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Plasminogen Activator Inhibitor 1 4G/5G Polymorphism and Coagulation Factor XIII Val34Leu Polymorphism: Impaired Fibrinolysis and Early Pregnancy Loss

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Background: A successful outcome of pregnancy depends on proper placental formation. In the very beginning of this process, trophoblast invasion and fibrin deposition into the wall of the decidual veins play an important part. Two polymorphisms, coagulation factor XIII (FXIII) Val34Leu and plasminogen activator inhibitor 1 (PAI-1) 4G/5G, interfere with fibrin cross-linking and regulation of fibrinolysis and may therefore contribute to early pregnancy loss.

Methods: We enrolled 49 unrelated Caucasian women with a history of two consecutive or three to six nonconsecutive early pregnancy losses and 48 unrelated parous healthy controls without a history of pregnancy loss and evaluated them for the following genetic variants: the factor V Leiden and prothrombin G20210A gene mutations, the methylenetetrahydrofolate reductase C677T and A1298C polymorphisms, and the PAI-1 4G/5G and FXIII Val34Leu polymorphisms.

Results: For the isolated occurrence of PAI-1 4G/5G or FXIII Val34Leu, we found no statistically significant difference between cases and controls. For homozygosity of either or compound carrier status of both mutations, the overall relative risk for early pregnancy loss was significantly increased (odds ratio = 2.4; 95% confidence interval, 1.1–5.5; $P = 0.032$). We observed no statistically relevant association of any of the other tested mutations with early pregnancy loss.

Conclusion: Homozygosity for PAI-1 4G or FXIII 34Leu polymorphisms as well as compound carrier status is associated with early pregnancy loss.

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Inherited and acquired thrombophilia can be found in more than 50% of women suffering from recurrent pregnancy loss of unknown cause (*1*). Thrombophilic risk factors are also frequent in women with other vascular placental pathologies, such as preeclampsia, intrauterine growth retardation, placental abruption, and late fetal loss (*2–10*). The common denominator for these pregnancy complications seems to be placental insufficiency (*11*), of which insufficient invasion of trophoblast and increased fibrin deposition are believed to be major pathologic components (*12–14*).

For successful implantation, invasion of the cytotrophoblast to the proper depth of the uterus is crucial. It provides anchorage for the conceptus and promotes adaptation of uteroplacental circulation (*13*). Urokinase plasminogen activator, its receptor, and plasminogen activator inhibitor 1 (PAI-1)[5] are believed to control proteolysis and remodeling of maternal tissue during trophoblast invasion (*7, 15*). On the other hand, the creation of the placental basal plate matrix (Nitabuch fibrinoid) involves the deposition of fibrin into the wall of the decidual veins at sites of trophoblast invasion by intravenous

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[5] Nonstandard abbreviations: PAI-1, plasminogen activator inhibitor 1; FXIII, coagulation factor XIII; MTHFR, methylenetetrahydrofolate reductase; and OR, odds ratio.

activation of the maternal procoagulant cascade (16, 17). In uncomplicated pregnancies, these systems appear to be temporally and spatially strictly regulated for a well-balanced modulation of extracellular matrix and fibrin formation. In addition, intravenous blood clots (17) and increased intervillous space fibrin (18) are frequent morphologic findings in spontaneous abortion tissue, indicating a dysfunction of hemostasis. This last fact draws attention to coagulation factor XIII (FXIII), which covalently cross-links fibrin by catalyzing the introduction of γ-glutamyl-ε-lysine peptide bonds between fibrin γ- and α-chains (19), and PAI-1, which plays a central role in controlling the fibrinolytic system.

Gris et al. (20) described higher PAI-1 concentrations in women suffering from their first unexplained primary early recurrent miscarriage and speculated that impaired plasmin-dependent proteolysis might favor recurrent abortion by limiting trophoblast development, promoting fibrin deposition in early placental circulation, or both (21).

Plasma PAI-1 concentrations have been related to a common guanosine insertion/deletion gene polymorphism, 4G/5G, 675 bp upstream from the start site of translation. Homozygosity for the deletion genotype (4G/4G) has been associated with PAI-1 concentrations higher than those associated with the insertion genotype (5G/5G), and hence with reduced fibrinolytic activity (22, 23).

An association with recurrent pregnancy loss has been reported not only for the PAI-1 polymorphism, but also for the Phe204Tyr polymorphism in exon 5 of the FXIII gene (24). This polymorphism, as well as the Val34Leu polymorphism in exon 2, has been associated with higher transglutaminase activity, but it has been demonstrated that this increase in FXIII activity partly reflects the presence of limiting thrombin concentrations in the test system (25). Nevertheless, at sufficient thrombin concentrations, increased catalytic efficiency and earlier cross-linking was clearly demonstrated for samples homozygous for the FXIII 34Leu allele. This leads to structural changes that produce a finer fibrin meshwork (19), which has reduced susceptibility to fibrinolysis and therefore influences fibrin degradation.

On the basis of these facts, we speculated that both the 4G/5G and Val34Leu polymorphisms promote early pregnancy loss and that compound heterozygosity may have an aggravating effect. We therefore evaluated the relationship between early pregnancy loss and the PAI-1 4G/5G polymorphism, the FXIII Val34Leu polymorphism, and compound carrier status for both polymorphisms.

Patients and Methods

STUDY POPULATION

For this case-control study, we enrolled 49 unrelated Caucasian women with a history of two consecutive or three to six nonconsecutive early pregnancy losses and 48 unrelated healthy controls. The patients were referred from local hospitals or gynecologists to the Department of Obstetrics and Gynecology, Division of Gynecologic Endocrinology and Reproductive Medicine, University of Vienna, for evaluation of common causative risk factors for recurrent pregnancy loss. For all patients we obtained complete medical histories, performed physical examinations and routine laboratory tests, and took cervicovaginal smears for microscopic examination of pathologic colonization and cultures for chlamydia, ureaplasma, and mycoplasma. Routinely timed hysteroscopy with endometrial biopsy under local anesthesia was performed for exclusion of both anatomic abnormalities and luteal-phase insufficiency. Extensive endocrinologic examinations, including day-night fluctuations and stimulation tests, were undertaken to identify even subclinical hormonal disorders. Immunologic tests for autoantibodies were added. Karyotyping was recommended to all women and their partners, but results were available for only one-half of the study population.

The exclusion criteria were as follows: anatomic abnormalities; endocrinologic dysfunction; balanced-type chromosomal translocation; autoimmune disease; arterial hypertension; liver function abnormalities; alcohol or drug abuse; inflammatory pelvic disease; overt evidence of inflammatory disease; use of medications that affect liver function or the blood coagulation system, including estrogen-containing medications; and a history of pregnancy complications related to multiple gestation or assisted reproduction.

Controls were healthy women who had delivered at least one healthy, term infant and had no history of pregnancy loss. Controls were matched for age, smoking status, and ethnic background. None of the controls used oral contraceptives, hormonal intrauterine devices, or any medication affecting liver function and/or blood coagulation.

The study was performed in accordance with the Declaration of Helsinki, and written informed consent was given by all participating women.

LABORATORY METHODS

Patients and controls were asked to undergo venous blood collection at least 3 months after pregnancy or cessation of lactation to rule out any pregnancy-related alterations of coagulation or fibrinolysis. After a 12-h fast, venous blood was collected in a 1:10 dilution of 0.11 mol/L trisodium citrate. Blood was centrifuged for 10 min at 2000g, and serum and plasma were frozen immediately after centrifugation and stored at -80 °C.

Plasma antithrombin activity, protein C and protein S activities, and fibrinogen were measured on an STA coagulation analyzer (Diagnostica Stago) with the Coamatic AT 400 (Chromogenix) and STA Protein C chromogen, STA Protein S Clotting, and STA Fibrinogen (all Diagnostica Stago) assays. Resistance to activated protein C was determined with the Coatest APC Resistance Assay (Chromogenix) on the same analyzer.

The presence of lupus anticoagulant was assessed according to the criteria of the International Society on Thrombosis and Hemostasis *(26)* by use of LA1 Screening Reagent, LA2 Confirmatory Reagent (Dade Behring), PTT-LA (Diagnostica Stago), and mixing studies with pooled normal plasma.

Anti-β_2-glycoprotein 1 was measured with the Varelisa β_2 Glycoprotein 1 Antibodies Screen (Pharmacia & Upjohn), which detects IgG, IgM, and IgA antibodies.

We classified antiphospholipid syndrome according to the criterion described by Gris et al. *(27)*, i.e., the presence of phospholipid-specific antibodies detected by immunoassays and/or coagulation-derived assays.

GENETIC ANALYSIS

Genomic DNA was isolated from peripheral blood leukocytes with use of a commercially available reagent set (GenXtract Blood DNA Extraction System; ViennaLab Labordiagnostika GmbH).

We used PCR and reverse hybridization to genotype samples for factor V Leiden, prothrombin G20210A, methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C, PAI-1 4G/5G, and FXIII Val34Leu, as described previously *(28, 29)*. DNA fragments were amplified in vitro, biotinylated in multiplex PCR reactions, and hybridized for 30 min at 45 °C to a membrane test strip presenting a parallel array of allele-specific, 15- to 20mer oligonucleotide probes for each mutation: factor V Leiden, 5'-GGACAGGC(G/A)AGGAATAC-3'; prothrombin, 5'-CTCTCAGC(G/A)AGCCTCAA-3'; MTHFR C677T, 5'-TGCGGGAG(C/T)CGATTTCA-3'; MTHFR A1298C, 5'-CAGTGAAG(A/C)AAGTGTCT-3'; PAI-1 4G/5G, 5'-ACACGTGG(−/G)GGAGTCAG-3'; and FXIII Val34Leu, 5'-TTCAGGGC(G/T)TGGTGCCC-3'. Specifically bound PCR fragments were detected by use of a streptavidin-alkaline phosphatase conjugate and color substrates. Control DNA samples, previously typed by direct DNA sequencing, were available for all mutations and were used to confirm the specificity of the assay. The entire hybridization and detection procedure was carried out in a fully automated device (profiBlot II T 30; TECAN Austria).

STATISTICAL ANALYSIS

The SAS statistical package, Ver. 8.2 (SAS Institute) was used for all statistical analyses. Depending on the type of data, the Pearson χ^2 test or the Fisher exact test (two-tailed) was used.

The relative risks for FXIII Val34Leu and PAI-1 4G/5G were estimated from odds ratios (ORs). Stepwise logistic regression was performed to rule out which genetic thrombophilic factor entered the model as an independent significant risk factor for early pregnancy loss.

Results

The study population included 49 Caucasian women with a history of two consecutive (5 participants) or three to six nonconsecutive (44 participants) early pregnancy losses and 48 healthy controls. Mean (SD) age at enrollment was 35.6 (6.0) years for cases and 36.6 (5.1) years for controls, with a range of 22–47 years. Patients and controls were matched for age within 3 years, for smoking status (14 smokers in cases vs 13 in controls), and all participating women were Caucasians. The controls had delivered 71 healthy term infants, whereas the number of overall pregnancies in women with early pregnancy loss was 196 with only 18 (7%) successful pregnancies and a mean (SD) of 3.6 (1.2) miscarriages per participant. Of the 178 first- and second-trimester pregnancy losses, 173 occurred between the 8th and 12th weeks of gestation and 5 between the 13th and 20th weeks. None of the women had experienced second-trimester pregnancy loss only. One of the enrolled patients had suffered a pregnancy-related thromboembolism (13th week of gestation), and one had developed a venous thrombosis after a miscarriage. Two other patients had suffered thrombophlebitis of superficial veins, but none was on anticoagulant therapy when included in the study.

Between cases and controls there was no difference in plasma concentrations of antithrombin [110.9 (12.1)% vs 110.9 (13.8)%], protein C [117.8 (21.7)% vs 111.3 (19.0)%], and protein S [95.1 (24.6)% vs 92.9 (22.9)%]. No participating woman had antithrombin or protein C concentrations below the laboratory's reference interval (80–120% for antithrombin and 70–125% for protein C). One case was deficient for protein S with a protein S value of 33% (reference interval, 65–120%). None of the controls was deficient for any of these coagulation inhibitors. The prevalence of the heterozygous prothrombin G20210A gene mutation did not differ significantly between women with or without pregnancy loss. We also found no significant difference between cases and controls for homozygosity of MTHFR C677T and A1298C mutations. We calculated the risk for the combined genotypes for the MTHFR C677T and A1298C mutations, including compound heterozygosity, compound homozygosity, and compound hetero-/homozygosity. There were no statistically significant differences between carriers of two or more mutant alleles compared with carriers of only one mutant allele for either polymorphism. For carriers of factor V Leiden, we found an approximately doubled, although not statistically significant, risk for early pregnancy loss (Table 1).

The prevalence of the PAI-1 4G/5G polymorphism in cases was 28 (57%) for 4G/5G and 12 (25%) for homozygous 4G vs 25 (51%) and 8 (17%), respectively, in controls. Twenty-one cases and 16 controls (43% vs 33%) were heterozygous for the FXIII Val34Leu polymorphism, and 4 cases were homozygous for FXIII 34Leu vs 1 control (8% vs 2%). We found no statistically significant difference for the isolated occurrence of one of these mutations. We also calculated the relative risks for the possible combinations of wild type, heterozygosity, and homozygosity for these polymorphisms. There was a continual increase of relative

Table 1. Prevalence of evaluated thrombophilic risk factors.

Thrombophilic risk factors	Cases, n (%)	Controls, n (%)	OR (95% CI)[a]	P[b]
Protein S deficiency	1 (2)	0 (0)	Not estimated	Not estimated
Antiphospholipid syndrome	4 (8)	0 (0)	Not estimated	0.043[b]
Factor V Leiden het.	8 (16)	4 (8)	2.2 (0.6–7.7)	0.232
Prothrombin G20210A mut. het.	2 (4)	3 (6)	0.6 (0.1–4.0)	0.629
MTHFR C677T hom.	8 (16)	5 (10)	1.7 (0.5–5.6)	0.393
MTHFR A1298C hom.	7 (14)	5 (10)	1.4 (0.4–4.9)	0.562
Combined MTHFR[c]	26 (53)	23 (48)	1.3 (0.6–3.0)	0.477
4G/4G + 4G/5G vs 5G/5G	40 (82)	33 (69)	2.0 (0.8–5.2)	0.212
4G/4G vs 4G/5G + 5G/5G	12 (25)	8 (17)	1.6 (0.6–4.4)	0.341
34Leu + Val34Leu vs Val34	25 (51)	17 (35)	1.9 (0.8–4.3)	0.121
High-risk vs low-risk group[d]	28 (57)	17 (35)	2.4 (1.1–5.5)	0.032[b]

[a] CI, confidence interval; het, heterozygous; mut, mutation; hom, homozygous.

[b] P values were obtained from χ^2 test, and values <0.05 were considered statistically significant.

[c] Because compound heterozygosity for MTHFR C677T and MTHFR A1298C is known to be as thrombophilic as homozygosity for one of these mutations, we also calculated the risk for the combined genotypes, including compound heterozygosity, compound homozygosity, and compound hetero-/homozygosity.

[d] To evaluate the risk for early pregnancy loss in carriers of both polymorphisms, we divided all participating women into low- and high-risk groups. Carriers of no or one mutant allele for either polymorphism were considered to be at low risk for early pregnancy loss. The high-risk group included the following genotype combinations: homozygosity for either polymorphism and compound heterozygosity or compound heterozygosity/homozygosity for both polymorphisms.

risks with an increasing number of PAI-1 4G and FXIII 34Leu alleles, respectively (Table 2).

To evaluate the risk for recurrent pregnancy loss in women homozygous for either polymorphism or who were carriers of both, we divided all participating women into low- and high-risk groups and then repeated the χ^2 test for cases and controls. The high-risk group included women who were either compound heterozygous or compound heterozygous/homozygous for both polymorphisms or homozygous for either 4G or 34Leu. All other women were considered to be at low risk.

For the high-risk group, the relative risk for early pregnancy loss was significantly increased (OR = 2.4; 95% confidence interval, 1.1–5.5; $P = 0.032$). Additionally, we performed a stepwise logistic regression analysis that included all determined thrombophilic risk factors except antiphospholipid syndrome, which could not be included for numeric reasons (n = 0 for controls). The only variable that entered the model as a significant and independent risk factor for early pregnancy loss when the significance level was 0.10 was the combination of PAI-1 and FXIII genotypes as described above.

Heterozygosity for factor V Leiden and homozygosity for the MTHFR 677 mutation were significantly more common in the high-risk group and were both associated with a 7.8-fold increase of risk for early pregnancy loss. We detected no relevant difference between the two groups for combinations with the other determined thrombophilic risk factors. However, there was a statistically significant difference in the prevalence of combined defects, which were defined as a combination of high-risk genotypes with at least one other established thrombophilic risk factor (Table 3).

Discussion

In this study, we examined the influence of selected genetic thrombophilic risk factors on early pregnancy loss, focusing on the PAI-1 4G/5G polymorphism and the Val34Leu polymorphism in the FXIII gene.

As seen for factor V Leiden in smaller studies, the isolated occurrence of either PAI-1 4G/5G or the Val34Leu polymorphism was not associated with a statistically significant difference between cases and controls. Possibly this is attributable to the small number of participating women, which did not allow detection of statistical differences for mild risk factors.

A higher first-trimester miscarriage rate was reported by Glueck et al. (11) in women carrying two gene mutations compared with women with only one. We therefore divided all participating women into low- and high-risk groups. Carriers of no or one mutant allele were regarded as low risk and carriers of at least two mutant alleles (homozygosity for one polymorphism, compound heterozygous or compound heterozygous/homozygous status) as high risk. Statistical analysis showed a significant difference between the high- and low-risk groups.

We also observed a significantly increased risk in women carrying the high-risk genotypes and an additional heterozygous factor V Leiden genotype. A similar interaction has been described by Glueck et al. (30) in

Table 2. Relative risks for early pregnancy loss in the PAI-1 4G/5G and FXIII Val34Leu polymorphisms.

	OR	95% CI[a]	P[b]
4G/5G vs 5G/5G	1.9	0.7–5.0	0.212
4G/4G vs 4G/5G	1.3	0.5–3.8	0.583
4G/4G vs 5G/5G	2.5	0.7–8.5	0.137
Val34Leu vs 34Val	1.7	0.7–3.9	0.217
34Leu vs Val34Leu	3.1	0.3–30.0	0.320
34Leu vs 34Val	5.2	0.5–49.3	0.119

[a] CI, confidence interval.

[b] P values <0.05 were considered statistically significant.

Table 3. Prevalence and relative risk of combined defects.

Thrombophilic risk factors	Cases, n (%)	Controls, n (%)	OR (95% CI)[a]	P[b]
High-risk genotype + APS	3 (6)	0 (0)	Not estimated	0.082
High-risk genotype + PS deficiency	0 (0)	0 (0)	Not estimated	Not estimated
High-risk genotype + FV Leiden het.	7 (14)	1 (2)	7.8 (0.9–66.3)	0.029[b]
High-risk genotype + Proth G20210A het.	2 (4)	3 (6)	0.6 (0.1–4.0)	0.629
High-risk genotype + MTHFR C677T hom.	7 (14)	1 (2)	7.8 (0.9–66.3)	0.029[b]
High-risk genotype + MTHFR A1298C hom.	1 (2)	2 (4)	0.5 (0.0–5.4)	0.545
Combined defects[c]	16 (33)	6 (13)	3.4 (1.2–9.6)	0.018[b]

[a] CI, confidence interval; APS, antiphospholipid syndrome; PS, protein S; FV, factor V; het, heterozygous; Proth, prothrombin; hom, homozygous.

[b] P values were obtained from χ^2 test, and values <0.05 were considered statistically significant.

[c] Combination of the high-risk genotypes with at least one other thrombophilic risk factor. Two cases and one control had combinations of a high-risk genotype with two other thrombophilic risk factors, and one case had a combination of a high-risk genotype with three thrombophilic risk factors.

serious late-pregnancy complications; the authors speculated that factor V Leiden-induced thrombosis in the placental bed with subsequent hypoxia might enhance the expression of PAI-1 and hence suppress fibrinolysis under conditions of low oxygen tension.

Additionally, we saw a clear increase in risk for the combination of high-risk genotypes with homozygosity for the MTHFR C677T mutation. This mutation has been described as a risk factor for venous thrombosis and has recently also been found to be a weak risk factor for recurrent pregnancy loss *(31)*. Similar to other studies *(10)*, we did not find a significant relationship between the isolated occurrence of this mutation and pregnancy loss, but the combination with the high-risk genotypes was associated with an OR of 7.8 for early pregnancy loss. However, for both factor V Leiden and MTHFR C677T in combination with the high-risk genotype, the confidence intervals are wide and data need to be interpreted in this context.

We observed no statistically significant difference between the high- and low-risk genotypes in combination with antiphospholipid syndrome or a deficiency for one of the determined coagulation inhibitors. For the combination with antiphospholipid syndrome, this may simply reflect the reduced statistical discrimination power resulting from the small number of participants. The fact that the determined coagulation inhibitors had no impact on our results may be attributable to their low prevalence in the general population.

Although our findings should be interpreted in the context of a small case-control study and require further confirmation by larger epidemiologic studies, we think that our results are valid and have clinically relevant impact for the following reasons: (*a*) Our results were not biased by established nonthrombophilic conditions associated with early pregnancy loss because women with gynecologic or endocrinologic risk factors were carefully excluded. (*b*) Genetic thrombophilia, for which an association with early pregnancy loss is established or at least in discussion, was taken into account by stepwise logistic regression analysis. However, in our study, these factors did not emerge as significant and independent risk factors for early pregnancy loss. (*c*) For the PAI-1 4G/5G and

FXIII Val34Leu polymorphisms, an increase in the number of mutant alleles was associated with an increasing risk for early pregnancy loss. This was clearly demonstrated by the ORs for the isolated occurrences of the two polymorphisms and the significant difference between women in the high- and low-risk groups. (*d*) There is also a rationale to how these two polymorphisms have additional effects in promoting early pregnancy loss: in homozygous FXIII 34Leu samples, a structural change of the fibrin meshwork was demonstrated, with thinner strands and reduced space between them. It appears that the earlier cross-linking of fibrin encoded by the FXIII 34Leu allele compared with FXIII encoded by the 34Val allele inhibits lateral aggregation of the fibrin fibers, producing a reduced mass/length ratio *(19)*. Recent experiments have shown that the fibrinolytic effectiveness of tissue plasminogen activator and urokinase plasminogen activator is enhanced on coarse, thick-stranded fibrin and that the higher water content and the large pore size in coarse fibrin facilitates penetration and diffusion of fibrinolytically active proteins *(32)*. Therefore, the changes in fibrin structure attributable to the FXIII 34Leu allele will have an antifibrinolytic effect.

Although there is no experimental proof, a protective effect of FXIII 34Leu in occlusive arterial disease is well established. However, the relationship to venous thrombosis is less clear *(25)*. It is noteworthy that Kohler et al. *(33)* described higher concentrations of PAI-1 and an increased frequency of the PAI-1 4G/4G genotype in patients with the FXIII 34Leu genotype and myocardial infarction, suggesting that impaired fibrinolysis negates the postulated protective effect of the FXIII 34Leu genotype. In contrast, our data suggest that the PAI-1 4G and FXIII 34Leu variants synergistically contribute to impaired fibrinolysis because of reduced activity of the fibrinolytic system and increased resistance of the fibrin network to fibrinolysis. In affected women this may then promote the development of early pregnancy loss by insufficient trophoblast invasion and unbalanced fibrin deposition in the early placental circulation.

In conclusion, our data indicate that carrier status for at least two mutant alleles of the PAI-1 4G/5G and/or FXIII

Val34Leu polymorphisms and, to a larger extent, compound carrier status with homozygosity for either polymorphism must be considered risk factors for early pregnancy loss.

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