polymorphism and coagulation factor XIII Val34Leu polymorphism: impaired fibrinolysis and early pregnancy loss. *Clin Chem* 2003; 49: 1081–6.
25. Glueck CJ, Phillips H, Cameron D, et al. The 4G/4G polymorphism of the hypofibrinolytic plasminogen activator inhibitor type 1 gene: an independent risk factor for serious pregnancy complications. *Metabolism* 2000; 49: 845–52.
26. Coulam CB, Jeyendran RS, Fishel LA, et al. Multiple thrombophilic gene mutations rather than specific gene mutations are risk factors for recurrent miscarriage. *Am J Reprod Immunol* 2006; 55: 360–8.
27. Martinelli I, Taioli E, Cetin I, et al. Mutations in coagulation factors in women with unexplained late fetal loss. *N Engl J Med* 2000; 343: 1015–18.
28. Ivanov P, Komsa-Penkova R, Konova E, et al. Combined thrombophilic factors among women with late recurrent spontaneous abortions. *Akušerstvo i ginekologija* 2011; 50: 8–12.
29. Glueck CJ, Kupfermanc MJ, Fontaine RN, et al. Genetic hypofibrinolysis in complicated pregnancies. *Obstet Gynecol* 2001; 97: 44–8.
30. Barcellona D, Fenu L, Cauli C, et al. Allele 4G of gene PAI-1 associated with prothrombin mutation G20210A increases the risk for venous thrombosis. *Thromb Haemost* 2003; 90: 1061–4.

Received: April 11, 2013

Accepted: May 15, 2013