Short Communication

Association of Inherited Thrombophilia with Recurrent Pregnancy Loss in A Population of Lebanese Women: A Case Control Study

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Abstract _____

Recurrent pregnancy loss (RPL) complication is a challenge of reproductive medicine due to its often unknown etiology. A case-control study was carried out between June 2019 and April 2020 to examine the correlation between RPL and inherited thrombophilia (IT), namely mutations in factor V Leiden (*FVL G1691A*), prothrombin (*FII G20210A*), and methylenetetrahydrofolate reductase (*MTHFR C677T*). A total of 120 Lebanese women with RPL was studied and compared, for the frequency of these mutations, to 100 healthy reproductive Lebanese women. The association between the zygosity status of the three tested mutations, the existence of more than one prothrombotic single nucleotide polymorphisms (SNPs), and the increased risk of RPL were examined using Chi-square or two-tailed fisher exact test, and the student t test. The predictive factors of RPL were analyzed using a multiple logistic regression model. P<0.05 was considered to be statistically significant. Our results showed statistically significant higher frequencies of *FVL G1691A* and *FII G20210A* mutations among the cases with RPL compared to the control group. Thus, RPL is associated with *FVL G1691A* and *FII G20210A* mutations. These mutations seem to increase the risk of RPL in the Lebanese women. Therefore, we suggest thrombophilia screening and adequate genetic counseling for women with RPL and at high-risk to plan for primary prevention, avoiding thromboembolic or obstetric accidents, and reducing the associated morbidity and mortality among Lebanese women.

Keywords: Abortion, Factor V Leiden (G1691A), Lebanon, MTHFR (C677T), Prothrombin (G20210A)

Recurrent pregnancy loss (RPL) is defined as the occurrence of two or more spontaneous pregnancy losses prior to 20 weeks of gestation. Approximately, 2 to 5% of women are affected by this clinical condition, which poses a challenging therapeutic dilemma in reproductive medicine (1). Even though the etiology of RPL is not clearly stated, anatomical abnormalities, autoimmune diseases, infections, genetic disorders, endocrine factors, and thrombophilia have been postulated as a possible root cause for RPL (2). Yet, more than 50% of the cases are classified as idiopathic RPL (3).

It was recently estimated that up to 50% of cases with RPL are due to thrombophilia (4). However, its implication in RPL varied between studies because of differences in the inclusion criteria and the ethnic origin of the subjects (5). Several studies have addressed the role of inherited thrombophilia (IT) as a risk factor for RPL (5, 6). The most common causes of IT include, factor V Leiden mutation (*FVL G1691A*), prothrombin gene mutation (*FII G20210A*), and homozygosity for the methylenetetrahydrofolate reductase deficiency (*MTHFR C677T*) (7).

In obstetrics, IT was shown to be a risk factor for maternal venous thromboembolism (TE) (8). Despite of the increasing number of studies that showed an association between RPL and IT, conflicting results exist (9). In addition, much uncertainty exists regarding the utility of thrombophilia testing in the routine investigation of RPL (3).

This study aimed to determine the frequency of *FVL G1691A*, *FII G20210A*, and *MTHFR C677T* mutations in a population of Lebanese women with RPL history and also, survey its correlation with RPL. Between June 2019 and April 2020, this case-control study was carried out in several Obstetrics and Gynecology clinics located in the nine governorates of Lebanon. The women with RPL; who experienced two or more pregnancy losses prior to 20 weeks of gestation participated in our case group (n=120). And a group of 100 healthy Lebanese women with no history of pregnancy loss and with at least 2 successful pregnancies made our control group in this study. Both cases and control subjects were Lebanese women. Women with anatomical abnormalities, vaginal infections, and systemic diseases were excluded from the case group, whereas women with

a history of pregnancy complications or miscarriage were excluded from the control group.

A standardized questionnaire was used to collect general data and to assess the medical history of all participants.

Ethical approval was obtained from the Ethics Committee of Beirut Arab University, Lebanon (IRB number: 2019H-0099-HS -R-0368). The procedures used in this study were in accordance with the ethical standards of Beirut Arab University institutional research committee. Written informed consent was obtained from all individual participants included in the study.

Three ml of venous blood was collected from each participant in Ethylene diamine tetra acetic acid tubes for DNA extraction. Genomic DNA was extracted using the Macherey-Nagel Nucleospin Blood kit (NucleoSpin blood; Macherey-Nagel GmbH & Co KG, (740951.50, Germany). Amplification reactions were performed using the MJ MiniTM Bio-Rad thermal cycler, according to the protocol described in the ThromboStrip- Opegen kit (3.117.016.53.000, Operon, Zaragoza, Spain). The following coagulation genes: *FVL G1691A, FII G20210A,* and *MTHFR C677T* were simultaneously amplified by polymerase chain reaction (PCR).

The detection of mutations was performed using the ThromboStrip- Opegen kit according to the manufacturer's instructions. Briefly, Hybridization of PCR products was performed at 42°C in a thermo-shaker adjusted to a speed of 450 rpm with a strip membrane bearing covalently-linked DNA probes that recognize each gene amplified by PCR. Following hybridization, several washes were done to eliminate nonspecific binding. The hybridization was then detected by incubating the membrane strip with a streptavidin-peroxidase conjugate, followed by the addition of peroxidase substrate (3,3′,5,5′-Tetramethylbenzidine or TMB). The probes for each gene, one for the normal sequence, one for the mutated sequence, and control probe lines of strip positioning, showed the pattern of each variant. Three possible results could be expected: no mutation, homozygous or heterozygous mutant.

Data were analyzed with a general linear model procedure of Statistical Package Software for Social Science (IBM SPSS, version 22.00, IBM Corp, Armonk, N.Y, USA). The Chi-square or 2-tailed Fisher exact test, and the student t-test were used to compare maternal characteristics and genotype frequencies between cases and controls. The predictive factors of RPL were analyzed using a multiple logistic regression model. P<0.05 was considered to be statistically significant.

A total of 220 study participants was assigned to two groups: cases (n=120) and controls (n=100). The mean age in both groups was 28.7 and 30.2 years, respectively. There were no significant differences by mean age, body mass index (BMI), smoking habits, parity, consanguinity, and family history of TE between these two groups (P>0.05, Table 1).

Hypertensive disorders and family history of TE were reported in both groups. However, individuals in the case group were more hypertensive in comparison with the control group (P=0.04, Table 1). Current medications undertaken by cases and controls have not been reported.

Table 1: General characteristics of our study participants

Characteristics	Cases (n=120)	Control (n=100)	P value
Age (Y)	28.7 ± 3.1	30.2 ± 2.7	0.97
BMI (kg/m²)	30.3 ± 2.1	28.9 ± 3.3	0.99
Smoking habits	48 (40)	33 (33)	0.28
Hypertension	19 (15.8)	7 (7)	0.04†
Parity	1.89 ± 1.7	3.1 ± 1.2	0.74
Consanguinity	13 (10.83)	7 (7)	0.32
Family history of TE	27 (22.5)	19 (19)	0.52

Data are presented as mean ± SD or n (%). Chi-square or two-tailed fisher's exact test, and the student t test were used. †; Statistically significant, BMI; Body mass index, SD; Standard deviation, and TE; Thromboembolism.

Higher frequencies of *FVL G1691A* and *FII G20210A* mutations were observed in the study cases in comparison with the control group (Table 2). In contrast, no significant difference was shown in the frequency of *MTHFR C677T* mutation between the two groups, respectively (66.66 vs. 62%, P=0.57).

Table 2: Prevalence of *FVL G1691A, FII G20210A*, and *MTHFR C677T* variants in cases with RPL and the control group

Variable	Cases (n=120)	Control (n=100)	P value
FVL G1691A mutation	25 (20.83)	9 (9)	0.01†
FII G20210A mutation	10 (8.33)	2 (2)	0.03†
MTHFR C677T mutation	80 (66.66)	63 (62)	0.57
>1 mutation	28 (23.33)	10 (10)	0.009†

Data are presented as n (%). Chi-square test was used. RPL; Recurrent pregnancy loss and †; Statistically significant.

The frequency of occurrence of more than one mutation in the same subject was significantly higher in the cases with RPL history compared to the control group, respectively (23.33% vs. 10%, P=0.009).

In addition, the frequency in heterozygous women for the FII (AG) mutation was significantly higher in the case group than the control group (6.66% vs. 1%, respectively), (P=0.03). In contrast, no statistical difference was observed between our groups in the *FVL* (AG) (14.16% vs. 8%, P=0.15) and *MTHFR* (CT) (56.66% vs. 57%, P=0.96) heterozygosity frequency, respectively (Table 3).

Moreover, the frequency of homozygotes (AA) was significantly higher in the cases with RPL than the control group (6.66% vs. 1%, P=0.03). However, no statistical difference was observed in the frequencies of homozygotes for the FII (AA) mutation (1.66% vs. 1%, P=0.67) and *MTHFR* (TT) mutation (10% vs. 5%, P=0.16) in the case and the control groups, respectively.

Multiple logistic regression was used to calculate the odds ratios (ORs) and to measure the predictive factors

of RPL. *FVL G1691A* and *FII G20210A* mutations seem to increase the risk of RPL by almost 3-fold and > 4-fold (OR: 2.70, 95% CI: 1.17 to 6.00; OR: 4.45, 95% CI: 0.95 to 20.82, respectively). The *MTHFR C677T* mutation was not associated with an increased risk for RPL (OR: 1.17, 95% CI: 0.67 to 2.04). Data are summarized in Table 4.

Table 3: Genotype distribution of *FVL G1691A*, *FII G20210A* and *MTHFR C677T* in women with RPL and the control group

Variable	Genotype	Cases (n=120)	Controls (n=100)	P value
FVL G1691A	GG	95 (79.16)	91 (91)	0.01[†]
	AA	8 (6.66)	1 (1)	0.03[†]
	AG	17 (14.16)	8 (8)	0.15
	Total mutation	25 (20.83)	9 (9)	0.01[†]
FII G20210A	GG	110 (91.66)	98 (98)	0.03[†]
	AA	2 (1.66)	1 (1)	0.67
	AG	8 (6.66)	1 (1)	0.03[†]
	Total mutation	10 (8.33)	2 (2)	0.03[†]
MTHFR C677T	CC	40 (33.33)	38 (38)	0.47
	TT	12 (10)	5 (5)	0.16
	CT	68 (56.66)	57 (57)	0.96
	Total mutation	80 (66.66)	62 (62)	0.47

RPL; Recurrent pregnancy loss and †; Statistically significant.

Table 4: Predictive factors of RPL in the multiple logistic regression analysis

Variable	Cases (n=120)	Control (n=100)	OR	95% CI
FVL G1691A mutation	25	9	2.70[†]	1.17-6.00[†]
FII G20210A mutation	10	2	4.45[†]	0.95-20.82[†]
MTHFR C677T mutation	80	63	1.17	0.67-2.04
> 1 mutation	28	10	2.73[†]	1.25-5.96[†]
Hypertension	19	7	2.49[†]	1.00-6.21[†]

RPL; Recurrent pregnancy loss, OR; Odds ratio, CI; Confidence interval, and †; Statistically significant.

In this study, a relatively high prevalence of *FVL G1691A, FII G20210A*, and *MTHFR C677T* variants has been observed in our groups, case and control, (20.83% vs. 9%, 8.33% vs. 2%, and 66.66% vs. 62%, respectively) which was in line with previous reports on the Lebanese population (10, 11). Similar results were seen in related ethnic populations such as Palestinian, Jordanian, Turkish, Syrian, Greek, and Greek-Cypriot, suggesting that eastern Mediterranean populations have a relatively high prevalence of these mutations (12-15).

Consistent with our results, the *FII G20210A* mutation was reported and identified as a risk factor for early RPL (16), and the *FVL G1691A* mutation as a common risk factor associated with early and late RPL (17, 18).

In addition, our results are supported by a meta-analysis whose findings show an increased risk of venous TE in pregnancy with *FVL G1691A* and *FII G20210A* carrier state (19).

Similarly, in Saudi Arabia, *FVL G1691A* and *FII G20210A* mutations were found to increase significantly the risk of RPL (20), which is in agreement with other findings in Iran and Turkey (17, 21). However, contradicting findings were reported in these same countries showing no correlation between the occurrence of RPL and mutations in *FVL* and *FII* (22, 23).

Moreover, regional and ethnic variations have been shown to affect the risk of RPL associated with *FVL G1691A* mutation. Indeed, a significant correlation has been found between *FVL G1691A* mutation and RPL in studies conducted in Asia, Africa, Europe, and the Middle-east, rather than Latin and North America (24). Our study supports this finding and identifies the *FVL G1691A* mutation as a risk factor for RPL in the Lebanese population.

Our case group showed a significant higher prevalence of heterozygous *FII* (AG) mutation in comparison with the control group, supporting Foka et al. (25) study that an increased frequency of *FII G20210A* was reported in women with RPL. Homozygous *FII* (AA) mutation was observed in our study cases with RPL and the control group at a prevalence of 1.66% and 1%, respectively. However, the difference was not statistically significant (P=0.67). Although, it is well established that the heterozygous and homozygous types of *FII G20210A* mutation predispose to a 3 to 8, and 18 to 80 times higher risk of thrombotic events, respectively (26), in our study homozygous *FII* (AA) mutation was not found to be a risk factor for RPL. This could be explained by the fact that RPL is a multifactorial condition, and one risk factor could not be enough for its occurrence.

Interestingly, when analyzing the frequency of women heterozygous for the *FVL* G1691A mutation in both groups, heterozygous *FVL* (AG) mutation alone, was not found to be a risk factor for RPL (P=0.1512). However, in contrast to our findings, a recent study conducted in Turkey, as well as other reports and meta-analyses, confirm that an increased risk of RPL was reported in women carriers of the *FVL G1691A* mutation (17, 18).

Our results suggest that homozygous *FVL* (AA) mutation could increase the risk of RPL, supporting previous study that showed an increased risk of developing venous TE during pregnancy with the *FVL G1691A* mutation, and largely when women bear the homozygous type of the mutation (8).

Assessing the prevalence of *MTHFR C677T* variant, it was not found to be a significant risk factor for RPL even in homozygosity pattern (P=0.57), that was in contrast to a study that showed homozygous women for the *MTHFR* (TT) mutation had a 2-3 fold-increased risk of early fetal loss in comparison to CC genotype women (27).

In addition, our study has shown an increased risk for RPL in women presenting more than one mutation, which was in agreement with previous findings that showed women with concurrent polymorphism for the three tested

mutations are at a greater risk for RPL in comparison with women with a single mutation (28).

In the present study, *FVL G1691A* and *FII G20210A* mutations were found to be associated with almost 3-fold and > 4-fold increased risk of RPL, respectively. However, the *MTHFR C677T* mutation was not associated with an increased risk for RPL. These data were in accordance with a previous report in which women with *FVL G1691A* or *FII G20210A* mutations, but not *MTHFR C677T* mutation had higher risks of developing RPL (24).

Surprisingly, our results are in contrast with a previous report on the Lebanese population, where no association has been found between adverse pregnancy outcomes and *FVL G1691A, FII G20210A*, and *MTHFR C677T* mutations (11). The described inconsistency could be due to differences in the type of obstetric complications, control selection, and inclusion and exclusion criteria. In addition, thrombophilia is a multifactorial disorder involving both genetic and environmental risk factors.

This gene-environment interplay could affect the pathogenesis of thrombophilia and could result in biased estimates even though confounding factors were controlled in our study. The influence of unknown confounders cannot be ruled out. This could be the most important limitation of our study, in addition to limited data collected from the study participants due to timing and convenience.

In our study, a statistically significant association has been found between RPL and mutations in *FVL* and *FII* in Lebanese women. However, even though an increasing number of studies have found such a correlation, yet, there is no consensus for genetic testing and counseling in the RPL cases. Altogether, our results could offer a strong argument in support of a change in current practices. Therefore, thrombophilia screening could be advocated for women at high risk of thrombotic episodes, allowing a better prognosis. Finally, early diagnosis of thrombophilia, genetic counseling, and gynecological monitoring could be of high benefit to prevent pregnancy complications in women with RPL and/or at high risk by proposing adequate therapeutic management and prophylaxis.

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Authors' Contributions

S.K., R.G.; Contributed to conception, design, and Drafted the manuscript. S.K.; Contributed to all experimental work, data, statistical analysis, and interpretation of data.

All authors read and approved the final manuscript.

References

1. ESHRE Guideline Group on RPL, Bender Atik R, Christiansen OB, Elson J, Kolte AM, Lewis S, Middeldorp S, et al. ESHRE guideline: recurrent pregnancy loss. Hum Reprod Open. 2018; (2): hoy004.
2. Patil R, Ghosh K, Vora S, Shetty S. Inherited and acquired thrombophilia in Indian women experiencing unexplained recurrent pregnancy loss. Blood Cells Mol Dis. 2015; 55(3): 200-205.
3. Kaiser J, Branch DW. Recurrent pregnancy loss: generally accepted causes and their management. Clin Obstet Gynecol. 2016; 59(3): 464-473.
4. Neamatzadeh H, Ramazani V, Kalantar SM, Ebrahimi M, Sheikhha MH. Serum immune reactivity against β2-Glycoprotein-I and anti-neutrophil cytoplasmic auto-antibodies by ELI-P-Complex Screening Technology in recurrent miscarriage. Minerva Ginecol. 2016; 68(3): 243-249.
5. López-Jiménez JJ, Porras-Dorantes Á, Juárez-Vázquez CI, García-Ortiz JE, Fuentes-Chávez CA, Lara-Navarro IJ, et al. Molecular thrombophilic profile in Mexican patients with idiopathic recurrent pregnancy loss. Genet Mol Res. 2016; 15(4): 15048728.
6. Pritchard AM, Hendrix PW, Paidas MJ. Hereditary thrombophilia and Rrecurrent pregnancy loss. Clin Obstet Gynecol. 2016; 59(3): 487-497.
7. Dautaj A, Krasi G, Bushati V, Precone V, Gheza M, Fioretti F, et al. Hereditary thrombophilia. Acta Biomed. 2019; 90(10-S): 44-46.
8. Croles FN, Nasserinejad K, Duvekot JJ, Kruip MJ, Meijer K, Leebeek FW. Pregnancy, thrombophilia, and the risk of a first venous thrombosis: systematic review and bayesian meta-analysis. BMJ. 2017; 359: j4452.
9. Lino FL, Traina É, Barreto JA, Moron AF, Mattar R. Thrombophilic mutations and polymorphisms, alone or in combination, and recurrent spontaneous abortion. Clin Appl Thromb Hemost. 2015; 21(4): 365-372.
10. Almawi WY, Finan RR, Tamim H, Daccache JL, Irani-Hakime N. Differences in the frequency of the C677T mutation in the methylenetetrahydrofolate reductase (MTHFR) gene among the Lebanese population. Am J Hematol. 2004; 76(1): 85-87.
11. Zahed LF, Rayes RF, Mahfouz RA, Taher AT, Maarouf HH, Nassar AH. Prevalence of factor V Leiden, prothrombin and methylene tetrahydrofolate reductase mutations in women with adverse pregnancy outcomes in Lebanon. Am J Obstet Gynecol. 2006; 195(4): 1114-1118.
12. Jarjour RA, Ammar S, Majdalawi R. Frequency of three prothrombotic polymorphisms among Syrian population: factor V G1691A, prothrombin G20210A and methylenetetrahydrofolate reductase C677T. Ann Hum Biol. 2017; 44(1): 70-73.
13. Attili R, Hussein A, Odeh H, Hejaz H. Prevalence of thrombophilia in palestine and the association of thrombophilic gene polymorphisms with recurrent pregnancy loss. Res J Obstet Gynecol. 2019; 12: 6-10.
14. Al-Zoubi N, Alrabadi N, Kheirallah K, Alqudah A. Prevalence and multiplicity of thrombophilia genetic polymorphisms of FV, MTHFR, FII, and PAI-I: A cross-sectional study on a healthy Jordanian population. Int J Gen Med. 2021; 14: 5323-5332.
15. Ekim M, Ekim H, Yılmaz YK. The prevalence of Factor V Leiden, prothrombin G20210A, MTHFR C677T and MTHFR A1298C mutations in healthy Turkish population. Hippokratia. 2015; 19(4): 309-313.
16. Gao H, Tao FB. Prothrombin G20210A mutation is associated with recurrent pregnancy loss: a systematic review and meta-analysis update. Thromb Res. 2015; 135(2): 339-346.
17. Barut MU, Bozkurt M, Kahraman M, Yildirim E, Imirzalioglu N, Kubar A, et al. Thrombophilia and recurrent pregnancy loss: The enigma continues. Med Sci Monit. 2018; 24: 4288-4294.
18. Sergi C, Al Jishi T, Walker M. Factor V Leiden mutation in women with early recurrent pregnancy loss: a meta-analysis and systematic review of the causal association. Arch Gynecol Obstet. 2015; 291(3): 671-679.
19. Ziakas PD, Poulou LS, Pavlou M, Zintzaras E. Thrombophilia and venous thromboembolism in pregnancy: a meta-analysis of genetic risk. Eur J Obstet Gynecol Reprod Biol. 2015; 191: 106-111.
20. Turki RF, Assidi M, Banni HA, Zahed HA, Karim S, Schulten HJ, et al. Associations of recurrent miscarriages with chromosomal abnormalities, thrombophilia allelic polymorphisms and/or consanguinity in Saudi Arabia. BMC Med Genet. 2016; 17 Suppl 1:69.

21. Kamali M, Hantoushzadeh S, Borna S, Neamatzadeh H, Mazaheri M, Noori-Shadkam M, et al. Association between thrombophilic genes Ppolymorphisms and recurrent pregnancy loss susceptibility in the iranian population: a systematic review and meta-analysis. Iran Biomed J. 2018; 22(2): 78-89.

22. Yengel I, Yorulmaz T, Api M. Association between FVL G1691A, FII G20210A, and MTHFR C677T and A1298C polymorphisms and Turkish women with recurrent pregnancy loss. Med Glas (Zenica). 2020; 17(1): 129-135.

23. Ahangari N, Doosti M, Mousavifar N, Attaran M, Shahrokhzadeh S, Memarpour S, et al. Hereditary thrombophilia genetic variants in recurrent pregnancy loss. Arch Gynecol Obstet. 2019; 300(3): 777-782.

24. Liu X, Chen Y, Ye C, Xing D, Wu R, Li F, et al. Hereditary thrombophilia and recurrent pregnancy loss: a systematic review and meta-analysis. Hum Reprod. 2021; 36(5): 1213-1229.

25. Foka ZJ, Lambropoulos AF, Saravelos H, Karas GB, Karavida A, Agorastos T, et al. Factor V leiden and prothrombin G20210A mutations, but not methylenetetrahydrofolate reductase C677T, are associated with recurrent miscarriages. Hum Reprod. 2000; 15(2): 458-462.

26. Chatzidimitriou M, Chatzidimitriou D, Mavridou M, Anetakis C, Chatzopoulou F, Lialiaris T, et al. Thrombophilic gene polymorphisms and recurrent pregnancy loss in Greek women. Int J Lab Hematol. 2017; 39(6): 590-595.

27. Tiwari D, Bose PD, Das S, Das CR, Datta R, Bose S. MTHFR (C677T) polymorphism and PR (PROGINS) mutation as genetic factors for preterm delivery, fetal death and low birth weight: A Northeast Indian population based study. Meta Gene. 2015; 3: 31-42.

28. Akdemir Y, Ayvaci H, Uludogan M. Effect of multiple thrombophilic gene mutations on uterine artery blood flow in nonpregnant recurrent pregnancy loss patients: are we searching enough? J Matern Fetal Neonatal Med. 2020; 33(14): 2466-2472.