Factor V Leiden and prothrombin G20210A gene mutations in women with a history of thrombosis during pregnancy

Relation to pregnancy outcomes for mother and fetus

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ABSTRACT

الأهداف: التحقق من وجود عامل ليدن الخامس (FVL)، و جين البروثرومبين (PRT)، وبروتين S، و بروتين C، في السيدات الحوامل بتاريخ الإصابة المسبقة بتجلطات الأوردة، و تقييم أثرها على نتائج الحمل و الجنين.

الطريقة: تم إجراء الدراسة في مستشفى جامعة عين شمس – مصر خلال الفترة من يناير 2006 حتى مارس 2008م. اشتملت الدراسة على 15 سيدة حامل بدون تاريخ مرضي للتجلطات بالأوردة العميقة (مجموعة التحكم) و 25 سيدة حامل بتاريخ مرضي لتجلطات الأوردة العميقة أثناء مرات الحمل السابقة (مجموعة المرضى). وتم دراسة معامل ليدن الخامس FVL وتمحور جين البروثرومبين PRT فيهن باستخدام طريقة سلسلة التفاعل المبلمر، وكذلك قياس نشاط بروتين S وبروتين C بواسطة إجراء مقياس التخثر.

النتائج: تم اكتشاف معامل ليدن الخامس FVL في 6.6%، وجد نقص في بروتين C في سيدة واحدة 6.6%. وبالنسبة لمجموعة المرضى، كان هنالك نمط ظاهري طبيعي لدى 13/25 52%، وظهر نمط ظاهري غير طبيعي لدى 12/25 48%، في معامل ليدن الخامس FVL، أو جين البروثرومبين PRT أو كليهما. وقد وجد نقص في بروتين C في12% 3/25 سيدة و أما بروتين S لدى 8% 2/25 سيدة فقط. وقد جاءت نتائج الحمل سلبية في 48% من السيدات في مجموعة المرضى و 33% من السيدات في مجموعة التحكم مع وجود فارق إحصائي واضح بين المجموعتين في تأخر نمو الجنين داخل الرحم (IUGR) أقل من عشر المائة. بينما لا توجد علاقة إحصائية في نتائج الحمل السلبية مثل تسمم الحمل، و الإجهاض، وتغيرات في المشيمة، أو تأخر نمو الجنين داخل الرحم IUGR أقل من خمس المائة. لم يرتبط معامل ليدن الخامس FVL بأي نتائج معاكسة بينما ارتبط جين البروثرومبين PRT مع تأخر نمو الجنين داخل الرحم IUGR أقل من خمس المائة.

خاتمة: أظهرت نتائج هذه الدراسة أن المراقبة الجيدة لنمو الجنين إلزامية لجميع نواقل جين البروثرومبين PRT.

Objectives: To investigate the presence of factor V Leiden (FVL), and prothrombin gene mutations (PRT), protein C and protein S in pregnant women with a previous history of thromboembolism, and evaluate their impact on maternal and fetal outcomes.

Methods: This study was carried out at Ain Shams University Hospital, Cairo, Egypt between January 2006 to March 2008. The study included 15 pregnant females without a history of thromboembolism (control group), and 25 pregnant females with a history of previous thromboembolism during pregnancy, and puerperium (patient group). Identification of FVL and PRT mutations by real-time quantitative polymerase chain reaction, and estimation of protein C and S activity by functional clotting assay were performed.

Results: Regarding the control group; one patient had FVL mutation (6.6%), and one had decreased protein C activity (6.6%). As regard the patient group 13/25 (52%) had normal genotype, and 12/25 (48%) expressed abnormal genotype either FVL or PRT G20210A, or both. Also 3/25 (12%) patients had decreased protein C activity, and 2/25 (8%) had decreased protein S. The intrauterine growth retardation (IUGR) less than the tenth percentile was more in the patients group (48%) compared to the control group (33%), while there was no statistically significant difference between both groups on preeclampsia, placental abruption, abortion, or IUGR less than the fifth percentile. The FVL was not associated with any adverse outcomes, while the PRT mutation was significantly associated with IUGR less than the fifth percentile.

Conclusion: The results of this study shows that good monitoring of fetal growth is mandatory for all carriers of the PRT gene mutation.

Saudi Med J 2010; Vol. 31 (2): 123-129

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Received 10th August 2009. Accepted 5th January 2010.

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Inherited thrombophilias are leading causes of maternal thromboembolism and may be associated with an increased risk of certain adverse pregnancy outcomes including intrauterine growth retardation (IUGR), and early onset of severe preeclampsia in addition to the thromboembolic complications, including pulmonary embolism, which is the major cause of death among women during pregnancy and purperium.[1] A point mutation in which adenine is substituted for guanine at nucleotide 1691 in the gene coding for coagulation factor V resulting in a substitution of a glutamine for an arginine at position 506 in factor V poly peptide.[2] Factor V Leiden (FVL) (G1691) is the most common inherited genetic abnormality in patients with thromboembolic diseases. It occurs in 20% of patients with first episode of venous thrombosis and up to 50% of those with recurrent venous thrombosis.[1] Heterozygosity of FVL mutation is present in 20-40% of non pregnant women with thromboembolism, and it contributes to around 40% of thromboembolic manifestations in pregnancy.[3] A guanine-to-adenine mutation in the prothrombin gene G20210A, is associated by means of elevated plasma prothrombin concentrations, and an increased risk of venous thrombosis. It accounts for 17% of thromboembolism in pregnancy. However, the actual risk of clotting in an asymptomatic pregnant carrier of this mutation is only 0.5%.[3] This study aimed to estimate the FVL (G1691A), and prothrombin gene G20210A mutations in pregnant women with previous history of thromboembolism during pregnancy, and to evaluate their impact on maternal and fetal outcomes.

Methods. Between January 2006 and March 2008, 25 pregnant females with a history of previous thromboembolism during pregnancy (deep venous thrombosis) attending for follow up at the obstetric outpatient clinic were referred to the Cytogenetic and Molecular Unit, Ain Shams University Hospital, Cairo, Egypt for detection of risk factors for thrombosis. Women diagnosed as antiphospholipid syndrome or was antithrombin III (AT III) deficiency were excluded from the study. The study also included 15 pregnant women without previous history of thromboembolism as a control group.

All women in the study were subjected to thorough history taking, especially for thrombotic attack(s) in the previous pregnancy, history of risk factors for thrombosis such as prolonged recumbency, intake of oral contraceptive pills, obesity, diabetes, and the treatment they received. Full obstetric history was taken, stressing recurrent fetal loss, IUGR, early onset of preeclampsia, and symptoms of pregnancy induced hypertension. Routine antenatal visits included serial body weight, blood pressure follow up, and also follow up for early signs of preeclampsia, (diastolic blood pressure more than 90 mm Hg on 2 occasions, 4 hours to 14 days apart, evident significant proteinuria [>300 mg protein in 24-hour urine]).[4] Also included in this routine visit is to diagnose IUGR or fetal death. Small for gestational age was defined as a birth weight less than tenth percentile derived from gender and race.[5] All participants were subjected to routine laboratory investigations including complete blood picture, prothrombin time (PT), partial thromboplastin (PTT), liver and renal function tests, and urine analysis. Informed consent was taken from all pregnant females included in the study. All methods were carried out during the first trimester.

This study was approved by the ethics committee of FMSU REC, Cairo, Egypt.

Isolation of genomic DNA. The DNA was extracted from ethylenediamine tetra-acetic acid (EDTA) peripheral blood automatically using (MagNA pure LC) instrument from Roch applied science, and MagNA pure LC DNA isolation kit (Roche Applied Science, Mannheim, Germany) The sample was lysed by lysis binding buffer then the cellular protein digested by proteinase K, then DNA were released. Magnetic glass particles were added and the DNA binds to their surfaces. Several washing steps with buffer removed the unbound substances. Finally, the purified DNA was eluted. One ml of venous blood was collected in EDTA. Tubes 50 µl of whole blood was pipetted into the sample cartridge, and placed in the MagNa pure LC instrument for extraction of DNA from whole blood automatically. The eluted purified DNA was stored in a sealed sample cartridge at -20°C until used.

Identification of FVL G1691A mutations and prothrombin. The procedure for identification of both gene mutations was carried out using a light cycler (Roche Diagnostics, Mannheim, Germany). A 222 bp fragment, and a 165 bp fragment of the factor V gene, and factor II gene were amplified from human genomic DNA using specific primers (F 5 for, 5>-TAATCT GTA AGA GCA GAT CC-3>, F 5rev, 5>-TGT TATCAC ACT GGT GCT AA-3> for FVL and F 2 for, 5>-CCG TCTG GTA TCAA AAT GGG-3>, 5>- CCG TCTG GTA TCAA AAT GGG-3> for prothrombin [PRT] gene mutation). The amplicon is detected by fluorescence using a specific pair of hybProbe probes (F5wt-probe5>-AAT ACC TGT ATT CCT CGC CTG TC-3>-FLU. For FVL and F2wt, 5>CTC AGC GAG CCT CAA TG-3> FLU for PRT gene mutation). The hybProbe probes consist of 2 different oligonucleotides that hybridize to an internal sequence of the amplified fragment during the annealing phase of the polymerase chain reaction (PCR) cycle.

Genotyping.[6] The HyProbe probes are used to determine the genotype by performing a melting curve analysis after the amplification cycles are completed, and the amplicon is present at increased concentration. The red 640-labeled HybPrbe probe hybridizes to a part of the target sequence that is not mutated and functions as an anchor probe. The fluorescein labeled HybProbe probe spans the mutation site (mutation probe). During the melting curve analysis, increasing temperature causes the fluorescence to decrease since the shorter of the 2 probes (mutation probe) dissociates first, and the 2 fluorescent dyes are no longer in close proximity. If the factor II (PRT) G20210A mutation or FVL is present, the mismatch of the mutation probe with the target destabilizes the hybrid so the decrease in fluorescence will occur at a lower temperature (65±2.5°C for FVL, or 59±2.5°C for PRT). With the wild type genotype, mismatches will not occur, and therefore, the heteroduplex DNA has a higher melting temperature (57±2.5°C for FVL or 49±°C for PRT). The heterozygous genotype exhibits a distinctive combination of properties.

Real time PCR protocol. The PCR was performed in glass capillary tubes in 20 µl final volume using 5 µl of isolated specimen DNA, 5 µl positive control, or 5 µl negative control, 11 µl diluent, 2 µl mutation detection mix, and 2 µl reaction mix. The PCR program is run as described by Roche Biodiagnostics, Mannheim, Germany. The mutation detection was achieved by melting curve analysis profiles for FVL, and PRT G20210A mutation.

Estimation of protein C and protein S activity.[7] They were measured by a functional clotting assay (Date Behring, Marburg, Germany, and Diagnostica Stago, Taverny, France).

Statistical analysis. We used Chi-square test to compare between categorical variables among the different groups. Odd's ratios (OR) were estimated. The OR is the ratio of the odds of an event occurring in one group to the odds of its occurring in another group. A *p*-value of <0.05 was considered significant.

Results. Results of this study are presented in **Tables 1-3**, and **Figures 1 & 2**. The study was carried out on 25 pregnant women with age range from 19-33 years, with mean±SD of 28.7±3.2. The women had previous history of thromboembolic manifestations during previous pregnancy or puerperium. The study included 15 normal pregnant females without previous history of any thromboembolic manifestations, they were age matched to the patient group. The patient group showed coagulation factor defect either as a sole defect or combined as more than one defect in 16 women out of 25 (64%) with a statistical significant difference between the patient group and the control group (**Table 1**). The FVL mutation was found with a relative risk of 5.4, with a statistical significant difference between the patient group and control groups. Five out of 9 cases were heterozygous genotype for FVL, 2 were double heterozygous for FVL and PRT (G20210A) mutation, and 2 cases were of homozygous genotype, one of the heterozygous gene type for FVL was associated with decreased protein C activity. Regarding prothrombin gene 20210A mutation, it was detected with a statistical significant difference between the patient group and control group (**Table 1**). Two cases had sole defects, 2 were associated with FVL mutation, and one case was associated with protein C decreased activity. All cases were of the heterozygous genotype (**Figure 2**). Protein C activity decreased in 3 cases, only one was the sole

Table 1 - Comparison of patient group and control group regarding all studied parameters.

Parameter	Patient group n= 25		Control group n=15		χ^2	*P*-value	Significance
	n (%)						
Patients with defects	16	(64)	2	(13)	3.8	0.038	S
Factor V Leiden	9	(36)	1	(6.6)	4.3	0.031	S
Prothrombin 20210	5	(20)	0	(0)	3.51	0.042	S
Protein C	3	(12)	1	(6.6)	0.296	>0.05	NS
Protein S	2	(8)	0	(0)	1.26	>0.05	NS
Pre-eclampsia	3	(12)	2	(13)	0.012	>0.05	NS
Abortion	3	(12)	1	(6.6)	0.024	>0.05	NS
Abruptio placenta	1	(4)	1	(6.6)	0.1110	>0.05	NS
IUGR <5	2	(8)	1	(66)	0.024	>0.05	NS
IUGR >5 - <10	4	(16)	0	(0)	2.97	0.048	S

IUGR - intrauterine growth restriction, S - significant, NS - not significant

Table 2 - Comparison of women with factor V Leiden mutation and patients without mutation regarding pregnancy outcome.

Parameter	Patient with FV Leiden (n=9)		Patients without mutations (n=16)		χ^2	*P*-value	Significance
	n (%)						
Pre-eclampsia	2	(22)	1	(6)	1.39	>0.05	NS
Abortion	1	(11)	2	(12.5)	1.011	>0.05	NS
Abruptio placenta	1	(11)	0	(0)	1.852	>0.05	NS
IUGR <5	1	(11)	0	(0)	1.852	>0.05	NS
IUGR >5 - <10	2	(22)	2	(12.5)	0.405	>0.05	NS

IUGR - intrauterine growth restriction, S - significant, NS - not significant

Table 3 - Comparison of patients with prothrombin 20210 A gene mutation, and patients without mutations regarding pregnancy outcome.

Parameter	Patient with prothrombin 20210 mutation (n=5)		Patients without mutation (n=20)		χ^2	*P*-value	Significance
	n (%)						
Pre-eclampsia	1	(20)	2	(10)	0.379	>0.05	NS
Abortion	0	(0)	2	(10)	0.543	>0.05	NS
Abruptio placenta	0	(0)	1	(5)	0.260	>0.05	NS
IUGR <5	1	(20)	0	(0)	3.965	0.039	S
IUGR > 5 - <10	4	(80)	0	(0)	4.21	0.001	S

IUGR - intrauterine growth restriction, S - significant, NS - not significant

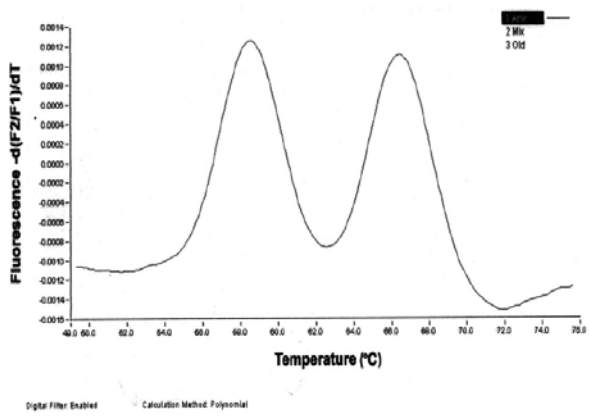

Figure 1 - The DNA analysis for genotype of factors V Leiden sequence using real-time polymerase chain reaction revealed heterozygous genotype.

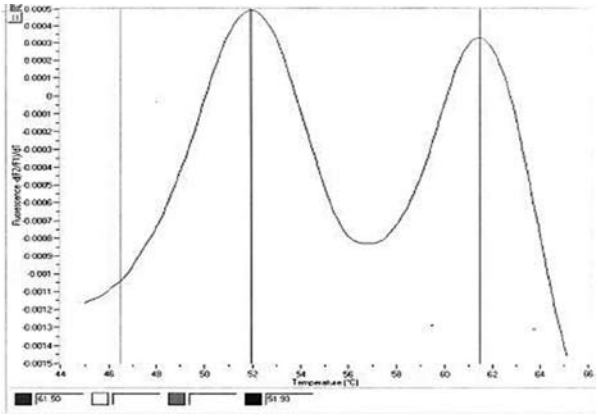

Figure 2 - The DNA analysis for genotype of prothrombin G20210 sequence revealed heterozygous phenotype.

anomaly; while protein S activity decreased in 2 cases, both were not associated with other genetic defects.

Relation to pregnancy outcome. Poor pregnancy outcome was detected in 12 out of 25 studied women (48%) with odds ratio (0.48:0.33) while 5 out of 15 (33.3%) in the control group exhibited poor pregnancy outcome. There was only a statistical significant difference between patients with previous history of thromboembolism, and pregnant women without history of previous thrombosis regarding IUGR less than tenth percentile while there was no statistical significant difference regarding pre-eclampsia, placental abruption, abortion, or IUGR less than fifth percentile (p>0.05) (**Table 1**). Comparing patients with VL mutation and patients without, there were no statistical association between presence of mutation and poor pregnancy outcomes (**Table 2**), while presence of PRT

gene 20210A mutation was only associated significantly with IUGR (**Table 3**).

Discussion. Pregnancy related venous thromboembolism is the most common cause of maternal death and a significant cause of maternal morbidity.[8] Congenital coagulation defects such as FVL, or PRT (G20210A) gene mutations are among the most common genetic factors that predisposes to thrombosis during pregnancy.[9]

The current study demonstrated that FVL was detected in 36% and PRT was detected in 20% (G20210A) gene mutations were detected among pregnant females with previous history of thromboembolism, while normal pregnant females showed only FVL mutation in one case (6.6%) and no one had PRT (G20210A). Protein C, and S decreased activities was detected in 12%, and protein S in 20% among females with previous history of thromboembolism. However, there was no significant difference between the patient group and normal pregnant females. These findings were comparable to Gerhardt and colleagues,[10] who found that the prevalence of FVL and PRT (F20210A) gene mutations was significantly higher among women with history of thromboembolism than normal pregnant females. It appears that there is a relation between presence of PRT (G20210A) gene mutations, and pregnancy related alterations in coagulation or fibrinolysis, that lead to thrombosis in women who are carriers of the PRT (G20210A) gene mutation.[11] In the current study, we found 7 cases out of 9 were of heterozygous genotype for FVL, and 2 were of homozygous gene type. Three of 7 cases were associated with an other abnormality, 2 were double heterozygous genotype for FVL, and PRT (G20210A) gene mutations, and one was associated with decreased protein C activity. However, all cases with PRT (G20210A) gene mutation were of heterozygous genotype, also one of them was associated with decreased protein C activity. As estimated risk for deep venous thrombosis proved to be 3 to 9 fold increase risk in those heterozygous for both FVL and PRT (G20210A) gene mutations or homozygous for FVL,[11,12] the risk of recurrent venous thromboembolism is 4-to-5 fold higher with hyperhomocysteinemia than individuals with FVL allele alone.[13] Bates and Ginsberg[14] found, that women with a prior history of venous thromboembolism probably have a higher risk of recurrence during pregnancy. The risk is likely higher in women with a prior spontaneous event and or coexisting genetic or acquired risk factors, especially FVL mutation. We detected poor pregnancy outcomes in 48% of the studied women with odds ratio (0.48:0.33), while healthy pregnant women exhibited poor pregnancy outcomes in 33%. There was a statistical significant difference between both groups only regarding IUGR less than tenth percentile, while there was no statistical significant difference regarding preeclampsia, placenta abruption, abortion, or IUGR less than fifth percentile (weight of the baby at 40 weeks of gestation being <2750 gm). Additionally, there was no association between presence of FVL mutation, and bad pregnancy outcomes while PRT (G20210A) gene mutation was significantly associated with IUGR less than fifth, and tenth percentile.

Dizon-Townson et al[15] studied the frequency of pregnancy related thromboembolic events among carriers of FVL and evaluated the impact of FVL mutation carriers or other thrombophilias on adverse pregnancy outcomes. They found that there were no differences in adverse pregnancy outcomes observed between FVL carriers, carriers of other coagulation disorders, and controls. Thus, maternal FVL mutation carriage was not associated with increased pregnancy loss, preeclampsia, placenta abruption or small for gestational age births. However, fetal mutation carriage was associated with more frequent preeclampsia among Africa-American (15%), and Hispanic women (12.5%) than white women.

It has been hypothesized that mothers or fetuses with acquired or genetic predisposition may have abnormal thrombosis and infection of uteroplacental circulation that might manifest clinically as adverse pregnancy outcomes.[15] However, retrospective studies and cross-sectional studies in this regard have been conflicting.[10,16,17] Accurate information regarding risks for thrombosis, and adverse pregnancy outcomes are important because of increasing number of individuals who have been tested and treated with anticoagulation therapy during pregnancy.[18] While some studies found a significant higher prevalence of FVL in women with preeclampsia (8-26%) compared to normal pregnancies (10-20%).[19] Another study showed that FVL has a stronger association with severe early onset of pre-eclampsia.[20] Other studies found no association of FVL mutation with preeclampsia.[21] This is comparable to our study as we did not found any association between presence of any of risk factors and occurrence of preeclampsia.

In contrary to the current study, Martinelli et al,[22] found a high prevalence of FVL heterozygosity in women with unexplained recurrent pregnancy loss. Another study reported miscarriage in 11% of FVL heterozygotes.[23] The timing of miscarriage is still controversial as some studies demonstrated that the risk of fetal loss is increased in the first trimester.[24] While Lissalde-Lavigne et al,[25] found that the risk of fetal loss with FVL mutation occurred in the second trimester.

They explained the late pregnancy losses by thrombosis of the placental vessels.

The data on the risk of fetal growth retardation are more limited. Howley et al,[26] found that the FVL allele and PRT (G20210A) gene mutations were associated with fetal growth retardation, and FVL may be associated with a significant 3 to 5 fold increased risk of fetal growth retardation. This may be due to infarctions of the placenta. The number of women with deficiencies of protein C, and protein S may be too low for it to be possible to identify the influence of each of them on pregnancy outcome.

The high frequency of FVL and PRT (G20210A) gene mutation (36% and 20%) among pregnant females with previous history of the thromboembolism indicates the need for screening for these abnormalities in every pregnant female with previous history of thromboembolism in the successive pregnancies, and their family members to identify those who might benefit from prophylaxis for recurrent attacks of thromboembolism. However, our findings indicate that the FVL mutation may have no adverse effects on pregnancy outcomes, while PRT (G20210A) gene mutations may affect intrauterine fetal growth, indicating the need for good monitoring of fetal growth in female carriers for PRT (G20210A) gene mutations.

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Authorship Entitlement

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