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Thrombophilia Gene Mutations in Relation to Recurrent Miscarriage

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ABSTRACT
The present study was undertaken to investigate the prevalence of thrombophilia- associated gene mutations (factor V Leiden,prothrombin gene G20210A and methylene-tetrahydrofolate reductase MTHFR C677T) in relation to recurrent miscarriage. Two hundred pregnant women divided into two groups were included in the study. Group I included 100 women with history of ≥ 3 unexplained consecutive pregnancy losses and group II included 100 age-matched controls with no history of recurrent miscarriage. Blood samples were collected from all pregnant women enrolled in the study for DNA extraction and genotype analysis based on polymerase chain reaction and reverse hybridization. Factor V Leiden and prothrombin gene mutations did not differ significantly between groups, whereas, MTHFR C677T mutations and combined thrombophilias (Factor V Leiden and MTHFR C667T) were significantly increased in group I compared to controls. Moreover, the total prevalence of gene mutations was significantly increased in group I (61%) compared to controls (21%). Homozygosity and heterozygosity did not differ significantly between groups , however , in group I, heterozygotes were significantly increased compared to homozygotes for each of the three gene mutations studied.

Keywords: *Recurrent miscarriage, thrombophilia, factor V Leiden, prothrombin gene G20210A, methylene-tetrahydrofolate reductase.*

INTRODUCTION

Pregnancy loss is a common occurrence among reproductive age women. Whereas, approximately 15% of all clinically recognized pregnancies result in spontaneous loss, there are many more pregnancies that fail prior to being clinically recognized. Only 30% of all conceptions result in a livebirth.[1]

Recurrent pregnancy loss is defined as three or more consecutive pregnancy losses prior to 20 weeks gestation with an incidence of approximately 1 in 300 pregnancies.2There exists a number of accepted etiologies for RPL including parental chromosomal abnormalities, untreated hypothyroidism, uncontrolled DM,uterine anatomic abnormalities, and antiphospholipid antibody syndrome. Other possible etiologies include additional endocrine disorders, heritable and/or acquired thrombophilia, immunologic abnormalities, infections and environmental factors.[2],[3]

Evidence addressed the association of inherited thrombophilia with recurrent pregnancy loss, focusing on tests for three genetic variants; factor V Lieden , prothrombin G 20210A and methylene-tetrahydrofolate reductase(MTHFR).4 Associations between these heritable thrombophilia variants and other serious pregnancy complications as fetal growth restriction, placental abruption, preeclampsia, eclampsia, prematurity and intrauterine fetal death have also been documented.5 The main underlying mechanisms seem to be inhibition of trophoblast differentiation/ invasion , and thrombosis of the maternal side of the placenta resulting in placenta mediated pregnancy complications and fetal loss. [6],[7]

Factor V Leiden mutation is a point mutation in the gene for clotting factor V. It has an autosomal dominant inheritance and is the most common inherited form of thrombophilia , with a prevalence in the general population ranging from 1-15%.It is a genetic disorder characterized by poor anticoagulant response to activated protein C and is the most common inherited mutation associated with increased risk of venous thromboembolism (VTE).[8]

The prothrombin G20210A has a prevalence of 2-5% in the general population. Although less frequent than factor V Leiden,it is detected in up to 17% of pregnant patients with VTE. Compared with women with only prothrombin G20210A mutation, those with combined thrombophilia have a threefold greater risk of VTE. Double heterozygosity for both factor V Leiden and prothrombin gene mutation occurs in approximately 1 in 1000 in the general population and in 1-3% of pregnant women with VTE.[9],[10]

Hyperhomocysteinemia is another thrombophilic anomaly which may be genetic or acquired and has been proposed as a potential cause of recurrent miscarriage.The most common form of genetic hyperhomocysteinemia results from C667 T polymorphism in the methylene-tetrahydrofolate reductase (MTHFR) gene.Suggested pathophysio-logic mechanisms for the effects of homocysteine include increased peroxidation injury, direct effect on endothelial cells, promotion of monocytic chemotaxis, promotion of clotting and activation of platelet aggregation.[11],[12]

However, the relationship between thrombophilia associated gene mutations and adverse obstetric outcome is controversial and data in the literature are inconsistent because of study heterogeneity,potential publication bias and sequential testing. [13],[14],[15]

OBJECTIVE OF THE STUDY

To investigate the prevalence of thrombophilia associated gene mutations namely,factor V (Leiden), prothrombin G20210A and methylene-tetrahydrofolate

reductase C 667 T) in relation to recurrent miscarriage in an attempt to identify candidates for anticoagulation to improve pregnancy outcome.

MATERIAL AND METHODS

A case-control study was conducted on 200 pregnant women between 9 -34 weeks of gestation attending the antenatal outpatient clinic for routine follow up.The study was undertaken to determine the prevalence of factor V Leiden,prothrombin G20210A and methylene –tetrahydrofolate reductase C667T in relation to recurrent miscarriage. All patients signed an informed consent to declare their agreement to be enrolled in the study, as agreed upon by the Research Ethics Committee.

INCLUSION CRITERIA

Group I:included 100 pregnant women with a history of three or more unexplained consecutive pregnancy losses.

Group II: included 100 healthy age – matched pregnant women with no history of recurrent miscarriages.

EXCLUSION CRITERIA

1. Induced abortions
2. Infections
3. Systemic diseases
4. Structural uterine anomalies
5. Personal or family history of thromboembolism.

All patients (group I and group II) were subjected to the following:-

1. Thorough history taking including:-
   - Age
   - Gestational age
   - Number and outcome of previous pregnancies
   - Number of previous miscarriages
   - Miscarriage stage (first or second trimester)
2. Physical examination
3. Ultrasound for fetal viability and gestational age.
4. Patients in group I (history of ≥ unexplained recurrent miscarriages) were selected based on complete work-up undertaken prior to pregnancy to exclude a cause for recurrent miscarriage and included the following investigations:-

- Fasting blood sugar.
- Hormonal assays:FSH,LH,prolactin and thyroid function tests.
- Anticardiolipin antibodies and lupus anticoagulant.
- Hysterosalpingography and /or hysteroscopy for the uterine cavity
- Karyotyping for chromosomal aberrations in both partners
- TORCH screening
- Transvaginal ultrasound for ovarian morphology.

5. Blood samples were collected from all pregnant women enrolled in the study for DNA extraction and genotype analysis. Factor V Leiden, prothrombin (PTH) and MTHFR gene mutations were assayed based on polymerase chain reaction (PCR) and reverse hybridization .The frequency of homozygous and heterozygous gene mutations, as well as, the co-expression of mutations were determined.

Analysis for Factor V-PTH-MTHFR mutations

The assay is based on the reverse hybridization principle and included three successive steps utilizing a commercially available kit.

- **DNA Extraction**

DNA was extracted from anticoagulated whole blood .Extraction started by incubating the blood and Lysis solution for 15 min followed by a binding step using the GENXTRACT Resin and another incubation as per the manufacturer guidelines.

- **Multiplex PCR**

- Factor V Leiden, prothrombin and MTHFR gene sequences were simultaneously amplified in vitro and biotin labeled in a single (multiplex) amplification reaction.
- The amplification products were selectively hybridized to a test strip which contained oligonuc-leotide probes (wild type and mutant specific) immobilized as parallel lines.
- Bound biotinylated sequences were detected using streptavidin-alkaline phosphatase and colour substrates.
- The assay covered three mutations namely, Factor V (G1691A), prothrombin (G20210A) and MTHFR (C677T) and the results were reported as normal genotype, heterozygous genotype or homozygous mutant genotype.

STATISTICAL METHODS

Data were analyzed using IBM SPSS Software 20.0.Mean, standard deviation, Chi-square test,t-test and Mann-Whitney tests were calculated using standard formulae. P value < 0.05 was considered significant.

RESULTS

Table 1: Comparison between groups as regards age

Age (years)	Group I (study group)	Group II (controls)
Range	23-36	24-37
Mean	28.65	27.61
S.D.	9.82	8.65
T- test	0.85	
P significance value	0.42	

The age of patients in group I ranged from 23 to 36 years with a mean age of 28.65±9.82 years, whereas, in group II the range was 24 to 37 years with a mean age of 27.61±8.65 years. There was no significant difference between groups.

Table 2: Comparison between groups as regards total number of pregnancies, gravidity and parity

	Group I	Group II	P value
Total number of pregnancies (live births +abortions)	421	390	0.743
Gravidity			0.002*
Range	3-9	2-5	
Mean	6.25	3.25	
S.D.	2.01	1.89	
Parity			0.087
Range	1-4	1-4	
Mean	2.01	2.68	
S.D.	1.36	1.04	

The total number of pregnancies in group I were 421 compared to 390 in controls. There was no significant difference between groups.. Gravidity in group I ranged from 3-9 with a mean of 6.25±2.01, whereas, in group II the range was 2-5 with a mean of 3.25±1.89. There was a significant increase in gravidity in group I compared to group II (p = 0.002). However, parity in group I and II ranged from 1- 4 with a mean of 2.01±1.36 and 2.68±1.04 respectively. There was no significant difference between groups.

Table 3: Comparison between groups as regards gestational age

Gestational age (weeks)	Group I	Group II
Range	9-34	12-33
Mean	24.3	21.4
S.D.	5.9	9.85
T test	1.25	
P significance value	0.236	

In group I, the gestational age at the time of blood sampling ranged from 9-34 weeks with a mean of 24.3± 5.9 weeks, whereas in group II, the range was 12-33 weeks with a mean of 21.4± 9.85 weeks.There was no significant difference between groups.

Table 4: History of live-births and miscarriages in the studied groups

	Group I	Group II	P value
Livebirths			
Number	53	370	0.001*
% out of total pregnancies	12.5	94.8	
Miscarriages			
Number	368	20	0.001*
First trimester	231 (62.7%)	11 (55%)	0.211
Second trimester	137 (37.3%)	9 (45%)	0.107

The number of livebirths in group I was 53 compared to 370 in group II. There was a statistically significant difference between groups (p=0.001).Furthermore, there was a statistically significant difference between groups as regards the total number of miscarriages.

Table 5: Prevalence of thrombophilia polymorphisms in the studied groups

	Group I	Group II	P value of mean
Factor V Leiden	13	10	0.562
Prothrombin gene G20210A	6	2	0.412
MTHFR C677T	42	8	0.001*
Total	61	20	0.003*

Considering each gene mutation individually, the prevalence of Factor V Leiden and prothrombin G20210A mutations did not differ significantly between groups, whereas, MTHFR C677T mutation and the total prevalence of the three gene mutations was significantly increased in group I.

Table 6: Analysis of thrombophilia gene mutations in the studied groups

	Group I		Group II		P value
	Number	*%*	*Number*	*%*	
Factor V Leiden Heterozygous Homozygous	 11 2	 84.6 15.4	 10 0	 100 0	 0.187
Prothrombin gene G20210A Heterozygous Homozygous	 6 0	 100 0	 2 0	 100 0	
MTHFR C667T Heterozygous Homozygous	 39 3	 92.8 7.1	 9 0	 100 0	 0.426
Combined Factor V Leiden and MTHFR	10	16.4	0	0	0.032*

Homozygosity and heterozygosity for each of the gene mutations studied did not differ significantly between groups.No homozygosity was detected for the prothrombin gene mutation nor for any gene mutation in group II.However, there was a significant difference between groups as regards combined thrombophilia involving Factor V Leiden and MTHFR C677T gene mutations.10 cases were detected in group I and none in group II.

Table 7: Analysis of thrombophilia gene mutations in group I

	Heterozygous		Homozygous		Total	
	Number	*%*	*Number*	*%*	*Number*	*%*
Factor V Leiden	11	84.6	2	15.4	13	13
Prothrombin gene G20210A	6	100	0	0	6	6
MTHFR C677T	39	92.8	3	7.1	42	42
X2 P	12.65 0.013*					

Homozygosity and heterozygosity were assessed for each of the three gene mutations studied and compared in group I.Heterozygotes were significantly increased compared to homozygotes.

DISCUSSION

Pregnancy is considered an acquired hypercoaguble state due to increased levels of coagulation factors, decreased levels of anticoagulants and decreased fibrinolytic activity.[16] The gradual increase in hypercoagulability during normal pregnancy predisposes to VTE and to gestational vascular complications including recurrent pregnancy loss, intrauterine growth restriction, eclampsia, preeclampsia and placental abruption. These adverse pregnancy outcomes affect up to 15% of gestations and are the major cause of maternal and fetal morbidity and mortality. [17],[18]

Pregnancy loss is a common medical problem among reproductive age women. However, relatively few women having one pregnancy loss experience multiple or recurrent pregnancy loss.[1]Mounting evidences point to a link between inherited thrombophilia and recurrent fetal loss being associated with abnormal placental vasculature and disturbances of haemostasis leading to inadequate feto-maternal circulation.[19]

This case-control study was conducted on 200 pregnant women divided into two groups .Group I included 100 pregnant women with a history of three or more unexplained consecutive miscarriages and

group II included 100 healthy age-matched pregnant women with uncomplicated previous pregnancies.

In the present study, maternal age did not differ significantly between groups (p=0.42). However, gravidity in group I was significantly increased compared to controls (p=0.002), whereas, the total number of pregnancies, parity and the gestational age at the time of blood sampling did not differ significantly between groups (p= 0.743 , 0.08 and 0.236 respectively) .

As regards the history of live births and previous abortions in the study groups, there was a significant increase in the number of live births per woman among controls and a significant increase in the total number of miscarriages among the patients group.

On assessing the individual prevalence of thrombophilia polymorphisms in the studied groups, it was found that Factor V Leiden and prothrombin gene G20210A mutations individually did not differ significantly between those with a history of recurrent miscarriage and controls. There is a large and contradictory body of literature on the association between maternal inherited thrombophilia and recurrent miscarriage[8],[9],[20]. Although most [8],[21],[22] but not all1[8],[23]] large prospective cohort studies have failed to establish a consistent association between inherited thrombophilia and early or late fetal loss, case -control and retrospective cohort studies have generally reported a link between factor V Leiden heterozygosity and possibly prothrombin gene mutation heterozygosity and fetal loss after 10 weeks and particularly for non recurrent loss after 20 weeks [14],[24],[25].This suggests that any association is limited to high risk populations and is modest. Small case-control or retrospective cohort studies involving heterogeneous populations have frequently reported contradictory results, in part because of the influence of various confounders (e.g. age, obesity) that are often not analyzed appropriately [14],[25].

In our study, MTHFR C 667T mutations were significantly increased in the patients group compared to controls. Several studies, [26],[27],[28] reported increasing evidence for a pathogenetic role of MTHFR gene polymorphism C677T in recurrent pregnancy loss , in particular, early loss. On the other hand, several authors [29],[30] found a negative association stating that MTHFR polymorphisms do not carry any risk in pregnancy. The different inclusion criteria and the different ethnic backgrounds of the selected patients may have contributed to the contradictory results.

In the current study, the total prevalence of the three gene mutations namely, Factor V Leiden, prothrombin gene G20210A and MTHFR C677 T were significantly increased in the patients group compared to controls. This is in accordance with some previous studies [30],[31],[32]. However, other large prospective studies [8],[21],[22] reported contradictory results stating that hypercoagulable thrombophilic gene mutations are not increased in women with recurrent miscarriage.

Infact, case- control and cohort studies reflect methodological diversity and clinical heterogeneity. Studies have been conducted in different countries, using different study designs and in routine care settings,as well as, high risk referral centres. Study limitations have included inadequately described and /or heterogeneous case and control groups and cohorts, insufficient information to adequately assess potential biases, and missing or incomplete information on

important covariates as maternal age and number and timing of losses.

In our study, there was a significant increase in the number of cases with combined thrombophilia in the patients group compared to controls. Combined thrombophilia included Factor V Leiden and MHTFR C677T and none of the cases involved prothrombin gene G20210A mutation. The same results were reported by previous studies [17],[26],[33],[34] that identified combined thrombophilic defects in women with recurrent pregnancy loss, both early and late. The study by Rozano-Gorelick et al 17 reported that combined thrombophilia exists when inherited and /or acquired prothrombotic factors are pooled and every combination carries a different risk of thrombosis. Furthermore, Sarig et al 18 proposed a scoring system for women with thrombophilia based on four major categories: obstetric history, previous thromboembolic events, family history of thrombosis or gestational vascular complications and type of thrombophilia. Combined thrombophilia was given a high score and subclassified as combined moderate (heterozygous for both factor V Leiden and prothrombin G20210A mutations) and combined severe (strong lupus anticoagulant and homozygous for factor V Leiden or antithrombin deficiency).The total score is calculated by summing up the scores of the four categories. Based upon the score achieved, the pregnancy risk for an individual woman may be stratified into four levels of risk: low ≤ 5, intermediate (score 6-10), high (score 11-14) and extremely high (score≥ 15).

Finally, the number of homozygous and heterozygous individuals were assessed for each of the gene mutations studied and compared between groups. No homozygosity was detected in controls and heterozygotes were significantly increased in the patients group compared to homozygotes. Couto et al35 reported a low prevalence of homozygotes for factor V Leiden and stated that the prothrombotic tendency during pregnancy and the risk of thromboembolic events is increased with antithrombin deficiency and homozygous factor V Leiden as single traits.Infact, the reported prevalence in the general population of factor V Leiden and prothrombin gene homozygotes is less than 1% with 2-4% risk of VTE per pregnancy increasing to around 17% in women with a previous history of VTE13,36.

Hence, our study and similar studies reported an association between some types of thrombophilia and recurrent miscarriage, but the absolute risk is small and varies considerably among reports. However, most large prospective cohort studies have failed to establish a consistent association between inherited thrombophilia and adverse pregnancy outcome.

According to recent evidence [13],[37], screening for inherited thrombophilia in women with a history of recurrent or non recurrent fetal loss, abruption, intrauterine growth restriction, or preeclampsia is not recommended. Moreover, there is mounting evidence that administration of prophylactic anticoagulation during pregnancy for the prevention of placenta-mediated pregnancy complications does not improve pregnancy outcome in affected patients.

CONCLUSIONS

The prevalence of Factor V Leiden and prothrombin gene G20210A mutations did not differ significantly between those with history of recurrent miscarriage and controls.

MTHFR C667T mutations and the total prevalence of the three gene mutations were significantly increased in the patients group compared to controls.

There was a significant increase in the prevalence of combined thrombophilia (Factor V Leiden and MTHFR C677T) in the patients group compared to controls.

Combined thrombophilia did not involve prothrombin gene G20210A mutations in either group.

No homozygosity was detected in controls for any of the gene mutations studied.

Heterozygotes were significantly increased in the patients group compared to homozygotes.

SUMMARY

Recurrent pregnancy loss is multifactorial involving clinical and biological risk factors. Evidence addressed the association of inherited thrombophilia with recurrent pregnancy loss and other serious pregnancy complications. However, the relation between thrombophilia associated gene mutations and adverse obstetric outcome is controversial and data in the literature are inconsistent.

The aim of the present study was to investigate the prevalence of thrombophilia gene mutations, namely, factor V Leiden, prothrombin G20210A and methylenetetrahydrofolate reductase C667T in relation to recurrent miscarriage.

This case-control study was conducted on 200 pregnant women divided into two groups. Group I included 100 pregnant women with history of three or more unexplained consecutive miscarriages and group II included 100 healthy age-matched pregnant women with uncomplicated previous pregnancies. Blood samples were collected from all pregnant women enrolled in the study for DNA extraction and genotype analysis based on polymerase chain reaction and reverse hybridization. The assay covered three mutations, factor V Leiden, prothrombin gene G20210A and MTHFR C667T.

The prevalence of Factor V Leiden and prothrombin gene G20210A mutations did not differ significantly between those with history of recurrent miscarriage and controls. However, MTHFR C667T mutations and the total prevalence of the three gene mutations were significantly increased in the patients group compared to controls.

There was a significant increase in the prevalence of combined thrombophilia (Factor V Leiden and MTHFR C677T) in patients group compared to controls. However, combined thrombophilia did not involve prothrombin gene G20210A mutations in either group.

Finally, the number of homozygous and heterozygous individuals were assessed for each of the gene mutations studied. No homozygosity was detected in controls and heterozygotes were significantly increased in the patients group compared to homozygotes.

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