Original Article

wiener klinische wochenschrift
the middle european journal of medicine
Printed in Austria

Wien Klin Wochenschr (2004) 116/17–18: 622–626

Thrombophilic polymorphisms – factor V Leiden, prothrombin G20210A, and methylenetetrahydrofolate reductase C677T mutations – and preterm birth

Bernhard Resch[1], **Siegfried Gallistl**[1,2], **Jörg Kutschera**[1], **Christine Mannhalter**[3], **Wolfgang Muntean**[1,2], and **Wilhelm D. Mueller**[1]

[1] Department of Pediatrics, University Hospital Graz,
[2] Ludwig Boltzmann Research Institute for Pediatric Thrombosis and Hemostasis, Department of Pediatrics, University Hospital Graz, Graz, and
[3] Department of Laboratory Medicine, University Hospital Vienna, Vienna, Austria

Summary. *Aim of the study:* To evaluate the influence of three common thrombophilic polymorphisms, factor V Leiden (FV), prothrombin G20210A (PT), and methylenetetrahydrofolate reductase (MTHFR) C677T mutations, on preterm birth of unknown cause.

Patients and methods: A single-centre case-control study of women with preterm infants ≤ 35 weeks of gestation, in whom obvious maternal, uterine, and fetal causes responsible for preterm birth were excluded (n = 35). The controls were 54 women with term infants hospitalised in the same ward.

Results: There were no significant differences between the groups of mothers in history of fetal loss, venous or familial thrombosis, or previous preterm birth. FV was found in 8.6% of the cases, PT in 5.7%, and MTHFR mutation (homozygous) in 4.8% compared with 5.4% (p = 0.292, OR 1.594, CI95% 0.303–8.384), 7.4% (p = 0.379, OR 0.758, CI95% 0.131–4.374), and 4.5% (p = 0.485, OR 1.050, CI95% 0.090–12.276), respectively, in the controls. Differences in the three thrombophilic polymorphisms in the two groups of infants were also not significant.

Conclusion: We could not demonstrate a distinct association between these thrombophilic polymorphisms and preterm birth.

Key words: Factor V Leiden, methylenetetrahydrofolate reductase C677T mutation, preterm birth, prothrombin G20210A variant.

Introduction

Inherited resistance to activated protein C is an autosomal dominant trait, mostly caused by a mutation consisting of a substitution of a G with an A at nucleotide position 1691 within the factor V (FV) gene. This mutation results in a replacement of arginine 506 with glutamine at the FV cleavage site for activated protein C [1]. The risk of thrombosis in carriers of this mutation, named FV Leiden, is 50 to 100 times higher in homozygotes and 5 to 10 times higher in heterozygotes than in non-carriers [2]. The genetic variant of prothrombin (factor II) is a polymorphism in the 3′-untranslated region of the prothrombin (PT) gene with a G to A transition at nucleotide 20210 and is associated with elevated levels of PT and an almost threefold increase in risk of venous thromboembolisms in adults [3]. The main genetic defect related to moderate hyperhomocysteinemia associated with arterial and venous thrombosis is a C to T substitution at nucleotide 677 of the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene [4].

Pregnancy is a physiological condition with an increased risk for thrombosis that is further increased in carriers of thrombophilic polymorphisms [5, 6]. Recent studies have demonstrated significant correlation between thrombophilic polymorphisms and fetal losses [7–9]. As an association with late abortions has been found, a correlation of thrombophilia with premature deliveries might possibly exist.

We therefore investigated the presence of these thrombophilic polymorphisms in women with preterm birth of unknown cause or caused by placental dysfunction and compared the prevalences with those in women with term infants. Thrombophilic polymorphisms were also investigated in the preterm and term infants.

Patients and methods

The study population consisted of all women with preterm infants of 35 weeks of gestational age or less consecutively admitted to the neonatal intensive care unit in the Department of Pediatrics Graz, a tertiary-care centre, between January 1998 and May 2000, in whom obvious maternal (chronic medical illness, infection, drug abuse), uterine (bicornate uterus, incompetent cervix), fetal (multiple gestation, erythroblastosis, non-immune hydrops) or other (premature rupture of membranes, polyhydramnios, iatrogenic) causes responsible for preterm birth were excluded [9]. Placental dysfunction, abruptio placentae, pre-eclampsia, and fetal distress – all clinical signs suggestive of possible placental thrombosis – were included. The controls were mothers of term infants (> 37 weeks of gestational age) in the same neonatal ward. Blood samples were taken

from both the mother and the baby at the time of discharge and were stored at –70 °C. Genomic DNA was prepared from the samples according to standard methods and subsequently analysed for the following three thrombophilic polymorphisms: FV Leiden mutation, PT G20210A mutation, and MTHFR C677T mutation. Because of a change in the study design, fewer patients were tested for MTHFR mutation: initially only analyses for FV and PT variants were performed, but after 10 months MTHFR analysis became available and was introduced into the study. Information on gestational age, birth weight, Apgar scores at 1 and 5 minutes, intra/periventricular haemorrhage, cystic periventricular leucomalacia, and age of the mother were collected, together with histories of thrombosis, abortion, familial thromboses and previous preterm birth. All mothers gave informed consent and the study, after approval by the local ethics committee, was carried out according to the Principles of the Declaration of Helsinki.

Factor V Leiden mutation

A 267-bp fragment of the FV gene containing base-pair 1691 was amplified using polymerase chain reaction (PCR) as described by Bertina et al. [1] with minor modifications. 0.25–0.50 µg of genomic DNA was amplified in 50 µg reaction mixture (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 0.8 mM each deoxynucleotide triphosphate (dNTP), 1 µM each primer) using 2.5 U of Ampli Taq (Perkin Elmer Cetus, Norwalk, CT) for 36 cycles (94 °C, 40 s; 72 °C 120 s) followed by 10 min at 72 °C. Digestion with the restriction endonuclease MnII of PCR products containing the 1691G allele results in 37-bp, 67-bp, and 163-bp fragments. The fragments were separated on 2% agarose gels and stained with ethidium bromide.

Prothrombin G20210A mutation

Genomic aliquots of approximately 200 µg DNA were amplified in 50 µl reaction volumes containing 0.2 mmol/l each dNTP, 10 mmol/l Tris-HCl (pH 8.3 at 25 °C), 50 mmol/l KCl, 0.3 µmol/l each primer, 1.5 mmol/l $MgCl_2$, and 1 unit Ampli Taq Gold polymerase (Perkin Elmer Cetus, Norwalk, CT). Primer sequences were chosen according to Poort et al. [3]. An initial denaturation step of 10 min at 95 °C was followed by 40 cycles of 1 min at 45 °C and 1 min at 72 °C. A final extension step of 10 min at 72 °C completed the reaction. Aliquots of 7 µl of each PCR product were digested with 10 units Hind III in 1xHind III buffer (Boehringer Mannheim, Mannheim, Germany) in a reaction volume of 30 µl. The digests were separated by electrophoresis on 6% polyacrylamide gels and stained with ethidium bromide. The presence of the rare A allele led to the generation of a Hind III cleavage site yielding a 322-bp product, whereas the 345-bp PCR product of the common G allele remained uncleaved by Hind III.

Methylenetetrahydrofolate reductase (MTHFR) C677T mutation

The PCR primers for amplification of MTHFR were chosen according to Frosst et al. [4] and generated a 198-bp fragment. The MTHFR polymorphism, a C to T substitution at bp 677, creates a *Hin*fl recognition sequence. If the mutation is present, *Hin*fl digests the 198-bp fragment into 175 bp and 23 bp fragments. Fragments were analyzed using polyacrylamide gel electrophoresis.

Statistical analyses

Yates' corrected χ^2 test and Fisher's exact test were used as appropriate for categorical data and the t-test for numerical data.

Results

The study group consisted of 35 mothers and the control group of 54 mothers. The perinatal characteristics of the infants are shown in Table 1. The perinatal characteristics of the mothers, including macroscopic description of the placentas, are shown in Table 2. Intra/periventricular haemorrhage was diagnosed in 5/35 (14.3%) of the preterm infants, and cystic periventricular leucomalacia in 3/35 (8.6%). There were no significant differences in histories of abortion, venous thrombosis, or familial thromboses between the groups of mothers, or in findings of placental dysfunction. None of the women had a history of tobacco smoking, and none had been given low-dose heparin during pregnancy.

Results for the thrombophilic polymorphisms are shown in Table 3. The differences in presence of FV Leiden, PT G20210A, and MTHFR mutations between

Table 1. Perinatal characteristics of the 35 cases (preterm infants ≤ 35 weeks gestational age) and 54 controls (term infants > 37 weeks gestational age)

	Cases		Controls		p-value
Gestational age in weeks*	31.2	(25–35)	39.6	(38–42)	< 0,001
Birth weight in grams*	1610	(610–2680)	3309	(2400–4225)	< 0,001
Small for gestational age	4	(11%)	0	(0%)	0,005
Apgar score at 1 minute*	6.6	(5–10)	7.6	(1–9)	< 0,001
Apgar score at 5 minutes*	8.4	(5–10)	8.9	(6–10)	0,084
Caesarean section	20	(57%)	12	(22%)	< 0,001
Breech presentation	2	(6%)	0	(0%)	0,039
Fetal distress	16	(46%)	14	(26%)	0,027
Asphyxia	2	(6%)	4	(7%)	0,379
Respiratory distress	18	(51%)	12	(22%)	< 0,001
Mechanical ventilation	27	(77%)	16	(30%)	< 0,001
Early-onset infection	5	(14%)	18	(33%)	0,023

* Mean; range.

Table 2. Maternal perinatal characteristics of 35 cases and 54 controls

	Cases	Controls
Maternal age (years)*	27 (19–39)	28 (18–37)
First pregnancy and first child	21 (60%)	36 (67%)
History of abortion	5 (14%)	8 (15%)
History of venous thrombosis	4 (11%)	2 (4%)
Familial thrombosis	4 (11%)	6 (11%)
History of previous preterm birth	1 (3%)	0 (0%)
Supplemental vitamins	27 (77%)	42 (78%)
Pre-eclampsia	2 (6%)	0 (0%)
Placental weight*	298 (180–450)	528 (420–600)
Abruptio placentae	3 (9%)	4 (7%)
Placental infarction	1 (3%)	2 (4%)

* Mean; range.

cases and controls were not significant. The FV Leiden and PT G20210A mutations identified in the study and control patients were all heterozygous. MTHFR mutations were homozygous in one case mother and two controls (Table 3) and heterozygous in eight case mothers and 16 controls (38 and 36%, respectively). One control mother was heterozygous for PT G20210A and MTHFR mutations; one control infant was heterozygous for FV Leiden and MTHFR mutations. None of the cases showed more than one thrombophilic polymorphism.

Discussion

Prematurity is a condition with various maternal, uterine, placental, and fetal causes, and with many associated risk factors including low socioeconomic status, single-parent families, teenage pregnancies, and close spacing of pregnancies [10]. We evaluated whether thrombophilic polymorphisms had an additional influence on its pathogenesis, but our results did not support an association between inherited thrombophilia and preterm birth.

In the history of venous thrombosis, thrombophilic polymorphisms have been found to be strongly associated with the complex nature of the multigenic pathogenesis of thrombophilia, but findings have not always indicated a single polymorphism as a thrombophilic risk factor. However, these observations suggest that screening patients for thrombophilic polymorphisms might be helpful in identifying patients at increased risk for thromboembolic events, and perhaps prophylactic therapy could be considered for selected groups [11]. Serious obstetrical complications such as severe pre-eclampsia, abruptio placentae, fetal growth retardation, and stillbirth are associated with intervillous or spiral-artery thrombosis. Recently women with such complications have been demonstrated as showing increased frequency of FV Leiden, PT G20210A, and MTHFR (homozygosis) mutations, and more frequent deficiencies of protein S and protein C, or antithrombin III or anticardiolipin antibodies [12]. Brenner et al. [13], reporting on an association of hereditary or acquired activated protein C resistance with recurrent fetal loss, found that only 34 of 184 gestations (18%) with this condition resulted in a live birth, with 11 of 34 (32%) being premature deliveries. Another recent study [14] examined whether hypofibrinolytic and thrombophilic gene mutations were associated with adverse pregnancy outcomes such as prematurity, miscarriage, stillbirth, intrauterine growth restriction, eclampsia, and abruptio placentae. Analysis of FV Leiden, MTHFR, PT gene mutations, and 4G/5G polymorphism of the plasminogen activator inhibitor type 1 found significantly more prematurity (10% v 4%), intrauterine growth restriction (3% v 0%), and total complications of pregnancy (37% v 21%) in 68 women with gene mutations. Both studies [13, 14] suggested some influence of thrombophilic polymorphisms on adverse pregnancy outcomes, and thus stimulated our search for an association between thrombophilia and prematurity. A German group investigated prothrombotic risk factors in cases of foetal growth restriction [15], and their retrospective analysis demonstrated that inherited risk factors, mainly FV Leiden, increase the risk for low birth

Table 3. Thrombophilic polymorphisms in 35 cases (mothers and preterm infants ≤ 35 weeks of gestation) and 54 controls (mothers and term infants > 37 weeks of gestation). Data are presented as number of patients (percentage)

	Cases (M) n = 35	Controls (M) n = 54	Cases (I) n = 35	Controls (I) n = 54
Factor V Leiden	3 (8.6%)	3 (5.6%)	2 (5.7%)	5 (9.2%)
p-value	0.292		0.275	
Odds ratio (CI 95%)	1.595 (0.303–8.384)		0.594 (0.109–3.245)	
Prothrombin G20210A	2 (5.7%)	4 (7.4%)	3 (8.6%)	2 (3.7%)
p-value	0.379		0.168	
Odds ratio (CI 95%)	0.758 (0.131–4.374)		2.438 (0.386–15.388)	
MTHFR*	1 (4.8%)	2 (4.5%)	0 (0%)	4 (9.1%)
p-value	0.485		0.079	
Odds ratio (CI 95%)	1.050 (0.090–12.276)		0	

MTHFR methylenetetrahydrofolate reductase (homozygous); *M* mother, *I* infant; * analyses performed in 21 cases and 44 controls.

weight. The effect was more pronounced in children with either homozygous defects or multiple combined prothrombotic defects. A very large study by Infante-Rivard et al. [16] appears to refute the idea that inherited thrombophilia is a clinically significant cause of fetal growth restriction; the study certainly has adequate statistical power to support such a negative conclusion as it compared 493 newborns with birth weight below the 10th percentile with 472 controls [17].

Our study design did not include histological examination of the placenta, and therefore it is difficult to argue to what extent we studied preterm birth of unknown cause or preterm birth caused by placental dysfunction. One might reasonably expect to find thrombotic lesions of the placenta if a woman with a thrombophilic state had an obstetrical complication, but some studies have reported on such lesions and others not, and all the studies have involved relatively small numbers of cases [18–20]. The study by Mousa et al. [20] examined the relationship between placental histology (thrombotic lesions including thrombosis of fetal stem vessels, fetal thrombotic vasculopathy, placental infarction, perivillous fibrin deposition, intervillous thrombosis and placental floor infarction) and thrombophilia status (including the three gene mutations analysed in our study) in women admitted with severe pre-eclampsia/eclampsia, placental abruption, intrauterine growth restriction or unexplained stillbirth. The authors could not identify any specific histologic pattern when groups that were thrombophilia positive and negative were compared, and therefore proposed a poor correlation between thrombophilia status and pathological changes in the placenta in these women. If placental pathology plays a major role in the pathogenesis of unexplained preterm birth, these results would further support our findings of a poor association with thrombophilic polymorphisms.

As there are many known causes and risk factors associated with preterm delivery [21], it was necessary to apply strict selection criteria in our study. This was best achieved through the prospective design, reporting on a consecutive cohort of infants fulfilling the criteria of preterm birth without obvious cause. The results for thrombophilic polymorphisms in such infants therefore more precisely reflect their influence on preterm birth. However, these strict selection criteria resulted in a small number of cases, and thus only strong risk factors can be expected to be associated with a statistically significant difference.

The 5.6% heterozygosity rate for FV Leiden mutation diagnosed in the controls is comparable to the expected prevalence of 4.6% in the Austrian population [22]. Göpel et al. [23] reported data on FV Leiden and the PT variant in a cohort of 205 infants < 1500 g birth weight and found a significant association between both polymorphisms and prematurity (16.6% compared with 7.3% in term controls). An additional analysis of 102 preterm infants of multiple pregnancies in their study could not confirm this association (9.8% vs 7.3%). In our opinion their results are limited by various factors – as reported above – that influence preterm birth and which have not been taken into account by simply screening a cohort of preterm infants. Thus, thrombophilic polymorphisms still play an uncertain role in this field.

In conclusion our study does not demonstrate a distinct association of FV Leiden, PT G20210A, or homozygous MTHFR C677T gene mutations with preterm birth of unknown cause or placental dysfunction.

References

1. Bertina RM, Koeleman BP, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, et al (1994) Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature 369: 64–67
2. Rosendaal FR, Koster T, Vandenbrouke JP, Reitsma PH (1995) High risk of thrombosis in patients homozygous for factor V Leiden (activated protein C resistance). Blood 85: 1504–1508
3. Poort SR, Rosendaal FR, Reitsma PH, Bertina RM (1996) A common genetic variation in the 3′-untranslated region of the prothrombin gen is associated with elevated plasma prothrombin levels and increase in venous thrombosis. Blood 88: 3698–3703
4. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, et al (1995) 5 A candidate genetic risk factor for vascular disease: a common mutation in methlenetetrahdrofolate reductase. Nat Genet 10: 111–113
5. Gerhardt A, Scharf RE, Beckmann MW, Struve S, Bender HG, Pillny M, et al (2000) Prothrombin and factor V mutations in women with a history of thrombosis during pregnancy and the puerperium. N Engl J Med 342: 374–380
6. Pabinger I, Grafenhofer H (2003) Pregnancy-associated thrombosis. Wien Klin Wochenschr 115: 482–484
7. Preston FE, Rosendaal FR, Walker ID, Briet E, Berntop E, Conard J, et al (1996) Increased fetal loss in women with heritable thrombophilia. Lancet 348: 913–916
8. Grandone E, Margaglione M, Colaizzo D, d'Addedda M, Capucci G, Vecchione G, et al (1997) Factor V Leiden is associated with repeated and recurrent unexplained fetal losses. Thromb Haemost 77: 822–824
9. Brenner B, Sarig G, Weiner Z, Younis J, Blumenfeld Z, Lanir N (1999) Thrombophilic polymorphisms are common in women with fetal loss without apparent cause. Thromb Haemost 82: 6–9
10. Stoll BJ, Kliegman RM (2000) The fetus and the neonatal infant. In: Behrman RE, Kliegman RM, Jenson HB (eds) Nelson textbook of pediatrics, 16th edn. Saunders, Philadelphia London Toronto Montreal Sydney Tokyo, pp 477
11. Watzke HH (2003) Clinical significance of gene-diagnosis for defects in coagulation factors and inhibitors. Wien Klin Wochenschr 115: 475–481
12. Kupferminc MJ, Eldor A, Steinmann N, Many A, Bar-Am A, Jaffa A, et al (1999) Increased frequency of genetic thrombophilia in women with complications of pregnancy. N Engl J Med 340: 9–13
13. Brenner B, Mandel H, Lanir N, Younis J, Rothbart H, Ohel G, et al (1997) 7 Activated protein C resistance can be associated with recurrent fetal loss. Br J Haematol 199: 551–554
14. Glueck CJ, Phillips H, Cameron D, Wang P, Fontain RN, Moore SK, et al (2000) The 4G/5G polymorphism of the hypofibrinolytic plasminogen activator inhibitor type 1 gene: an independent risk factor for serious pregnancy complications. Metabolism 49: 845–852
15. Von Kries R, Junker R, Oberle D, Kosch A, Nowak-Göttl U (2001) Foetal growth restriction in children with

prothrombotic risk factors. Thromb Haemost 86: 1012–1016
16. Infante-Rivard C, Rivard GE, Yotov WV, Genin E, Guiguet M, Weinberg C, et al (2002) Absence of association of thrombophilia polymorphisms with intrauterine growth restriction. N Engl J Med 347: 19–25
17. Roberts D, Schwarz RS (2002) Clotting and hemorrhage in the placenta – a delicate balance. N Engl J Med 347: 57–59
18. Many A, Schreiber L, Rosner S, Lessing JB, Eldor A, Kupferminc MJ (2001) Pathologic features of the placenta in women with severe pregnancy complications and thrombophilia. Obstet Gynecol 98: 1041–1044
19. Sikkema JM, Franx A, Bruinse HW, van der Wijk NG, de Valk HW, Nikkels PG (2002) Placental pathology in early onset preeclampsia and intra-uterine growth restriction in women with and without thrombophilia. Placenta 23: 337–342
20. Mousa HA, Alfirevicl Z (2000) Do placental lesions reflect thrombophilia state in women with adverse pregnancy outcome? Hum Reprod 15: 1830–1833
21. Lockwood CJ (2002) Predicting premature delivery – no easy task. N Engl J Med 347: 282–284
22. Födinger M, Mannhalter C, Pabinger I, Koizar D, Rintelen C, Hörl WH, et al (1996) Resistance to activated protein C (APC): mutation at Arg^{506} of coagulation factor V and vascular access thrombosis in haemodialysis patients. Nephrol Dial Transplant 11: 668–672
23. Göpel W, Kim D, Gortner L (1999) Prothrombotic mutations as a risk factor for preterm birth. Lancet 353: 1411–1412

Correspondence: Dr. Bernhard Resch, MD, Division of Neonatology, Department of Pediatrics, University Hospital Graz, Auenbruggerplatz 30, 8036 Graz, Austria,
E-mail: bernhard.resch@meduni-graz.at

(Received March 8, 2004, accepted after revision May 18, 2004)