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# THE ALLELIC DISTRIBUTIONS OF SNPS FROM THROMBOSIS ASSOCIATED GENES IN PATIENTS WITH MISCARRIAGES IN ERBIL, IRAQ

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**ABSTRACT**

*Abortion is the most common complication during pregnancy. Given the importance of thrombophilia in pregnant women and it is association with recurrent pregnancy loss. Polymorphism analysis of the genes involved in thrombophilia may be useful.*

*There are many factors that affect abortion and recurrent miscarriage such as Female age and embryonic aneuploidy, Antiphospholipid syndrome, Parental structural chromosomal abnormalities, Uterine structural abnormalities, Endocrine factors: thyroid function, PCOS and prolactin, Immunological factors and progesterone supplementation, TLC, infection, lifestyle and sperm DNA damage and one of the factors is Inherited thrombophilia that include several genes that may have an impact on recurrent miscarriage. We have analyzed seven genes in the Iraq-Erbil population to determine the relationship between these genes and the number of abortions, age and family history.*

*Blood samples were taken from 50 women with recurrent miscarriages and another 30 women who did not have any abortions as control cases and using PCR-RFLP, researchers analyzed and extracted DNA from samples for carrying Methylene Tetrahydrofolate Reductase (MTHFR) C677T and A1298C, Plasminogen Activator Inhibitor-1 (PAI-1), Factor V Leiden, Prothrombin G20210A , Factor XIII and Fibrinogen beta chain polymorphisms.*

*There was no association between recurrent miscarriage with age however the two genes MTHFR C677 and MTHFR A1298C were related to family history. After comparing the genes among themselves, the static analysis was that MTHFR C677T and PAI-1 (4G/5G) are associated with recurrent pregnancy loss. But after comparing the genes with control cases, the static result of the analysis was that all genes are associated with recurrent miscarriage and are significant.*

*However, further investigate on large scale populations perhaps needed to recognize new genetics diverse.*

***Keywords:*** *Recurrent miscarriage, MTHFR C677, MTHFR A1298C, PAI-1, gene.*

## INTRODUCTION

The spontaneous abortion refers to the miscarriage phenomena taking place within 20 weeks of the gestation (1).The American College of Obstetricians and Gynecologists (ACOG) has estimated the rate of premature delivery for all pregnancies as 26%, while the rate is determined as 10% for clinically monitored pregnancies(2-4).

While 15% of the clinical pregnancies end in unconstrained premature birth, the rate of recurrent pregnancy loss (RPL) was determined to be approximately 5%, and is identified as one of the most prominent female infertility causes(5, 6). World Health Organization (WHO) has determined that three or more consecutive premature deliveries before 20th week of gestation is defined as RPL(7).

There are many factors that affect recurrent pregnancy loss, such as uterine structural abnormalities, endocrine factors like (thyroid function, polycystic ovary syndrome and prolactin anomalies), immunological factors and progesterone supplementation, infection, lifestyle, sperm DNA damage and inherited thrombophilia(8). Thrombophilia has been associated with RPL in many studies showings it as the main factor, making up for 40% of the cases, particularly during the early periods of the pregnancy(9-11). For inherited thrombophilia, on the other hand, number of single nucleotide polymorphisms (SNPs) have been associated with inherited thrombophilia as the risk factors for VTE cases(7). The three most common ones are Factor V Leiden (FVL), Prothrombin G20210A (PT G20210A), and methylene tetrahydrofolate reductase (MTHFR)(11, 12). Seven single nucleotide polymorphisms (SNPs) associated with inherited thromboembolism; methylene-tetra-hydro-folate reductase (MTHFR), MTHFR 1298 and 677, factor V leiden, PAI-1 Factor XIII, Fibrinogen beta chain and FII prothrombin; are considered as risk factors for RPL.

The aim of this study was to investigate the allelic distribution of SNPs that are associated with thrombosis, including factor V Leiden (R506Q), prothrombin (G20210A), factor XIII (V34L), fibrinogen beta chain, plasminogen activator inhibitor-1 and the methylene tetrahydrofolate reductase (MTHFR, C677T) and (MTHFR A1298C), in women with miscarriages in Erbil, Iraq.

## MATERIALS AND METHODS

The instruments used for this study are listed in table 2.1 and the materials are listed in table 2.2.

Table (2-1) Utilized instruments, brands and manufactures

| NO. | Equipment | Brand | Origin |
|---|---|---|---|
| 1 | Laminar air flow hood | Bioquel | UK |
| 2 | Water bath | Thermostatic | Taiwan |
| 3 | Sensitive balance | Sartorius | Germany |
| 4 | Nano Drop Spectrophotometer | Thermo Scientific | USA |
| 5 | Micropipettes | Eppendorf | Germany |
| 6 | Eppendorf tube | Eppendorf | Germany |

| | | | |
|---|---|---|---|
| 7 | Centrifuge 13500 | Datthan Scientific | Korea |
| 8 | Autoclave | Binder | Germany |
| 9 | UV-Documentation | IVB | Germany |
| 10 | Gel electrophoresis instrument | GFL | Germany |
| 11 | PCR system (Polymerase Chain Reaction) Thermo cycler | Eppendorf | Germany |

| **No.** | **Chemicals** | **Brands** | **Origin** |
|---|---|---|---|
| 1 | Genomic DNA (High pure PCR TemplatePreparationKit)(blood/culture cell) | Roche | Germany |
| 2 | Isopropanol | Merck | Germany |
| 3 | Agarose (Analytical Grade) | Cinna Gen | Iran |
| 4 | TBE Buffer 10X | Thermo Scientific | USA |
| 5 | DNA Safe Stain | Cinacolo | Iran |
| 6 | Primers | Metabion | Germany |
| 7 | Taq 2x Master Mix Red, 1.5mm $MgCl_2$ | Ampliqon | Denmark |
| 8 | DNA Ladder | Thermo Scientific | USA |

Table (2-2) Table listing the chemicals including kits, brands and manufactures.

**METHODS**

1. Sample collection:

The blood samples were collected from the Maternity Teaching hospital. The study included 50 females with recurrent abortion in Erbil city-Iraq.

2. DNA extraction from whole blood

Three to four ml of blood sample was withdrawn aseptically from 50 patients with venous thrombophilia diseases and 30 health subjects in sterile vacutainer tube coated with ethylene diamine tetra acetic acid (EDTA). The blood samples were stored at -20 until processed.

Genomic DNA extraction from whole blood was performed by DNA Kit (High Pure PCR Template Preparation Kit), according to the protocol which designed for extraction of Genomic DNA from the whole blood.

3. Primer Design

A primer is strand of nucleic acid that serves as a starting point for DNA synthesis. Selection of primers was depending on the target region in the sequence. Six pairs of primers were designed by

Online primer program (http://www.nlm.nih.gov/tool/primer-blast) targeting the SNPs of interest.

4. PCR (polymerase chain reaction) for DNA amplification

PCR was used to amplify the regions of interest for each gene. The PCR amplification of the genes was performed with the designed pairs of primers spanning the target region. Each PCR was performed in a final volume of 25µl and the final concentrations of the primers were 0.5 µM.

5. Restriction Fragment Length Polymorphism (RFLP) by using Restriction enzyme for DNA cutting at specific site.

RFLP is a molecular method of genetic analysis that allows identification based on unique patterns of restriction enzyme that cuts in specific regions of DNA with known variability. It can be used to genotype polymorphisms to distinguish between individuals. Hinf 1 (10 u/µl.2000 unit) restriction enzyme was used to genotype the (MTHFR) C677T genes. Hind III (10µ/ µl.5000unit) restriction enzyme was used to genotype the Factor V Leiden and Prothrombin G20210A genes.

Hpyf3I (10 µ/ µl.500 unit) restriction enzyme was used to genotype the Factor XIII genes. Bsur1 (10 µ/ µl.3000 unit) restriction enzyme was used to genotype the Fibrinogen beta chain genes. The product was analyzed using 2% agarose gel electrophoresis.

A touchdown is the method of PCR requires starting by high annealing temperature and progressive decreasing the annealing temperature for each PCR cycle. the upper annealing temperatures within the primary cycles of a touchdown confirm that only much specific base pairing it happens between the DNA and thus the primer, hence the primary sequence to be amplified is presumably to be the sequence of interest, this method used for MTHFR A1298C gene, The 10 µl of PCR product was analyzed using 2% agarose gel electrophoresis.

(ARMS) is a method of PCR during which DNA is amplified by specific allele primers. It is an acutely useful method for recognition of point polymorphisms, this method used for PAI-1 gene, the 10 µl of PCR product was analyzed using 2% agarose gel electrophoresis.

6. Agarose Gel Electrophoresis Two grams agarose gel (Cinna Gencompanies) in 1X1000 TBE (Tris-Borate EDTA) buffer was prepared to analyses the RPLF products. One in ten dilution with D.W. and 5µg/ml ethidium bromide was used to visualize the PCR products. Five µl of PCR product and six µl of 100 bp molecular weight DNA Ladder were added to the wells. The gels were visualized by UV gel visualization systems. The equipment used is electron small tank, power splay, balance and micro wave.

## BIOSTATISTICS

The IBM SPSS software 25th edition was used for analyzing the data. The statistical significance between the genotype of polymorphisms for each gene and family history or recurrent miscarriages was investigated using the Pearson-chi square test.

The statistical significance between the genotype of polymorphisms for each gene, number of pregnancy loss and the age of patients was investigated using the ANOVA (CRD design) test.

The statistical analysis depending on type of data and P-values < 0.05 were considered to be statistically significant while P-values>0.05 were not significant.

## RESULT

This study included 50 women who were experiencing miscarriages in the first trimester of pregnancy and 30 women who were the control case. All RPL women were in the age range of 19 to 43 years and number of miscarriages ranging from 2 to 10. The allelic frequencies for *MTHFR C677T* gene were 64.0% for C/C wild type, 24.0% for heterozygote C/T and 12.0% for homozygote T/T, respectively. and the wild type allele for *MTHFR A1298C* was lower compared to the wild type *MTHFR C677T* gene, in such A/A wild type was observed in 36.0% of the females with RPL, whereas the heterozygote A/C was 54.0% and the homozygote C/C was 10.0%, respectively. The wild type G/G

genotype of *Prothrombin G20210A* were observed for all the patients are Wild type. The allelic frequencies of *Factor XIII* ratio for V/V wild type was 78.0% and for heterozygote V/L was 32.0% with no homozygote L/L genotype. The allelic frequency of Fibrinogen beta chain was higher for the wild type G/G with 68.0% followed by the heterozygote A/G genotype with 22.0%. Similarly, homozygote A/A genotype was not observed for these patients either. On the contrary, there was no wild type genotype of 4G/4G detected for *PAI-1*, however the heterozygote 4G/5G was detected to be 84.0%, and the homozygote 5G/5G was 16.0%. All the patients with the *Factor V Leiden* were detected to have the wild type G/G genotype.

There was a statistical significance between the allelic frequencies of heterozygote and homozygote alleles of the *MTHFR C677T* (p.=0.039) and *MTHFR A1298C* (p.= 0.049). There was no statistical significance between the family history of miscarriage and the genotypes of the *Factor XIII*, Fibrinogen beta chin and PAI-1 ($p>0.05$). No statistical analysis was performed for the *prothrombin G20210A* and *Factor V Leiden* a since all the genotypes were the wild type. Generally, the result demonstrates that only *MTHFR C677T* and *MTHFR A1298C* have a relationship with family history.

Further investigation involved biostatistical analysis of genotypes of each SNP and the maternal age, There was no statistical significance between the maternal age and the genotypes of the *MTHFR C677T, MTHFRA1298C, Factor XIII, Fibrinogen beta china* and *PAI-1* ($p>0.05$). No statistical analysis was performed for the *prothrombin G20210A* and *Factor V Leiden* a since all the genotypes were the wild type.

Further investigation involved biostatistical analysis of genotypes of each SNP and number of abortion, there was statistical significance between the number abortion and the genotype of the *MTHFR C677T* ($p = 0.005$) and *PAI-1* ($p = 0.018$) and there was no statistical significance between the number abortion and the genotypes of the *MTHFRA1298C, Factor XIII and Fibrinogen beta china* ($p>0.05$). No statistical analysis was performed for the for *the prothrombin G20210A and Factor V Leiden* a since all the genotypes were the wild type. The results detected that only the *MTHFR C677T and PAI-1* had a relationship with number of abortions.

Statistical analysis was performed to investigate each genotype of the SNPs within the genes in RPL and the control group, The results showed that the wild type (*MTHFR C677T, MTHFRA1298C, Factor XIII, Fibrinogen beta china , PAI-1, prothrombin G20210A* and *Factor V Leiden)* genotype was observed significantly more in the control group for SNP (*MTHFR C677T, MTHFRA1298C, Factor XIII, Fibrinogen beta china , PAI-1, prothrombin G20210A* and *Factor V Leiden*).

The outcome of all of the genes show a communication between each genotype of the SNPs within the genes in RPL and the control group.

## DISCUSSION

Many factors are believed to contribute to the spontaneous abortion cases, such as embryo aneuploidies, hormonal problems, or uterine anomalies(13, 14). The aim of this project was to investigate the polymorphisms within seven genes which had effective connection with number of abortion when it has been compared with control cases(10, 11, 15). In addition, the significance of the thrombophilia cases in women over the pregnancy and its combination with recurrent miscarriages were also analyzed with regards to the polymorphisms of the genes. Studying and analyzing these genes in different locations related to the thrombophilia cases can help develop strategies for early diagnosis and treatment of recurrent pregnancy loss cases(11).

However, there have been a number of studies performed on different populations which claim that there is no correlation between recurrent pregnancy loss and such polymorphisms(16, 17).In clinical basis, the SNPs associated with thrombophilia are routinely genotyped in females with miscarriages in Iraq. Since the association between these polymorphisms and the RPL has been reported to be population specific(15).the aim of this study was to investigate the frequency and correlationof seven

polymorphisms associated with thrombophilia and RPL in aged matched Iraqi population.

In the present paper, the analysis of data collected regarding the gene known as the MTHFR C677T has shown that the parental polymorphisms of the gene have a statistically significant relationship with recurrent pregnancy loss (RPL) cases, as suggested by(18).(19)suggests that, spontaneous abortion cases in humans occur as a result of sophisticated interactions between a range of genetic factors and certain environmental conditions. One of the most prominent examples of such interactions is the clear correlation between the heightened homocysteine content and deficiencies in the neural tubes. This phenomenon has led the researchers to believe that increased homocysteine levels are potentially embryotoxic, could have negative effect on fetal viability. The researchers also claimed that polymorphic TC genotypes of rs1801133 of *MTHFR* structures are amongst the risk factors for a series of developmental diseases like neural tube defects, orofacial clefts, and even Dawn syndrome. The results of this study showed that CT genotype *MTHFR C677T* had a significant impact on the number of abortions among the patients. Studies have shown that out of 50 women, 32 had the CC (wild type) genotype, while the number of patients with heterozygote and homozygote was 12 and 6 respectively, which had a great relationship with the family history of miscarriage. In concordance with the results of this study investigation of 50 patients in Duhok-Iraq showed that there is a relationship between (CT and TT) genotype *MTHFR C677T* and RPL(20).

In this study, concerning (AC and CC) genotype *MTHFR A1298C*, has not been communication with recurrent pregnancy loss when compared with non-control cases, our results was coincident with the findings of(21)which they conducted a study on the effects of *MTHFR A1298C*on recurrent abortion. The researchers used a total of 1163 RPL cases and a control group of 1061 individuals, and analyzed the random-effect rates for CC versus total genotypes (P = 0.3456), for CC + AC versus total genotypes (P = 0.0833) and for C versus total alleles(P = 0.7112). As such, the researchers claimed no correlation between MTHFR A1298C polymorphisms and RPL cases. This finding is in line with our own findings regarding this specific gene polymorphism.

On the other hand, in this study when*MTHFR A1298C*dealing with control cases it has great communication with RPL and according study by(22)100 Syrian women who have had recurrent abortion, they findings indicate that RPL women with CC genotype for *MTHFR A1298C* either alone or compound CT genotypes have a high risk of RPL in Syrian women, that is consistent with our result.

In this study, the patients had the GG (wild type) allele for the *Prothrombin G20210A* and *Factor V Leiden*. These results are in agreement with the results of recent studied reporting a total of 4,167 cases were utilizable for tested and were analysis for the prothrombin G20210A polymorphisms. they found only of 157 (3.8%) women in their study group had the Prothrombin gene polymorphism (156 heterozygous and one homozygous)(23). The researchers have reported that the rate of pregnancy loss was similar whether the women had the Prothrombin G20210A mutation or not(23).This result was cross analyzed with multivariance analysis where numerous factors like patient age, race, RPL history, Small of Gestational Age (SGA) neonate events, and thromboembolism history were considered, all of which returned irrelevant statistical relationship(23).

The researchers therefore concluded that Prothrombin G20210A polymorphism had no relationship with RPL, preeclampsia, or abruptions. In our study, a very small variance has occurred for women with heterozygotes and homozygotes of this polymorphism(23, 24).This data indicates that it might be irrelevant to monitor women in terms of these polymorphism., particularly if they don't have a history of thrombosis.

However, for support our result according to the (20)having done research on 50 patients in Duhok-Iraq and (25)having done research on 70 cases, 40 of which were patient and 30 were in control in Baghdad-Iraq, and both study groups have reported that there were no statistical connections between

*Prothrombin G20210A* polymorphism and RPL cases.

In this study *Factor XIII* has not been communication with recurrent pregnancy loss when compared with non-control cases, but when dealing with control cases it has great communication with RPL. This finding is also supported by the study of(26),who worked on 140 women in Iran they have recurrent abortion, and showed a relationship between Factor XIII and recurrent pregnancy losses.

The results of this study showed that the*factor V Leiden* polymorphisms had not been associated with pregnancy loss when compared with control and non-control cases. According the results (20)and (25) in their research on the Iraqi population they have concluded that there was no correlation between *factor V Leiden* and recurrent pregnancy loss in concordance with these results. However, These results are also confirmed by (27)showing that they appraised 78 pregnant Indian women for FVL polymorphisms, among them 50 woman had a history of recurrent abortion. Only one (2%) woman tested positive for heterozygous FVL polymorphism.It has been presented that there is no significant association between *FVL* polymorphism and URPL.

According to the result on this study plasminogen activator inhibitor-1 *(PAI-1)* −675 4G/5G, *beta fibrinogen (BF)* −455G/A have communication with recurrent pregnancy loss when compared with control cases. In concordance with these results, the correlation of the plasminogen activator inhibitor-1 *(PAI-1)* 675 4G/5G, *beta fibrinogen (BF*;−455G/A, integrin beta 3 (ITGB3; 1565T/C & 1298A/C) polymorphisms with RPL were performed previously showing an association between *BF* −455G/A genotype and RPL(28). Consequently, they concluded that it is important to check thrombophilia screening in patients with RPL.

Moreover, in this study *beta fibrinogen (BF)* −455G/A has not been communication with recurrent pregnancy loss when compared with non-control cases., for support our result according to the(29)having done research on 220 Iranian woman cases, 110 of which were patient and 110 were in control , they have concluded that there is no association between GA genotype *beta fibrinogen (BF)* with RPL.

In conclusion, more studies are needed to classify all DNA mutations in Iraqi population. Further studies on more samples are needed to better understand the role of *(MTHFR) C677T, (MTHFR) A1298C, Prothrombin G20210A, Factor XIII, Fibrinogen beta chain, Plasminogen activator inhibitor-1 (PAI-1)* and *Factor V Leiden* genes with recurrent miscarriage especially since these polymorphisms are routinely checked in the hospitals and clinics in RPL patients.

Patients with risk factor such as thromboembolism should be screened for thrombophilia and referred to a specialist physician and treated. The results of this study are aimed to form the basis of allelic frequencies of thrombolysis associated gene polymorphisms with RPL patients.

## CONCLUSIONS

The results of this study clearly reveal a significant correlation between RPL cases and thrombophilia-related gene polymorphisms like *MTHFR (C677T and A1298C), Prothrombin (G20210A), Factor XIII, Fibrinogen beta chain, Plasminogen activator inhibitor-1 (PAI-1),* and *Factor V Leiden.*

The study also investigated a series of other variables like age, family history, and number of previous miscarriages, with the above-mentioned polymorphisms and RPL occurrence. The results of this analysis show that there are no statistical correlations between patient age and polymorphisms of genes but a relationship was discovered between two *MTHFR* polymorphisms (namely the C677T and A1298C) and family history miscarriages. Inter-relationships between the genes were also examined, revealing that the presence of *MTHFR C677T* and *Plasminogen activator inhibitor-1(PAI-1)* was in fact correlated with RPL.

Comparison of all the genes individually with the control group, on the other hand, revealed that all genes are correlated with RPL cases in a statistically meaningful manner.

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