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Thrombophilic Gene Mutation and Correlation to Recurrent Miscarriage: An Egyptian Study

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Abstract

Background: The most common complication in pregnancy is spontaneous abortion. Recurrent pregnancy loss (RPL) is three or more consecutive pregnancy loss before the 25 th week of gestation. Genetical, environmental, viral, endocrinal, hematological and anatomical reasons are assumed to have a role. In spite of the continuous advancement in discovering the major reasons for fetal miscarriage, it is still one of the most important medical challenges. Some researchers claim that thrombophilia plays a big role in placenta related problems, intrauterine complications, disruption in fetal circulation and miscarriage. Pregnancy is a hyper-coagulable state, so any disruption or increase in thrombotic factors will result in maternal deep venous thrombosis on one hand, and clot formation, as well as disruption of the fetal circulation on the other hand, hence miscarriage. Discovering important correlations between coagulation factors and RPL may, in turn, decrease the rate of miscarriage via the use of antithrombotic measures.

Aim of Study: Is to determine the association between factor five (FV), factor two (FII), Methylenetetrahydrofolate reductase (MTHFR) 677, (MTHFR) 1298, Methionine synthase (MTR) 2756 and Methionine synthase reductase (MTRR) 66 polymorphisms and the unexplained RPL among Egyptian women in their child bearing period.

Patients and Methods: In this research, fifty women of reproductive age (19-45 years) with history of at least 3 unexplained miscarriages until the 24 weeks of gestation were included as cases, while 50 age-matched females as controls. Screening coagulation factors are tested for pregnant women with recurrent abortion such as Protein C, Protein S, the IgG & IgM for both ACLA and Beta-1-Glycoprotein, antithrombin III and lupus anticoagulant. This research studied the most common thrombophilic genes such as FII (1691), FV (20210), MTHFR (677 & 1298), MTR 2756 and MTRR 66 and were analyzed for heterogenicity or homogeneity to determine their role if any in miscarriage.

Results: Abnormalities of screening coagulation factors such as Anticardiolipin antibody (ACLA) (IgG & IgM), Beta-1-Glycoprotein (IgG & IgM), antithrombin (AT) III, lupus anticoagulant, protein C (PC) and protein S (PS) deficiencies when determined, they showed no statistical significance. Similarly, polymorphisms of FII, FV and MTHFR 677 showed no significance difference. In contrast, other thrombophilic gene mutationssuch as MTR 2756, MTHFR 1298 & MTRR 66 showed significant difference. MTR 2756 polymorphism revealed significant difference for heterozygous G/A with p-value 0.005 [Odds Raio (OR) 4.0, 95%CI 1.5-10.4]. In comparison MTHFR 1298 as well as MTRR 66 showed high significant difference with p-value less than 0.001, with G allele increasing the risk for miscarriage. For instance, MTHFR 1298, combined G/A and G/G genotypes increases risk for RPL (OR7.7, 95%CI 2.8-20.8 and OR 16.0 95%CI 3.2-80.1 respectively). Similarly, MTRR 66 Odds ratio revealed that G allele increases risk (G/A OR 26.1, 95%CI 7.0-96.8 and G/G OR 57.0 95%CI 13.0-249.7). Therefore, MTHFR 1298 & MTRR 66 revealed the highest significant difference with percentage of polymorphisms among cases 70% and 92%, respectively. Thus, one can conclude that G allele of those genes could be considered as susceptibility marker of RPL in Egyptian women.

Key Words: *Thrombophilia – Recurrent pregnancy loss – MTHFR 677 mutation – MTHFR 1298 mutation – MTR 2756 mutation – MTRR 66 mutation – Antithrombotic measures – Screening coagulation factors – Egyptian women – Polymorphism.*

Introduction

RPL is a very common medical problem that affects 3 to 5% of pregnant women, the real cause, however, is still unknown for almost 50% of miscarriage [1]. Viral, genetical, anatomical, endocrinal, hematological are considered causes for RPL [2]. Since pregnancy is a hyper-coagulable state, thrombophilic gene mutation may play a great role in placental complications such as intrauterine fetal death, disruption of vascular supply, intrauterine fetal growth restriction, hence disruption in fetal circulation and eventually miscarriage [3]. Disruption in feto-maternal circulation whether inherited or acquired may have a consequential role on pregnancy loss. Literature is still in conflict about the main etiology of RPL. In the last decades, researchers studied the most common thrombophilic genes and their being heterogenic or homogenic

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to investigate their role in miscarriage. However, while some scientists supported the presence of strong correlation between miscarriage and thrombophilic gene polymorphisms, others denied this association and considered it insignificant **[2,4]**. The most common mutations are factor five, factor two and homozygosity of C677T mutation in the MTHFR gene with 2-15%, 2-3%, and 1 1 %, respectively **[4-10]**. More mutations are studied such as MTHFR 1298, MTRR 66 and MTR 2576 and their correlation with RPL is still debatable.

Patients and Methods

Patients and controls:

This case-control study was conducted in the Obstetrics and Gynecology and Clinical Pathology Departments; Faculty of Medicine, Cairo University, from January 2021 to July 2022. For every patient included in the study, an informed consent was obtained before data collection and after explanation of the study objectives. In this research, fifty women of reproductive age (19-45 years) with history of at least 3 unexplained miscarriages until the 24 weeks of gestation were included as cases, while 50 age-matched females as controls. The most common proteins that can play role in coagulation are tested for women with recurrent abortion such as Protein C&S, the IgG & IgM for both ACLA and Beta- 1 -Glycoprotein, antithrombin III and lupus anticoagulant. As mentioned earlier, since researchers have declared that thrombophilic gene polymorphisms may be a cause for miscarriage, this research studied the most common thrombophilic genes such as FII (1691), FV (202 10), MTHFR (677 & 1298) and MTR 275 6 & MTRR 66 and were analyzed for heterogenicity or homogeneity for cases with recurrent pregnancy loss.

Controls are those with at least one live-born children and no personal history of pregnancy loss. Those excluded are thehypertensive patients or thosetaking any anticoagulant therapy whether pregnant or diabetic woman. Data were collected directly by the researcher for each participant with regard to age, occupancy, medication, chronic illness and obstetric history.

Laboratory evaluation:

5.0mL vacuette blood collection tubes; serum sterile tubes, tubes withanticoagulant to avoid any hindering of PCR, for example, salt of ethylenediaminetetraacetate (EDTA) at a final concentration of 2.0mg/mL and sodium citrate anticoagulant tubes. In order to obtain platelet free plasma, the sample was centrifuged twice at 2500g for 15min at room temperature then frozen and stored in small aliquots at 20°C until tested. EDTA blood was immediately stored at 40°C.

Women in both the control and study groups underwent the below-mentioned tests: ACLA (IgG & IgM), Beta- 1 -Glycoprotein (IgG & IgM), antithrombin III, lupus anticoagulant, Protein C&S. Furthermore, Real-Time PCR (DNA Technology) was conducted to detect any mutation for FV, FII, MTHFR 677, MTHFR 1298, MTR 2756, MTRR 66. Extraction for the target DNA template followed by its amplification is performed. The PCR mix includes two different labelled allele-specific probes having reporter fluorescent dyes (Fam and Hex) for each variant of polymorphism. Each specific segment has its own specific melting temperature as demonstrated in (Error! Reference source not found) and (Fig. 1), hence its fluorescence intensity. This is measured by Real time PCR thermal cycler which is analyzed graphically by a specific software.

Table (1): Genotypes and melting temperatures (only for DTlite, DTprime instruments) **[11]**.

Polymorphism	Genotyping	FAM	HEX
FV: 1691 G>A	GG	54.4	48.7
FII: 20210 G>A	GG	59.5	47.0
MTHFR 677 C>T	CC	56.3	46.0
MTHFR 1298 A>C	AA	55.9	48.5
MTR 2756 A>G	AA	54.1	46.3
MTRR 66 A>G	AA	51.8	41.0

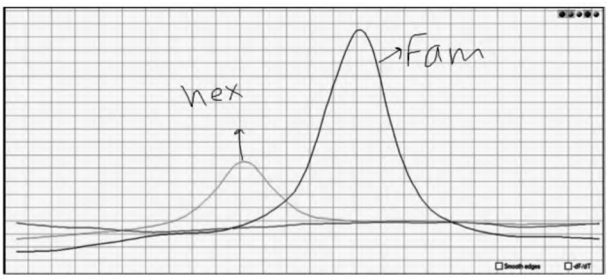

Fig. (1): Example of human genomic DNA amplification (Folate Metabolism Real-Time PCR Genotyping Kit, DNA Technology) **[11]**.

Statistical analysis:

The statistical package for the Social Sciences (SPSS) version 28 (IBM Corp., Armonk, NY, USA) was used to code and enter data. Frequencies (number of cases) and relative frequencies (percentages) were used to summarize data. Logistic regression was used to compare genotype frequenciesbetween the disease and the control groups. Odds ratio (OR) with 95% confidence intervals was calculated **[12]**. p-values less than 0.05 were considered as statistically significant and <0.001 highly statistically significant.

Results

Screening coagulation factors conducted for all women suffering from recurrent miscarriage showed no significant differences. To illustrate, the relative frequency of abnormalities for Protein C&S, ACLA (IgG & IgM), Beta- 1-Glycoprotein (IgG & IgM) or AT (III) results are represented in Table (2) as well as Table (3) and are demonstrated in Figs. (2,3). Frequency of abnormality in Protein C and lupus anticoagulant is negligible almost (0%) while Protein S and ATIII (1.8%) as shown in Table (2). Although, ACLA and Beta-1-Glycoprotein show relatively more abnormality, in other words, the former is 0% while the latter revealed around 4% and 5% for the IgG and IgM respectively as demonstrated in Fig. (3) and Table (3). No abnormalities are noticed in the control with regards to these parameters.

Table (2): Screening tests results (Protein C, Protein S, Anti-thrombin III and Lupus Anticoagulant) in the case group.

	Protein C	Protein S	Antithrombin III	Lupus Anticoagulant
Below Normal	0	1.8	1.8	0
Normal	96.4	89.1	81.8	100
Above Normal	3.6	9.1	16.4	0

Table (3): Screening tests results [ACLA (IgM and IgG) in the case Beta-1-Glycoprotein (IgM and IgG)].

	ACLA IgG	ACLA IgM	Beta-1-Glycoprotein IgG	Beta-1-Glycoprotein IgM
Negative	100	93	96	89
Equivocal	0	7	3	7
Positive	0	0	3	4

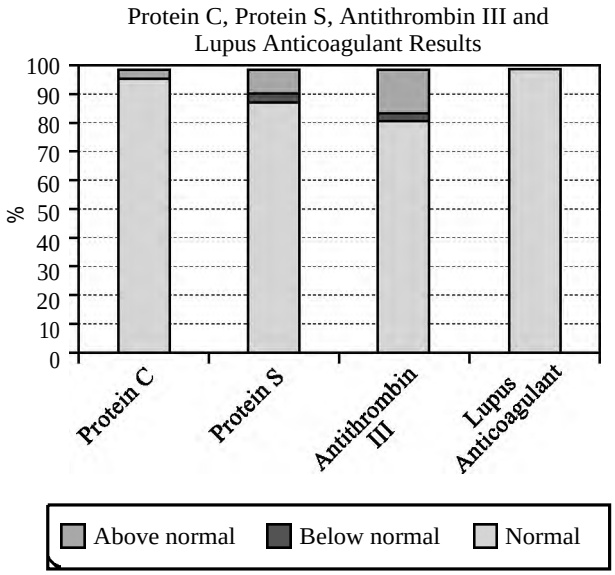

Fig. (2): Screening tests results (Protein C, Protein S, Anti-thrombin III and Lupus Anticoagulant) in the case group.

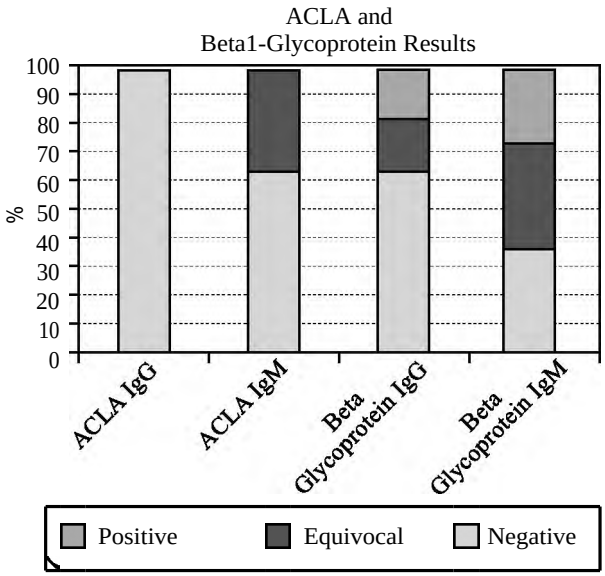

Fig. (3): Screening tests results [ACLA (IgM and IgG) in the case Beta- 1-Glycoprotein (IgM and IgG)].

Regarding gene mutations, the following results are demonstrated in Table (4). To start with, both FII and FVpolymorphismsare only present in 2 cases in our sample (4%). Similarly, MTHFR677 polymorphisms showed no significant difference, p-value 0.4, as per Fig. (4). In comparison a higher percentage is noticed for MTR2756 A/G almost 40% with p-value 0.005 (OR 4.0, 95%CI 1.53-10.45), however, the mutant showed no statistical significance as per Table (5). Finally, the highest polymorphisms were noticed with high significant difference (p-value less than 0.001) for MTHFR 1298 as well as MTRR 66having high mutation frequency (70%) and (92%) respectively as per Table (4) and Fig. (4). MTHFR 1298, combined G/A and G/G genotypes revealed high risk (OR 7.7, 95%CI 2.8-20.8 and OR 16.0 95%CI 3.2-80.1 respectively). Similarly, MTRR 66 Odds ratio demonstrated an increased risk of the G allele (G/A OR 26.1, 95%CI 7.0-96.8 and G/G OR 57.0 95%CI 13.0-249.7), as per Table (5).

Table (4): Distribution of gene mutations in women with RPL.

	FV	FII	MTHFR 677	MTHFR 1298	MTR 2756	MTRR 66
Normal %	96	96	70	26	50	8
Homozygous%	0	0	8	28	10	48
Heterozygous%	4	4	22	46	40	44

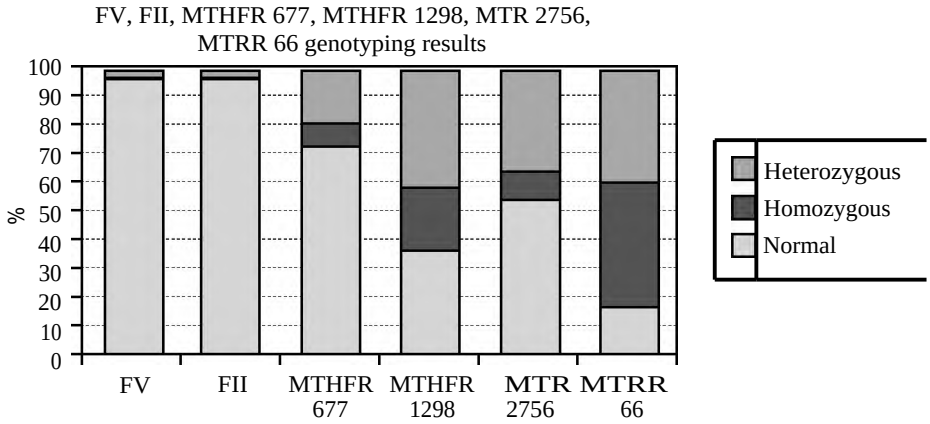

Fig. (4): Distribution of gene mutations in women with RPL.

Table (5): Statistical Analysis and comparison between MTHFR 677, MTHFR 1298, MTR 2756, MTRR 66 genotyping.

	Cases		Control		*p*-value	OR	95% CI	
	Count	%	Count	%			Lower	Upper
MTHFR 677:								
Homozygous	4	8.0	2	4.0	0.356	2.286	0.394	13.245
Heterozygous	11	22.0	8	16.0	0.384	1.571	0.568	4.347
Normal	35	70.0	40	80.0		Reference		
MTHFR 1298								
Homozygous	12	24.0	2	4.0	0.001	16.000	3.197	80.067
Heterozygous	23	46.0	8	16.0	<0.001	7.667	2.821	20.833
Normal	15	30.0	40	80.0		Reference		
MTR 2756:								
Homozygous	5	10.0	2	4.0	0.113	4.000	0.720	22.210
Heterozygous	20	40.0	8	16.0	0.005	4.000	1.531	10.449
Normal	25	50.0	40	80.0		Reference		
MTRR 66:								
Homozygous	24	48.0	4	8.0	<0.001	57.000	13.013	249.678
Heterozygous	22	44.0	8	16.0	<0.001	26.125	7.049	96.829
Normal	4	8.0	38	76.0		Reference		

Discussion

In spite of studying the impact of the most common thrombophilic mutations in fetal loss in different populations, their main role is still debatable. Hence, it is important to find out the correlation between these genes' polymorphisms and RPL in Egyptian women. A number of thrombophilic-factors were determined such as Protein C, Protein S, Antithrombin III, Beta- 1-Glycoprotein & ACLA on one hand, and mutations in thrombophilic genes on the other hand.

In our research, thrombophilic factors did not show statistically significant abnormality. To illustrate, the frequency of abnormalities ranges from 0% to 5.5% with the highest noticedwith the Beta-1-Glycoprotein. With respective to Protein C and S, Gris et al. and Parand et al., found that the latter deficiency is more frequently noticed than the former in association with pregnancy loss **[13,14]**, which is in agreement with ourpresented results, as per Fig. (2). In agreement to our research, Mekaj et al., reported that the highest abnormality reached, while screening hematological coagulation factors

abnormalities among RPL, was only 5.77% of the total number of cases in his research [15]. In contrast, an Iranian study showed abnormality ranging from 30.5% to 8.5% for Protein C and for lupus anticoagulant (42.7%) [16]. Such discrepancy in results might be due to geographical or ethnic variation, however it is still debatable if these factors havea consequential role in miscarriage.

Prothrombin level is considered a high risk of thrombosis and has been considered as a potential risk for pregnancy loss by many researchers [17]. Factor V is a common thrombotic risk as it plays a role in activated protein C resistance. Although, itis associated with inherited mild-activated protein C resistance, its association with recurrent pregnancy loss is still debatable [4,5,9,18-20]. In the present study, it was found that the frequency of polymorphisms for both FII and FV is 1.8% of the control and 3.6% of cases with heterozygous form and none was found in the homozygous form. Almost the same finding was observed in a study conductedon a larger scale of 4167 cases where only 3.8% of the women tested showed FII mutation, and their abortion rate was similar to those with normal genes. Hence, in concordance with our research, Silver et al., concluded that there was no association between FII and abortion [21].

Since polymorphism may be affected by regional and ethnic variation as mentioned earlier, we started comparing our results to previousresearch in the middle east. For example, in Algeria, Nassour-Mokhtari et al., noticed that both FII and FV were detected in almost 8.33% of patients, while in the control sample, the former frequency was 11.11%, the latter showed no abnormality [22]. However, in Tunisia, Mahjoub et al., reported that FII polymorphism (0.0100 vs. 0.0225) was less than that of FV (0.1400 vs. 0.0276) in cases than controls, respectively [23]. In Saudi Arabia, another research showed that FII and FV homozygous and heterozygous mutations were statistically significant suggesting an association for RPL [24,25]. Therefore, such variation and disagreements may be due to geographical differences as mentioned earlier. In agreement, Liatsikos et al., noticed that FII and FV mutations are lowest in Africa (0-0.3%) and (0-0.6%) and highest in Southern Europe (3%) and (7%) respectively. Hence, geographical allocation may have arole in mutation variation [26].

Furthermore, the research investigated more correlations with other thrombophilic mutations, such as the methylenetetrahydrofolate reductase (MTHFR 677T and MTHFR 1298T). Many researchers declare that MTHFR 677C/T mutation might result in drop of MTHFR activity [20,27-29]. In the presented work, MTHFR 677 didnot show statistical significant difference with p-value 0.4 In concordance to our finding, Dell'Edera et al., reported absence of correlations between pregnancy loss and C677T [30]. In contrast, Cao et al. and Govindaiah et al., denoted an association between MTHFR C677T and the unexplained recurrent pregnancy loss [31,32]. Nevertheless, such variation may be attributed to geographical and ethnic changes.

On the other hand, MTHFR 1298T revealed high significant difference with p-value less than 0.001 denoting that combined G/A (46% of cases) and G/G (24% of cases) genotypes increase risk for RPL (OR 7.7, 95%CI 2.8-20.8 and OR 16.0 95%CI 3.2-80.1, respectively). Similarly, in another research by Mosin et al., the authors declared the presence of MTHFR1298 mutations inheterozygous state (30.7%) and homozygous state (15.3%), though the percentages are relatively lower than our research, still a correlation is denoted similar to ours [33].

Furthermore, MTR 2756 and MTRR 66 were studied to evaluate their impact in pregnancy loss. To illustrate, mutations in these genes may lead to elevated plasma homocysteine level which in turn may cause fetal loss or fetal abnormalities and sometimes associated with breast cancer [34-37]. There are a number of studies that demonstrate a strong correlation between MTRR and miscarriage [38-40]. MTR 2756 polymorphism showed a frequency of 50% of cases with G/A 40% and A/A 10%, hence revealing a significant difference for heterozygous G/A only with p-value 0.005 (OR 4.0, 95%CI 1.531-10.449) but homozygous was statistically insignificant with p-value 0.113. In contrast, MTRR 66 showed high significant difference p-value <0.001. In our research, which is in concordance with Talwar et al., the genotypic frequency distribution of MTRR 66 genetic polymorphism was obviously noticeable among cases rather than control [41]. In our research, relative frequency of MTRR 66 polymorphism was 92% (GG 48% & AG 44%) of the cases, which is remarkably high. As a consequence, MTRR66 polymorphisms revealed high significant difference denoting that G allele increases risk of RPL(G/A OR 26.13, 95%CI 7.0-96.8 and G/G OR 57.0 95%CI 13.0-249.7. Therefore, while the role of thrombophilic mutation is still controversial, our research reported that the highest frequency was noticed for MTHFR 1298 & MTRR 66 at 70% and 92% respectively with a p-value less than 0.001 denoting an association with miscarriage.

Conclusion:

This Egyptian study examines the implication ofa number of thrombotic coagulation factors as well asinherited thrombophilic gene mutationson the occurrence of RPL. Screening tests for abnormalities via analyzing the thrombotic factors didnot show any significant difference from the control group. For example, the percentage of abnormality ranged from 0% to 5.5% with Beta- 1 -Glycoprotein showing the maximum discrepancy from normal. Based on literature, screening tests' role in miscarriage is debatable. However, thrombophilic gene polymorphisms results are worth consideration. To start with, FV, FII and MTHFR 677 showed very low percentage of abnormalities among cases and their p-value revealed no significant difference. MTR2756 heterozygous mutations showed significant difference with p-value (0.005) while the homozygous was insignificant. On the other hand, studying MTHFR1298 and MTR66 polymorphisms revealed high statistical significance with p-value less than 0.001 reporting high mutation frequency 70% and 92% respectively. Thus, one can conclude that G allele of MTHFR 1298 and MTRR 66 could be considered as susceptibility marker of RPL in Egyptian women. Nevertheless, this study carries a number of limitations, as all cases were conducted in one lab and on one ethnic group (the Egyptian women). In addition, due to financial constraints, the study was restricted to a relatively small sample, yet our results are still in concordance with many previous research. Further studiesare needed to investigate the role of anticoagulant measures as a prophylactic or therapeutic measure for pregnant women with different doses and at different stages of pregnancyto reach optimal management. This study should be extended to include more ethnic groups, larger sample and women from different countries.

Conflict of Interest:

Authors declare no conflict of interest.

Author contributions:

- Conceptualization and study design: Mervat El Ansary, Irene Bishai.
- Methodology and technique: Mervat El Ansary, Rasha Sayed, Irene Bishai.
- Acquisition, analysis, and interpretation of the data: Mervat El Ansary, Mariam A. M. Dawood, Rasha Sayed, Hend Tamim, Irene Bishai.
- Writing - original draft preparation: Irene Bishai.
- Writing - review and editing: Mervat El Ansary, Mariam A. M. Dawood, Rasha Sayed, Irene Bishai.
- Supervision: Mervat El Ansary.

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طفرة الجينات الخثارية وارتباطها بالإجهاض المتكرر: دراسة مصرية

المضاعفات الأكثر شيوعاً فى الحمل هى الإجهاض التلقائى. فقدان الحمل المتكرر (RPL) هو ثلاثة أو أكثر من حالات فقدان الحمل المتتالية قبل الأسبوع الخامس والعشرين من الحمل. من المفترض أن يكون للأسباب الوراثية والبيئية والفيروسية والغدد الصماء والدموية والتشريحية دور. على الرغم من التقدم المستمر فى إكتشاف الأسباب الرئيسية لإجهاض الجنين إلا أنه لا يزال من أهم التحديات الطبية. يدعى بعض الباحثين أن أهبة التخثر تلعب دوراً كبيراً فى المشكلات المتعلقة بالمشيمة والمضاعفات داخل الرحم واضطراب الدورة الد موية للجنين والإجهاض. الحمل حالة شديدة التجلط لذا فإن أى اضطراب أو زيادة فى عوامل التخثر سيؤدى إلى تجلط وريدى عميق للأم من جهة وتشكيل جلطة بالإضافة إلى اضطراب الدورة الدموية للجنين من جهة أخرى ومن ثم الإجهاض. قد يؤدى إكتشاف الارتباطات المهمة بين عوامل التخثر و RPL، بدوره، إلى تقليل معدل الإجهاض عن طريق استخدام التدابير المضادة للتخثر.

الهدف الرئيسى من هذه الدراسة هو تحديد الارتباط بين العامل الخامس (FV) و العامل 2 (FII) وMethylenetetrahydrofolate reductase (MTHFR) 677 و MTHFR 1289 و Methionine synthase (MTR) 2756 وMethionine synthase reductase (MTRR) 66 تعدد الأشكال و RPL غير المبررة بين النساء المصريات فى فترة الإنجاب. شذوذ عوامل تجلط الدم مثل مضادات الكارديوليبين (ACLC) IgG (IgM)، بيتا 1 بروتين سكرى (IgM & IgG)، مضاد الثرومبين III (AT)، الذئبة المضادة للتخثر، البروتين (CPC) ونقص البروتين S (PS) عند تحديده لم يظهروا أى أهمية. أشار هذا البحث إلى أنه بينما أظهرت الأشكال المتعددة للعامل FII و3.6 FV%، لوحظ فرق معنوى أعلى مع تعدد الأشكال فى MTHFR 677 و MTR 2756 27.2% و 45.5% على التوالى. تم الإبلاغ عن أعلى طفرة لـ MTHFR 1298 و MTRR66 بنسبة 63.6% و83.6% على التوالى. لذلك قد تترافق الأشكال المتعددة الأخيرة مع RPL بين النساء المصريات ولكن هناك حاجة لمزيد من الدراسات وعينة أكبر لتعميم مثل هذا الارتباط مع الإجهاض.