Original Article

Combined Parental Thrombophilia Gene Mutation Defects in Couples with Repeated Pregnancy Loss

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Abstract

Background: Several genetic mutations in female thrombotic defects have recently been shown to affect recurrent pregnancy loss (RPL); however, it is unclear which common parental mutations are involved in thrombosis-associated repeated pregnancy loss RPL. **Aims:** In this study, the prevalence of some combined parental thrombophilia gene mutation defects was studied in couples with RPL. **Settings and Design:** The observational study was done in babol infertility research center (Iran) in 2022. **Materials and Methods:** Sixty-two infertile women with a history of RPL and their male partners (124 individuals) participated in this study. The frequencies of common defects associated with methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C, factor V Leiden, protein C, protein S and homocysteine were analysed in these couples. **Statistical analysis used:** The data were statistically analysed using the Mann–Whitney test. **Results:** Sixty-two couples (124 individuals) were analysed. 56.2% of couples with a history of RPL had MTHFR C677T and 23.1% had MTHFR A1298C. Forty percent of couples showed homocysteine deficiency and 12.5% protein C deficiency. Other genes tested were only observed in the mother or father but not both. **Conclusions:** Results obtained with RPL couples demonstrate the importance of further investigating combined parental thrombophilia gene mutation defects (not only maternal).

Keywords: *Factor V Leiden, homocysteine, pregnancy, protein C, thrombophilia*

Introduction

Recurrent pregnancy loss (RPL) is defined as two or more failed clinical pregnancies before 20 weeks of gestation.[1] Some of the miscarriages result from thrombophilia disorders.[2] Growing clotting factors or reducing anticoagulant factors are the result of thrombophilia defects. Failure in maternal-foetal immune homeostasis arises from venous and arterial thromboembolism leading to rejection of the embryo during the 1st week of pregnancy.[3] It seems genetic disproportion plays a role in thromboembolism, as the prevalence of thrombotic disorder varies by race and ethnicity.[4,5] The frequencies of maternal thrombophilia defects in RPL have been mostly reported within various ethnicities of Iran.[6-10] A systematic review was performed by Kamali *et al*. in Iran to investigate thrombophilia gene polymorphisms in women with RPL. The results of Kamali's study showed a significantly increased miscarriage rate in all genotypes of the Iranian population.[7] Another study found that methylenetetrahydrofolate reductase (MTHFR) gene C677T and A1298C had the highest rate (50%) of common mutations, and F2 G20210A and F5 Leiden G1691A were 20% in Iranian women with RPL.[11] Foetuses carry paternal genes as well as maternal genes; however, one of the most frequently stated problems is the combined parental thrombophilia defects that related studies in Iran have not focused on. A study conducted

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Received: 16-10-2023 **Revised:** 02-11-2023
Accepted: 19-12-2023 **Published:** 29-12-2023

Access this article online

Quick Response Code:

Website: www.jhrsonline.org

DOI: 10.4103/jhrs.jhrs_137_23

How to cite this article: Kashifard M, Basirat Z, Ramezani F, Ghofrani F, Golsorkhtabaramiri M. Combined parental thrombophilia gene mutation defects in couples with repeated pregnancy loss. J Hum Reprod Sci 2023;16:352-7.

amongst different ethnicities in Turkey (owing to ethnic similarities to Iran) resulted that RPL couples showed greater combined parental thrombophilia gene mutations versus fertile couples.[12] Unfortunately, we found a few studies that investigated combined parental thrombophilia gene mutation defects in couples with RPL. Given the importance of parental thrombophilia disorder in RPL, the objective of this research was to determine the frequency of some thrombophilia genes in Iranian couples with repeated pregnancy loss.

Methods

This observational study was conducted in Babol in 2022. The research received ethics code: IR.mubabol.rec.1400.046 from the Ethics Committee of Babol in 2021.

Eligibility criteria included infertile women in ages of 20–35, having ≥2 miscarriages, without diabetes or hypertension, non-smokers and the ones had not used heparin for 6 months before the study. In addition, the patient's hysterosalpingography, hysteroscopy or sonohysterography should be without uterus malformation such as a unicorn or bicorn uterus, any filling defect in the upper two-thirds of the uterus, polyp or fibroid >1 cm, septum >1 cm in width and depth of the uterus and other abnormality like Asherman syndrome. Patients who did not wish to participate in the study or did not agree to share information were excluded. No sample size calculation was performed.

Recurrent pregnancy loss (RPL) is defined as the loss of at least two clinical pregnancies ≤24 weeks of gestation.[1]

A complete medical history and physical examination of all eligible couples was performed after obtaining written informed consent. According to their records, the demographic-obstetrical checklist was completed. More information about the thrombophilia tests could be found in the second checklist.

All couples underwent thrombophilia-related tests, including protein C activity, protein S activity, resistance to activated protein C, factor V Leiden (FVL) mutation, factor II mutation, factor 7, 8, 9 and 11, homocysteine, antinuclear antibody, anticardiolipin, antiphospholipid, antithrombin III, lupus anticoagulant and beta-2 glycoprotein. MTHFR gene polymorphism testing was performed in individuals with high fasting homocysteine levels.

Laboratory procedure

All tests were done in a laboratory by a practiced technician. The venous blood collected was placed in a tube (containing 3/3 cc of citrate and 5 CC of blood) and was sent immediately to the laboratory for testing thrombophilia factors. The samples were centrifuged at 2500–3000 rpm for 10 min, and the separated plasma was kept at −70°C until the beginning of the test. DNA was extracted from the blood sample by salting out methods.[13] It was broken with sodium dodecyl sulphate, and leucocytes and proteinase K were recovered from cell lysis. DNA was purified using phenol, chloroform, and ethanol vapor. Ultraviolet spectroscopy was used to confirm DNA purity and DNA content, and agarose gel electrophoresis was used to assess the integrity of the extracted DNA. The extracted DNA was stored at −20°C. The presence or absence of mutations in the genes MTHFR C677T, A1298C, FVL, antithrombin III and factor XIII was detected by polymerase chain reaction technique with primers.[14,15] For polymorphisms A1298C and C677T, we examined heterozygous and homozygous alleles with and without mutations in the couples. Homozygotes with no mutation were considered normal. Homozygotes and heterozygotes with mutation were considered abnormal.

Statistical analysis

The Mann–Whitney test was performed using the SPSS version 19 software (SPSS Inc., Chicago, IL) to compare the thrombophilia genes in males and females. Statistical significance was set at $P < 0.05$.

Results

Sixty-two women with their male partners (124 individuals) were included in the study. 96.7% of women experienced ≥2 miscarriages. The mean of miscarriages in women was 2.47 ± 1.18. 27. 4% of the couples were in a consanguinity marriage.

Characteristics of women with RPL and their male partners are shown in Table 1. Table 2 compares all thrombotic factors in females and males. Amongst all factors, men showed significantly greater homocysteine value than women ($P < 0.001$).

Table 3 presents the frequencies of thrombophilia factors examined in women and their partners individually. MTHFR C677T and MTHFR A1298C had the most

Table 1: Characteristics of the women with recurrent implantation failure and their partners

	Female	Male
Age	29.65±5.94	31.50±5.13
FSH (mlu/mL)	7.06±4.41	6.50±6.31
LH (IU/L)	5.97±5.36	4.87±2.35
TSH (mlu/mL)	3.67±4.97	2.66±2.37
Anti-TPO (IU/mL)	39.72±11.63	15.44±1396

FSH=Follicle-stimulating hormone, LH=Luteinizing hormone, TSH=Thyroid-stimulating hormone, Anti-TPO=Anti-thyroid peroxidase

frequency factors amongst other factors examined in women and men (25.1% and 16.1%) [Table 2]. Examination of C677T polymorphism in the MTHFR gene revealed that seven couples were both heterozygous, 39 couples were both homozygous without mutation and 1 of 16 couples were abnormal (homozygotes and heterozygotes without mutation). There is no significant difference between protein S and protein C in women and men (P = 0.47 and P = 0.07). The mean of prothrombin 3 in women was 28.64 ± 12.61 and in men was 24.03 ± 5.32, which was significant (P = 0.006).

The frequencies of combined paternal thrombophilia factors in couple or one of them (only maternal or paternal) are shown in Table 4. In the couples with a history of RPL, 48% (36) of women and 52% (39) of men had at least one of thrombotic factors such as protein C, homocysteine, factor 7, factor 8, factor 9, factor 11, MTHFR C677T mutation and MTHFR A1298C mutation. 56.2% (9) of couples with a history of RPL had MTHFR C677T, and 23.1% (3) had MTHFR A1298C. Forty percent (2) of couples showed homocysteine deficiency and 12.5 showed protein C deficiency. Other thrombophilia gene mutations have not been seen in both parents (only maternal or only paternal). The concordance rate for gene mutations in males and females with RIF is shown in Table 5. Thirty-nine couples from 62 couples showed MTHFR C677T homozygote without mutation. Other couples showed a mutation in MTHFR C677T. Furthermore, in MTHFR A1298C, 45 couples of 62 couples had homozygotes without mutation, and others showed mutation.

Table 2: Comparison of thrombophilia factors amongst women and men in recurrent implantation failure

	Female	Male	*P*
ANA	10.16±20.32	4.57±4	0.2
Homocysteine (μmol/L)	8.87±3.27	13.18	<0.001
Anticardiolipin IgG (pg/mL)	2.39±2.27	2.01±2.52	0.01
Anticardiolipin IgM (MPL/mL)	2.47±3.79	1.23±0.74	0.07
Antiphospholipid IgG (U/mL)	1.88±1.5	1.61±1.1	0.16
Antiphospholipid IgM (U/mL)	1.73±1.1	1.55±1.4	0.04
Beta-2 glycoprotein 1 IgG (EU/mL)	1.61±1.02	1.56±0.62	0.61
Beta-2 glycoprotein 1 IgM (EU/mL)	1.61±1.3	1.39±0.75	0.78

ANA=Antinuclear antibody, IgG=Immunoglobulin G, IgM=Immunoglobulin M

Discussion

The most important relevant finding was that the important combined parental thrombophilia defects in couples suffering RPL are gene polymorphisms MTHFR C677T, MTHFR A1298C, protein C mutation deficiency and homocysteine abnormality.

In the present study, MTHFR A1298C disorder was similar in males and females (16%). Toth *et al*.'s study

Table 3: The frequencies of thrombophilia factors in infertile couples with recurrent implantation failure

	Abnormal rate			*P*
	Women (62), *n* (%)	Men (62), *n* (%)	Total (124), *n* (%)	
ANA	5 (8.1)	-	5 (4)	0.02
Protein C	8 (12.9)	8 (12.9)	16 (12.9)	0.08
Protein S	3 (4.8)	-	3 (2.4)	0.99
Homocysteine	5 (8.1)	7 (27.4)	12 (9.7)	0.005
Anticardiolipin IgG	-	1 (1.6)	1 (0.8)	0.5
Anticardiolipin IgM	1 (1.6)	-	1 (0.8)	0.5
Antithrombin III	2 (3.2)	-	2 (1.6)	0.15
Antiphospholipid IgG	1 (1.6)	-	1 (0.8)	0.5
Antiphospholipid IgM	1 (1.6)	-	1 (0.8)	0.5
Lupus anticoagulant	1 (1.6)	-	2 (1.6)	0.99
Factor 5 Leiden (heterozygote)	1 (1.6)	-	1 (0.8)	0.99
Factor 5 Leiden (homozygote)	62 (100)	61 (98.4)	123 (99.2)	
Factor 7	4 (6.5)	4 (6.5)	8 (6.5)	0.99
Factor 8	11 (17.7)	6 (9.7)	17 (13.7)	0.19
Factor 9	4 (6.5)	1 (1.6)	5 (4)	0.17
Factor 11	2 (3.2)	6 (9.7)	8 (6.5)	0.14
Beta-2 glycoprotein 1 IgG	-	-	-	-
Beta-2 glycoprotein 1 IgM	-	-	-	-
MTHFR C677T*	16 (25.8)	16 (25.8)	32 (25.8)	0.99
MTHFR A1298C*	13 (21%)	7 (11.3)	20 (16.1)	0.22

*Abnormal factors consist of heterozygote with mutation and homozygote mutation. ANA=Antinuclear antibody, IgG=Immunoglobulin G, IgM=Immunoglobulin M, MTHFR=Methylenetetrahydrofolate reductase

in a case–control study revealed that the frequency of mutations in the partners of women with RPL was less than men in the control group.[16] De Galan-Roosen *et al*. showed that more than one thrombophilia abnormality was observed in 55% of couples with a history of perinatal death compared with the controls (17%). The result showed that the risk of developing thrombophilia in men whose wives experienced perinatal deaths was twice that of fathers whose wives had completed the pregnancy successfully.[17]

Single-nucleotide polymorphisms in genes encoding enzymes that regulate important metabolic pathways like MTHFR are considered one of the important factors in the formation of thrombophilia. MTHFR enzymes play a central role in the metabolism of folate, methionine and homocysteine. Homocysteine has proatherogenic and prothrombotic properties that lead to increased intimal thickness and vascular smooth muscle hypertrophy, platelet aggregation and clot formation. Foetal health is directly related to blood circulation. Factors that disrupt this relationship are harmful to the foetus. It seems that the formation of thrombi in the capillaries of the placenta disrupts the communication process between mother and foetus and ultimately leads to pregnancy loss.[18] Khaleghparast *et al*. concluded that neither of the two MTHFR polymorphisms C677T and A1298C could be associated with the RPL in the women studied,[19] while Bigdeli *et al*., Barut *et al*. and Chatzidimitriou *et al*. found that polymorphism of both is directly related.[10,20,21]

There are little published data on both parental thrombophilia disorders in Iran. Apparently, genetic predispositions in various ethnicities or races interfere in thromboembolism.[4,5] Iran has some similar ethnicity with Turkey. A study was conducted on 543 Turkish women with pregnancy loss and 327 of their male partners compared with a control group of 106 fertile couples. The authors resulted that RPL couples showed greater maternal and paternal thrombophilia gene mutations versus fertile couples.[12]

One unanticipated finding in this study was that abnormal homocysteine was greater in men than in women. This finding was also reported by Hoek *et al*. and Dai *et al*. who concluded that hyperhomocysteinemia leads to DNA damage to sperm and polymorphisms of related genes in homocysteine metabolism and results in pregnancy complication.[22,23] It is notable that many thrombophilia defects and variation in homocysteine concentration in women are related to geographic and cultural diversity.

Another notable finding of this study was that couples with RPL showed protein C mutation. Nahas *et al*. studied thrombotic factors in women with a history of RPL who were subsequently treated with antithrombotic medicines. Protein C and S mutation rates were 19% in these patients. Women who received enoxaparin had higher live birth rates, especially if they had a history of four or more miscarriages.[24] Our findings on protein C mutations are consistent with their observations. No protein S mutations were found in males, and it was <5% in females. Hansda *et al*. also revealed that protein S mutation was the most common defect seen in women with RPL; however, the association was not significant.[25] Further research is needed to determine whether protein is a risk factor for couples with RPL.

In the present study, only the women had heterozygous FVL thrombotic mutations, unlike their male partners.

Table 4: The frequencies of thrombophilia factors in one or both infertile couples with recurrent implantation failure

	Abnormal rate in both couples, *n* (%)	Abnormal rate in women with normal partner, *n* (%)	Abnormal rate in men with normal partner, *n* (%)
Protein C	1 (12.5)	7 (87.5)	7 (13)
Protein S	-	3 (100)	-
Homocysteine	2 (40)	3 (60)	5 (26.3)
Antithrombin III	-	2 (100)	-
Factor 5 Leiden	-	1 (100)	-
MTHFR C677T*	9 (56.2)	7 (43.8)	7 (15.2)
MTHFR A1298C*	3 (23.1)	10 (76.9)	4 (8.2)

*Abnormal factors consist of heterozygote with mutation and homozygote with mutation. MTHFR=Methylenetetrahydrofolate reductase

Table 5: Rate for methylenetetrahydrofolate reductase gene mutations in couples with recurrent implantation failure

Female	Male partners					
	MTHFR A1298C			MTHFR C677T		
	Heterozygote with mutation, *n* (%)	Homozygote without mutation, *n* (%)	Homozygote with mutation, *n* (%)	Heterozygote with mutation, *n* (%)	Homozygote without mutation, *n* (%)	Homozygote with mutation, *n* (%)
Heterozygote with mutation	7 (50)	5 (35.7)	2 (14.3)	3 (23.1)	10 (76.9)	-
Homozygote without mutation	5 (10.9)	39 (84.8)	2 (4.3)	3 (6.1)	45 (91.8)	1 (2)
Homozygote with mutation	-	2 (100)	-	-	-	-

In another study by Nassour-Mokhtari *et al*., a significant correlation was found between FVL in women with RPL in the second trimester,[26] while Abu-Asab *et al*. showed that FVL had no significant relationship with RPL, but it was higher in RPL patients.[27] The results of this study indicate that in couples with a history of miscarriage, men should be evaluated along with women for factors affecting RPL. The role of women remains a major factor; however, the factor studied is common to men and their women, and this could open a new window of opportunity in terms of how RPL can be treated considering both parents.

In this study, there were no couples with children or a history of RPL as controls for comparison of thrombotic factors. This is the main limitation of the present study. Furthermore, more than 25% of the couples were in a consanguinity marriage. This could influence the partners' genetic results. Another limitation is the small sample size of the study. However, with the above limitation, caution should be applied, as the findings may not be generalisable. Further research considering these weaknesses, will need to be undertaken. Notwithstanding the relatively limited sample, this work provides valuable insights into the presence of paternal thrombotic defects in RPL and raises many questions that require further investigation with a greater focus on male thromboembolic disorders. It is suggested to study the combined parental (and not just maternal) thrombolytic gene mutations in women with recurrent miscarriages.

Conclusions

The presence of combined thrombotic genetic defects in couples with repeated miscarriages needs more research on fathers as well.

Author's contributions

MK - Concept, design, definition of intellectual content, data acquisition and analysis, manuscript editing and review; ZB - Concept, design, manuscript preparation, and editing; FR - Data and statistical analysis; FG - Data acquisition and experimental studies; MG- Manuscript preparation. In addition, MK and MG have done literature search and all authors have critically reviewed the manuscript.

Acknowledgements

We are grateful to Dr. Molood Aghajani Delavar for assisting to design the manuscript.

Financial support and sponsorship

This study was funded by the Babol University of Medical Science.

Conflicts of interest

The authors declared no conflicts of interest. The Chancellor of Research and Technology of Babol University of Medical Science supported us financially.

Data availability statement

The data set used in the study is available with corresponding author.

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