The polymorphism of platelet membrane integrin $\alpha_2\beta_1$ ($\alpha_2$807TT) is associated with premature onset of fetal loss

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Summary

Inherited thrombophilia could increase susceptibility to adverse pregnancy outcomes such as fetal loss. We determined the G1691A mutation of the factor V gene (FVL), the G20210A mutation of the prothrombin gene, the C677T polymorphism of the methylenetetrahydrofolate-reductase (MTHFR) gene, the HPA-1 polymorphism of the β_3 subunit of the platelet integrin $\alpha_{IIb}\beta_3$ and the C807T polymorphism of the α_2 subunit of integrin $\alpha_2\beta_1$ in 104 women with fetal loss and 277 normal women. In a subgroup analysis of women with recurrent early fetal loss (n=34), the *prevalence* of the genetic markers did not differ significantly between the women with early fetal loss and the normal women. However, in this subgroup of patients the *onset* of fetal loss was significantly earlier in women with the $\alpha_2$807TT genotype (7.1 ± 1.9 vs. 8.8 ± 1.5 weeks, p=0.001). No such significant difference was observed in carriers of the other genetic markers. In the subgroup analysis of women with late fetal loss (n=70), only the prevalence of heterozygous FVL was significantly associated with late fetal loss (odds ratio 3.2, p=0.002). There was no significant association of any genetic risk factor with premature fetal loss in the subgroup analysis of women with at least one late miscarriage. This study demonstrates a significant association of the $\alpha_2$807TT genotype of the platelet membrane integrin $\alpha_2\beta_1$ with premature onset of early fetal loss. It appears that this risk factor does not induce the pathomechanism, but modulates the course of fetal loss. Furthermore, our study confirms the association of FVL with late fetal loss.

Keywords

Fetal loss, platelet receptor polymorphisms, HPA-1/PI^A, $\alpha_{IIb}\beta_3$, glycoprotein IIb-IIIa, $\alpha_2$807TT genotype, $\alpha_2\beta_1$, glycoprotein Ia-IIa

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Introduction

In pregnancy, a successful outcome is highly dependent on appropriate placental development and sustained placental function. These processes, in turn, require the formation of an adequate feto-maternal circulatory system (1). Obstetrical complications such as recurrent miscarriage, stillbirth, severe preeclampsia, abruptio placentae, and fetal growth retardation are associated with abnormal placental vasculature and inadequate placental maternal-fetal circulation (2–7).

The hemostatic pathways are intimately involved in ovulation, implantation and placentation (8). Pregnancy per se is associated with a hypercoagulable state (8). Although recurrent fetal loss is a heterogeneous condition and no single abnormality can account for all cases of fetal loss, the hypothesis has been developed that many cases of miscarriage, both early and late, are caused by an exaggerated maternal hemostatic response leading to thrombosis of the uteroplacental vasculature and subsequent fetal loss (1, 9–14).

Established thrombophilic risk factors, most of them are well known risk determinants of venous thrombosis, include the G1691A mutation of the factor V gene (factor V Leiden), the G20210A mutation of the prothrombin-gene, hyperhomocysteinemia and deficiencies of antithrombin, protein C and protein S. The degree of the association between thrombophilia and fetal loss varies in the published studies, related to type of fetal loss and the type of thrombophilia (14). The association between homozygosity for the C677T polymorphism of the methylene-

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tetrahydrofolate-reductase (MTHFR)-gene and venous thrombosis or fetal loss is controversial (14).

In the case of a vascular alteration primarily located in the arterial system, a risk factor of arterial thrombogenicity may be of importance. Platelets play a pivotal role in the pathogenesis of arterial thrombosis, which has been well documented for acute coronary syndromes and cerebrovascular events.

As shown in preliminary studies, two platelet receptor genotypes, the HPA1-b allele of the β_3 subunit of the essential platelet integrin $\alpha_{IIb}\beta_3$, also known as glycoprotein IIb-IIIa, and the 807TT genotype of the α_2 subunit of integrin $\alpha_2\beta_1$, also known as glycoprotein Ia-IIa, are risk factors for increased platelet thrombogenicity (15–21). In accordance with this observation, these genetic variants may lead to thrombotic occlusion in the presence of a predisposing vascular alteration resulting in premature fetal loss.

We used a case-control design to assess hereditary risk factors of venous and arterial thrombosis as risk determinants for recurrent fetal loss and a case-only design to assess these risk factors as determinants for premature onset of fetal loss.

Materials and methods

Subjects

We retrospectively studied 104 consecutive women who had recurrent fetal loss without known cause, who were referred for thrombophilia screening to the Düsseldorf University Medical Center between January 1999 and July 2003. To avoid a referral bias, all women with prior evaluation of genetic risk factors were excluded. Women were also excluded if they had the antiphospholipid-syndrome. Inclusion criteria were either recurrent early fetal loss (three or more consecutive fetal losses at < 12 weeks gestation and no late fetal loss, n=34), or at least one late fetal loss ($\geq$ 12 weeks gestation, n=70) according to previously published classifications (11).

Only women with post-embryonic loss after ultrasonic disappearance of fetal pulse from the intrauterine fetal pole were included in the study. Documented first trimester preclinical and blighted ovum abortions, as well as fetal losses that were the result of documented fetal malformation or the result of an infectious complication, were excluded. None of the women received an antithrombotic prophylactic therapy (e.g. low molecular weight heparin) or an antiplatelet agent during the pregnancy complicated by fetal loss.

The women enrolled with recurrent fetal loss had no previous history of venous or arterial thromboembolic disease, diabetes mellitus, chronic hypertension, thyroid dysfunction, systemic lupus erythematosus, intrauterine growth retardation, pregnancy-induced hypertension, or preeclampsia. They all had a detailed investigation which was negative for potential causes of fetal demise including fasting glucose, basal FSH, LH and estradiol levels on day 3 of a natural cycle, TSH and prolactin levels and antinuclear factor.

In addition, transvaginal scanning was performed to verify ovarian morphology. Women with three or more first trimester or one or more second or third trimester pregnancy losses underwent a hysterosalpingography and/or hysteroscopy to confirm uterine cavity normalcy, and both partners were also investigated for chromosomal aberrations.

As control subjects, 277 normal women with at least one previous pregnancy, no previous fetal loss or late pregnancy complications, and no history of previous arterial or venous thromboembolism were recruited by the Heinrich Heine University Blood Donation Center. The normal women were from the same geographic region as the women with recurrent fetal loss, but were unrelated to them.

Personal histories were obtained from all women with the use of a standardized questionnaire. The study was approved by the ethical committee of the Faculty of Medicine, Heinrich Heine University, Düsseldorf, and written informed consent was given by all women.

Laboratory tests

Genomic DNA was extracted from peripheral blood leukocytes according to standard protocols using the Chelex system (BIO-RAD, München, Germany) or Qiagen system (Qiagen, Hilden, Germany). The presence or absence of factor V Leiden and the G20210A mutation in the prothrombin gene were identified using an allele-specific restriction-enzyme analysis as reported (21, 22). Analysis for the presence of the MTHFR C677T polymorphism was performed by the method described by Frosst et al. (23). Determination of the HPA-1 polymorphism of the β_3 subunit of $\alpha_{IIb}\beta_3$ and the C807T polymorphism of the α_2 subunit of integrin $\alpha_2\beta_1$ was performed as previously described (24, 25)

The activities of plasma protein C and protein S were measured by a functional clotting assay (Instrumentation Laboratory, Milano, Italy), and total protein S antigen and free protein S antigen by an ELISA Kit (Diagnostic International, Schriesheim, Germany). Antithrombin activity was measured with the use of Berichrom (Dade Behring, Liederbach, Germany).

Statistical analysis

The association of genetic variants with an increased risk for fetal loss was evaluated in a case-control design. Differences in proportions were tested by the Fisher exact test and relative risk was estimated by the odds ratio. The results of continuous variables were calculated as means ± standard deviation (SD). Continuous variables were compared by Wilcoxon two sample exact test. The analyses were performed in subgroups of women with early and late fetal losses.

The association of genetic variants with premature onset of fetal loss was evaluated in two ways. First, the weeks of gestation at onset of the earliest fetal loss were analyzed using the Wilcoxon two sample exact test and a multivariate regression analysis based on the Cox proportional hazards model to identify independent predictors of premature fetal loss. Variables included in the analyses fulfilled the proportional-hazards assumption, since the results of tests of these variables for nonproportional hazards were not significant. Since only patients with fetal loss were evaluated in this model, no individuals were censored and the proportional hazards model represents a multivariate analysis evaluating premature onset of fetal loss and not an increased risk of fetal loss.

Second, the complete data of *all* early fetal losses (n=132) in the subgroup of women (n=34) with early fetal losses and *all* late fetal losses (n=91) in the subgroup of women with late fetal loss (n=70) over time were analyzed regarding the weeks of gestation at fetal loss using a mixed model with unequally spaced repeated measurements (26). In this analysis, each pregnancy represents a new measurement. This model allows a longitudinal analysis of the influence of a between-subjects factor (e.g. genetic variant) on the week of fetal loss, the influence of a within-subjects factor (number of fetal losses) on the week of fetal loss, and the interaction of the genetic variant and number of fetal losses representing a change of risk factor-dependent differences over time (number of fetal losses).

Subjects with HPA-1a/1b or HPA-1b/1b genotypes were classified as HPA-1b-positive, those with HPA-1a/1a as HPA-1b-negative (dominant model). In the C807T polymorphism, the 807TT genotype was compared with the 807CC+CT genotypes (recessive model). This classification is based on the results of previous functional and clinical studies (16). P values of less than 0.05 were considered to be significant. All confidence intervals were calculated at the 95 percent level. All statistical analyses were performed with SAS software (version 8.2, SAS Institute Inc., Cary, North Carolina).

Results

Study patients

The study population comprised 34 women with recurrent early fetal loss (three or more consecutive miscarriages at < 12 weeks gestation and no late fetal loss), and 70 women with late fetal loss (at least one late miscarriage at ≥ 12 weeks gestation). The median age of the women with recurrent fetal loss was 35.1 years ± 7.7 vs. 33.6 years ± 12.4 of the normal women. In total, 104 women with recurrent fetal loss had 262 previous pregnancy losses (mean 2.5, range 1 to 7). The number of fetal losses among the 34 women with early fetal loss was 132 (mean 3.9, range 3–7); of these women, 17 (50 %) had at least one successful pregnancy. The number of fetal losses among the 70 women with late fetal loss was 130 with 91 late fetal losses (mean 1.30, range 1 to 4) and 39 early fetal losses (mean 1.3, range 1 to 2); of these women, 40 (61.4 %) had at least one successful pregnancy.

Risk association with recurrent fetal loss using a case-control design

The prevalence of heterozygous factor V Leiden was significantly higher among the women with recurrent fetal loss (n=104) than among the normal women (Table 1). In contrast, no significant difference in the prevalences of the G20210A prothrombin gene mutation and of the MTHFR 677TT genotype as well as the prevalences of HPA-1b and $\alpha_2$807TT genotype were found in women with recurrent fetal loss and in normal women (Table 1). None of the women had a combined heterozygous or homozygous defect of the factor V Leiden or the G20210A prothrombin gene mutation and none of the women had a deficiency of antithrombin, protein C, or protein S.

To assess a possible difference between risk factors of early and late fetal loss, additional subgroup analyses in women with three or more consecutive miscarriages at < 12 weeks gestation and those with at least one late miscarriage at ≥ 12 weeks gestation were performed.

Women with early fetal loss

In the subgroup analysis of women with three or more consecutive miscarriages at < 12 weeks gestation, the prevalence of the genetic markers did not differ significantly between the women with recurrent fetal loss (n=34) and the normal women (n=277) (Table 1).

Table 1: Risk association with recurrent fetal loss using a case-control design.

Genetic Defect	Women With Recurrent Fetal Loss (n=104) %	(no. with defect/ total no.)	Normal Women (n=277) %	(no. with defect/ total no.)	P Value	Odds Ratio	95% Confidence Interval
Factor V Leiden heterozygous							
All fetal losses (n=104)	15.4	16/104	7.9	22/277	0.036	**2.1**	(1.0-4.2)
Early fetal loss (n=34)	2.9	1/34			0.49	**0.4**	(0.1-2.7)
Late fetal loss (n=70)	21.4	15/70			0.002	**3.2**	(1.5-6.5)
Prothrombin G20210A heterozygous							
All fetal losses (n=104)	1.9	2/104	2.2	6/277	1.0	**0.89**	(0.2-4.5)
Early fetal loss (n=34)	-	-			-	-	-
Late fetal loss (n=70)	2.9	2/70			0.67	**1.3**	(0.3-6.7)
677TT MTHFR genotype							
All fetal losses (n=104)	13.5	14/104	10.1	28/277	0.36	**1.4**	(0.7-2.7)
Early fetal loss (n=34)	14.7	7/34			0.38	**1.5**	(0.6-4.3)
Late fetal loss (n=70)	12.9	9/70			0.52	**1.3**	(0.6-2.9)
HPA – 1b- positive *							
All fetal losses (n=104)	21.2	22/104	28.5	79/277	0.15	**0.7**	(0.4-1.2)
Early fetal loss (n=34)	20.6	7/34			0.42	**0.7**	(0.3-1.6)
Late fetal loss (n=70)	21.4	15/70			0.29	**0.7**	(0.4-1.3)
$\alpha_2$807TT genotype							
All fetal losses (n=104)	15.4	16/104	13.0	36/277	0.62	**1.2**	(0.6-2.3)
Early fetal loss (n=34)	14.7	5/34			0.79	**1.2**	(0.4-3.2)
Late fetal loss (n=70)	15.7	11/70			0.56	**1.2**	(0.6-2.6)

* Subjects with the HPA-1a/HPA-1a genotype were classified as HPA-1b-negative and subjects with the HPA-1a/HPA-1b genotype or HPA-1b/HPA-1b genotype were classified as HPA-1b-positive.

Table 2: Premature fetal loss in women with three and more early abortions according to genetic risk determinant (case-only design).

Genetic Defect	Analysis Using Earliest Fetal Loss as Event Time in 34 Women						Analysis Using all Early Abortions (n=132) as Event Time in 34 Women				
	Genetic Marker Negative		Genetic Marker Positive		P Value Univariate (Wilcoxon Exact Test)	P Value Multivariate *	Genetic Marker Negative		Genetic Marker Positive		P Value (Mixed Model Multivariate)
	No.	Week of abortion Mean ± SD	No.	Week of abortion Mean ± SD			No.	Week of abortion Mean ± SD	No.	Week of abortion Mean ± SD	
Factor V Leiden heterozygous	33	7.7 1.6	1	6.0 0	0.47	0.13	129	8.6 1.6	3	7.0 1.0	0.27
Prothrombin G20210A heterozygous	34	7.7 1.7	-		-	-	132	8.5 1.6	-	-	-
677TT MTHFR genotype	29	7.9 1.6	5	6.6 1.5	0.13	0.14	114	8.6 1.5	18	7.9 2.1	0.18
HPA – 1b- positive †	27	7.8 1.7	7	7.1 1.5	0.36	0.82	109	8.6 1.6	23	8.1 1.6	0.75
$\alpha_2$807TT genotype	29	8.1 1.4	5	5.4 0.9	0.001	<0.0001	111	8.8 1.5	21	7.1 1.9	0.001

* Multivariate analysis: including age, factor V Leiden, 677TT MTHFR genotype, HPA-1 genotype, and $\alpha_2$807TT genotype.
† Subjects with the HPA-1a/HPA-1a genotype were classified as HPA-1b-negative and subjects with the HPA-1a/HPA-1b genotype or HPA-1b/HPA-1b genotype were classified as HPA-1b-positive.

Women with late fetal loss

In contrast, in the subgroup analysis of women with at least one late miscarriage (≥ 12 weeks gestation) (n=70), the prevalence of heterozygous factor V Leiden was significantly higher among the women with fetal loss than among the normal women (Table 1). No significant difference in the prevalences of the G20210A prothrombin gene mutation and of the MTHFR 677TT genotype as well as the prevalences of HPA-1b and $\alpha_2$807TT genotype were found in women with late fetal loss and in normal women (Table 1).

Assessment of premature fetal loss using a case-only design

To assess an association of the different genotypes with premature fetal loss, a case-only design was used (Table 2).

Women with early fetal loss

In the subgroup analysis of women with early fetal loss (three or more consecutive miscarriages at < 12 weeks gestation), the onset of the earliest fetal loss was significantly earlier in women with the $\alpha_2$807TT genotype (Wilcoxon exact test p=0.001). No such significant difference was observed in carriers of the other genetic markers (factor V Leiden, the G20210A prothrombin gene mutation, the MTHFR 677TT genotype, HPA-1b) (Table 2). These results were confirmed using the mixed model analysis for evaluation of all early fetal losses over time. The $\alpha_2$807TT genotype was significantly associated with premature fetal loss using univariate and multivariate analysis (Table 2). There was no significant influence of the number of fetal losses and no significant interaction between any risk factor and the number of fetal losses on the week of fetal loss (Table 2).

Table 3: Premature fetal loss in women with at least one late fetal loss (± 12 weeks gestation) according to genetic risk determinant (case-only design).

Genetic Defect	Analysis Using Earliest Fetal Loss as Event Time in 70 Women						Analysis Using all Late Abortions (n=91) as Event Time in 70 Women				
	Genetic Marker Negative		Genetic Marker Positive		P Value Univariate (Wilcoxon Exact Test)	P Value Multivariate *	Genetic Marker Negative		Genetic Marker Positive		P Value (Mixed Model Multivariate)
	No.	Week of abortion Mean +- SD	No.	Week of abortion Mean +- SD			No.	Week of abortion Mean +- SD	No.	Week of abortion Mean +- SD	
Factor V Leiden heterozygous	55	18.9 8.7	15	16.8 5.7	0.36	0.52	72	18.6 8.7	19	16.6 5.4	0.26
Prothrombin G20210A heterozygous	68	18.8 8.2	2	12.0 0	0.12	0.112	89	18.3 8.1	2	12. 0 0	0.13
677TT MTHFR genotype	61	18.9 8.5	9	15.6 4.5	0.58	0.50	79	18.4 8.5	12	16.5 5.0	0.42
HPA – 1b- positive †	55	18.1 7.8	15	19.8 9.6	0.79	0.67	71	18.1 7.9	20	18.3 8.9	0.89
$\alpha_2$807TT genotype	59	19.2 8.6	11	14.5 4.0	0.095	0.09	79	18.4 8.2	12	16.4 7.8	0.21

* Multivariate analysis: including age, factor V Leiden, prothrombin G20210A mutation, 677TT MTHFR genotype, HPA-1b allele, and $\alpha_2$807TT genotype.
† Subjects with the HPA-1a/HPA-1a genotype were classified as HPA-1b-negative and subjects with the HPA-1a/HPA-1b genotype or HPA-1b/HPA-1b genotype were classified as HPA-1b-positive.

Women with late fetal loss

There was no significant association of any genetic risk factor with premature fetal loss in the subgroup analysis of women with at least one late miscarriage (≥12 weeks gestation), neither in the evaluation of the earliest fetal loss nor in the evaluation of all late fetal losses using the mixed model (Table 3).

Discussion

This study demonstrates a significant association of the $\alpha_2$807TT genotype of the platelet membrane integrin $\alpha_2\beta_1$ with premature onset of early fetal loss. Furthermore, our study confirms previous findings indicating an association of factor V Leiden, a risk factor for venous thrombosis, with late fetal loss (14).

The number of platelet $\alpha_2\beta_1$ receptor copies varies up to 10-fold among normal individuals, whereas the levels of other integrins do not (27–29). Three allelic differences in the α_2 gene are associated with expression levels on the $\alpha_2\beta_1$ integrin on the platelet surface: allele 1 (807T/837T/873A/Br^b), abbreviated α_2 807T, is associated with increased levels of $\alpha_2\beta_1$, allele 2 (807C/837T/873G/Br^b) and allele 3 (807C/837C/873G/ Br^a), abbreviated α_2 807C, are each associated with lower levels of $\alpha_2\beta_1$ (27). The rate of platelet adhesion to collagen is proportional to the density of $\alpha_2\beta_1$ receptor copies on the platelet surface (27). Because $\alpha_2\beta_1$ mediates platelet adhesion to collagen in vivo, variation in its expression levels has a significant impact on platelet function, contributing to an increased risk of thrombosis or to bleeding (30).

In accordance with evidence of a prothrombotic phenotype for the $\alpha_2$807T allele, the results of our study have several major implications: First, since the $\alpha_2$807TT genotype is not related to fetal loss itself, as shown in the case-control design (Table 1), premature onset of early fetal loss can be explained by an effect associated with this genotype modulating the course of the underlying disease process. Thus, the $\alpha_2$807TT genotype appears to be a risk factor for premature onset of fetal loss in patients with already existing feto-maternal malfunction, but not a risk factor for the development of the disease per se. In contrast to the $\alpha_2$807TT genotype, factor V Leiden shows an increased odds ratio for late fetal loss, indicating an increased rate of fetal loss associated with this risk determinant (Table 1).

Placental vascular growth begins as early as 21 days post conceptionem and continues throughout gestation. Disturbances in the placental vascular development are associated with fetal loss in pregnancy (31). The pathomechanisms of early and late fetal loss comprise placental vascular abnormalities, imposing primarily as endothelial inflammation, and thrombotic occlusion of the placental vasculature (9, 32, 33). Since endothelial dysfunction can act as a major stimulus of hemostasis, it is likely that a risk determinant of thrombogenicity will be without consequence in regular non-altered vessels. Comparing cases with vascular alterations and controls who do not have vascular alterations will identify risk determinants associated with the disorder itself, but not risk determinants of thrombogenicity leading to premature fetal loss.

Second, the different results for early and late fetal loss are possible indicators for distinct pathophysiological mechanisms. The increased risk for late fetal loss associated with factor V Leiden, a risk determinant for venous but not for arterial thrombosis, may indicate vascular placental occlusions primarily located in the venous system. In contrast, the association of the $\alpha_2$807TT genotype, a potential risk determinant for arterial thrombotic occlusion, with premature early fetal loss may indicate vascular placental occlusions primarily located in the arterial system.

Considering the small sample size, our positive results for an association of the $\alpha_2$807TT genotype with premature early fetal loss needs confirmation in further studies. Moreover, we cannot rule out, that a significant association with the other genotypes might be observed in a larger study.

Possibly the $\alpha_2$807TT genotype is linked to another yet unidentified genetic marker, which represents the true risk determinant. Furthermore, it may be the case that cellular sources other than platelets are being affected, since the integrin $\alpha_2\beta_1$ also serves as a collagen receptor on fibroblasts and pos-sibly other cells involved in early fetal development (34, 35).

If the $\alpha_2$807TT genotype-associated premature fetal loss is induced by an increased platelet thrombogenicity, our results may have implications in terms of the therapy with antiplatelet agents. The modulating influence of the $\alpha_2$807TT genotype on the onset of early fetal loss indicates that a platelet-dependent pathomechanism may be involved. Consequently, it will be of importance to examine whether the critical subgroup of patients could benefit from prevention therapy with a specific single or combined antiplatelet agents.

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