Birzeit University

Master's Program in Clinical Laboratory Science

# Genetic and Acquired Risk Factors of Thrombophilia among Palestinian Women with Recurrent Pregnancy Loss

By

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العوامل الوراثية والمكتسبة المؤثرة على تخثر الدم وعلاقتها
بخسارة الحمل المتكرر عند النساء الفلسطينيات

By

Laura Batmani

This thesis was successfully defended and approved on June 2nd, 2015

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*I proudly dedicate this thesis to my supportive parents Bassam and Hiyam Batmani, without whom none of my success would be possible.*

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# List of Abbreviations

- **RPL** Recurrent Pregnancy Loss
- **FVL** Factor V Leiden
- **FII** Factor II
- **MTHFR** Methyl-TetraHydroFolate Reductase
- **APS** Antiphospholipid Syndrome
- **LA** Lupus Anticoagulants
- **ACA** Anti-Cardiolipin Antibodies
- **AGPA** Anti $\beta_2$-Glycoprotein I Antibodies
- **TF** Tissue Factor
- **AT** AntiThrombin
- **APC** Activated Protein C
- **PS** Protein S
- **APCR** Activated Protein C Resistance
- **FVa** Factor V activated
- **Arg** Arginine
- **Gln** Glutamine

# Abstract

Recurrent pregnancy loss (RPL) is a distressing experience that affects 1% of pregnancy cases. The etiology of RPL is not well characterized but thrombophilia is a major factor. There are genetic as well as acquired factors that may be involved in RPL. This study was conducted on 50 cases with RPL and 50 controls. Samples were collected between May and December 2014. The genetic factors that were evaluated included point mutations on Factor V (G1691A), FII (G20210A), and Protein S deficiency. The acquired factors that were evaluated included Antiphospholipid antibodies syndrome (APS), detected as lupus anticoagulant (LA), anticardiolipin antibodies (ACA) and anti-$\beta_2$ glycoprotein I antibodies (AGPA). The aim of this project was to investigate the association between thrombophilia and RPL.

In this study group, mutation of the FVL was 32%, 10% (p=0.007, OR= 4.235) for cases and controls respectively. The rate of FII 20210 was 6%, 4% (p=0.500, OR= 1.532) for cases and controls respectively. Deficiency of total and free Protein S was detected in 38% and 34% of cases as compared to 20% and 16% controls respectively (P=0.047, P=0.038). The anticardiolipin antibodies were not detected in both cases and controls. However, the anti β2-glycoprotrein I antibodies for IgM only were positive at a rate of 10% of the cases and 6% of controls with no statistical significance.

In conclusion, it is apparent that FVL and PS deficiency are associated with RPL while the other factors tested cannot be associated with RPL. This may be due to the sample number; more comprehensive study may clarify the association between RPL and thrombophilia.

# ملخص


يعتبر فقدان الحمل المتكرر (RPL) تجربة نفسية مؤلمة تؤثر على 1٪ من حالات الحمل. مسببات فقدان الحمل المتكرر لا تزال غير واضحة بشكل جيد ولكن زيادة تخثر الدم يعتبر عاملا رئيسيا. هناك عوامل وراثية وأخرى مكتسبة يمكن أن تؤدي الى فقدان الحمل المتكرر. لقد أجريت هذه الدراسة على 50 حالة تعاني من فقدان الحمل المتكرر و50 حالة ذات حمل طبيعي . تم جمع العينات ما بين مايو وديسمبر 2014. لقد شملت العوامل الوراثية التي تم تقييمها في هذه الدراسة وجود الطفرة G1691A على العامل الخامس للتخثر، والطفرة G20210A على العامل الثاني للتخثر، ونقص بروتين PS . اما العوامل المكتسبة التي تم تقييمها فقد ركزت على وجود الأجسام المضادة في الدم ل Antiphospholipid antibodies syndrome (APS) عن طريق فحص وجود الاجسام المضادة لمرضlupus anticoagulant (LA)، والأجسام المضادة للكارديوليبين (ACA) والأجسام المضادةل anti-$\beta_2$ glycoprotein I antibodies (AGPA) . وكان الهدف من هذا المشروع اثبات وجود علاقة بين زيادة قابلية تخثر الدم و فقدان الحمل المتكرر.

كانت نسبة الطفرات للعامل الخامس للحالات المرضية وغير المرضية في هذه الدراسة 32% و 10% (P = 0.007) ، (OR = 4.235) على التوالي. كان نسبة العامل الثاني 20210 للحالات المرضية وغير المرضية في هذه الدراسة 6% و 4% (P = 0.500)، (OR = 1.532) على التوالي. تم الكشف عن نقص من إجمالي والحر من البروتين S في 38٪ و 34٪ من الحالات المرضية بالمقارنة مع 20٪ و 16٪ من الحالات غير المرضية على التوالي (P = 0.047) ، (P=0.038). لم يتم العثور على الأجسام المضادة للكارديوليبين في كلتا الحالتين المرضية وغير المرضية. بالنسبة ل β2 glycoprotein, لم يتم العثور على مضادات الاجسام من نوع G بينما نوع M تم الثور على مضادات له بنسبة 10% للحالات المرضية و6% للحالات الغير مرضية مع عدم وجود اهمية احصائية.

ختاما، من الواضح وجود علاقة بين الطفرة G1691A على العامل الخامس للتخثر ونقص PS مع فقدان الحمل المتكرر بينما لم توجد علاقة بين جميع العوامل الاخرى التي شملتها الدراسة مع فقدان الحمل المتكرر. ربما يرجع ذلك إلى حجم العينة. لذا لا بد من القيام بدراسة اشمل لتوضيح امكانية وجود علاقة بين فقدان الحمل المتكرر وزيادة قابلية تخثر الدم .

# 1. General Overview

## 1.1 Introduction

Successful pregnancy requires the adjustment of a woman's body to a number of interacting factors. An efficient utero-placental circulation is a vital one which may be affected by disorders of haemostasis [1]. Pregnancy is a prothrombotic state that has likely evolved to protect women from hemorrhage at the time of miscarriage or childbirth [2]. The risk of venous thrombosis is 6-10 folds higher during pregnancy than in non-pregnant women of similar age [3-5]. Thrombophilia or hypercoagulability is a genetic or acquired disorder that predisposes and increases the tendency of blood to clot. It is a multi-factorial disorder involving several risk factors which include family history, surgery, immobility, obesity, smoking, estrogen therapy, and pregnancy [1, 6].

Recurrent pregnancy loss (RPL) is distressing experience that affects 1% of pregnancies [7, 8]. The etiology of RPL in some cases can be identified while remain ambiguous in others. There are many factors that may contribute to RPL such as genetic, endocrine, anatomic, immunologic factors, infectious factors and environmental factors [9]. The main genetic factors involved in maternal thrombophilia that can be related to RPL include point mutations in the genes of Factor V (FV), prothrombin (factor II) and methyl-tetrahydrofolate reductase (MTHFR) [1, 10, 11]. Deficiencies in protein C and protein S are rare hereditary factors that may increase the risk of thrombophilia. It has been reported that increasing age, immobilization, surgery, and oral contraceptives are acquired factors that may lead to thrombophilia and contribute to RPL [12]. However, the primary factor for acquired thrombophilia is the antiphospholipid syndrome (APS) detected as lupus anticoagulant (LA), anticardiolipin antibodies (ACA) and anti-$\beta_2$ glycoprotein I antibodies (AGPA) [13, 14].

Haemostasis refers to the process whereby blood coagulation is initiated and terminated in a tightly regulated fashion. In healthy individuals, a balance between procoagulant, anticoagulant and fibrinolytic activity is maintained so that neither excessive bleeding nor clotting occurs. A scheme of coagulation is illustrated in Fig.1. Antithrombin, protein C and protein S have major roles in the regulation of coagulation, thus they are referred to as natural anticoagulants. Antithrombin down regulates thrombin, and activated protein C (APC), together with its cofactor protein S, inactivates factors Va and VIIIa [15].

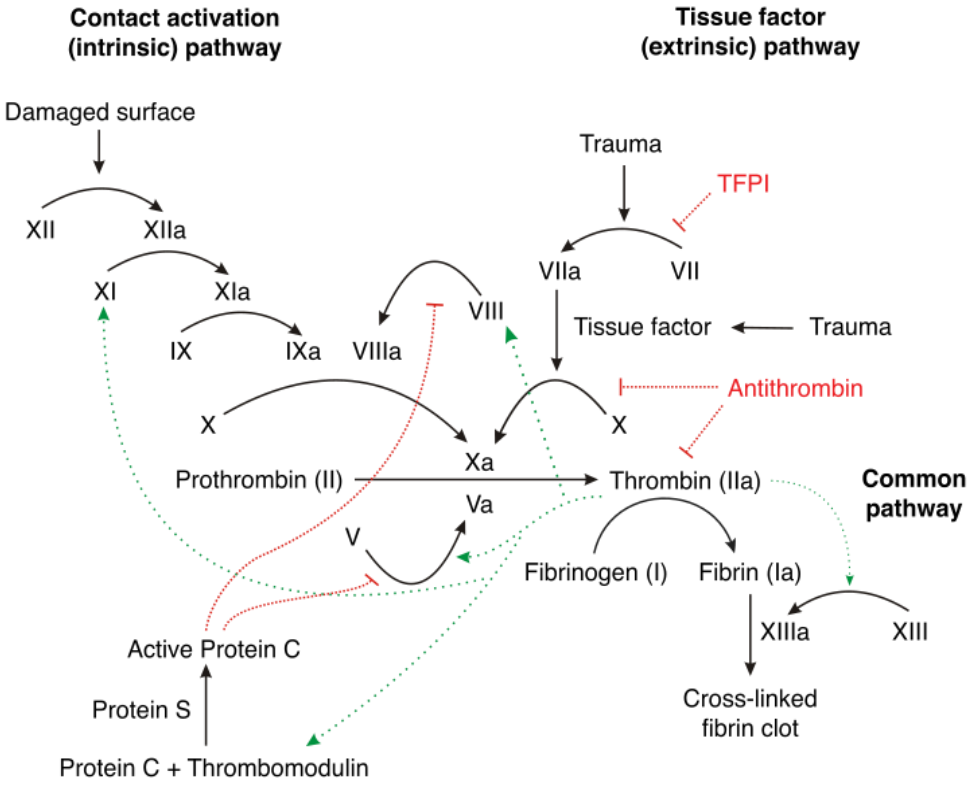


**Fig.1:** Blood Coagulation scheme. Coagulation is initiated by a tissue factor (*TF*)—factor VIIa complex that can activate factor IX or factor X, leading to formation of the key enzyme thrombin (*factor IIa*). The coagulation system is regulated by the protein C pathway. Thrombin activates protein C in the presence of thrombomodulin. Together with protein S (*PS*), activated protein C (*APC*) is capable of inactivating factors Va and VIIIa, which results in a down-regulation of thrombin generation and consequently in an up-regulation of the fibrinolytic system. The activity of thrombin is controlled by the inhibitor antithrombin (*AT*). The green arrows indicate activation (positive feedback) and the red arrows inhibition (negative feedback). *Adopted from Joe D. (2007) more in-depth version of the coagulation cascade.*

Virchow had set the concept of hypercoagulability and postulated that thrombosis is the result of three interrelated factors that have an effect on the haemostasis process [16]. Pregnancy is considered a prothrombotic state with all three components of Virchow's triad [2, 17]. Venous stasis results from both a hormonally induced decrease in venous tone and obstruction of venous blood flow by the enlarging uterus. Endothelial damage in pelvic veins can occur at the time of delivery or from venous hypertension. Intrinsic alterations in the nature of the blood itself by the increased levels of several procoagulant factors (I, II, VII, VIII, IX and XII), a progressive fall in natural anticoagulants levels (protein C, protein S), an acquired resistance to activated protein C and impaired fibrinolysis [17, 18]. Accordingly, in women suffering from pregnancy complications, it is suggested that placental insufficiency due to vascular thrombosis and/or trophoblast growth restriction is central to the problem [8, 19]. Further investigation of the Genetic and acquired risk factors associated with hypercoagulability in women with unexplained RPL can help in the earlier diagnosis and treatment.

## 1.2 Literature review

RPL of 2-3 times or even more is a frustrating experience for patients, families and physicians. There are consensus among clinicians that genetics and acquired factors as etiological agents. Several theories have been suggested to explain the relation between thrombophilia and RPL. Since the beginning of the 80's, the thrombosis theory attempted to explain the cause and effect events between thrombosis and RPL [20].

The pathogenesis of thrombosis was first recognized in 1856 when Virchow in his triad related thrombophilia to alterations in the blood flow, changes in the constitution of blood, and changes in the vessel wall [16]. During the last 50 years, the role of genetics as risk factors in thrombophilia has been considered. Mutations in the genes encoding anticoagulant proteins such as Antithrombin (AT), protein C, and protein S have been identified. Single point mutation in FVL and prothrombin genes have been found to be common in the general population and constitute major genetic risk factors for thrombosis [21]. It is apparent that thrombosis is the result of more than one factor. Regarding single point mutation on FVL gene, patients may be asymptomatic until oral contraceptives are administered [22]. In cohort studies during the 1980's, the role of AT, protein C, and protein S deficiencies in causing thrombosis was recognized. These studies reported a rate of Thrombophilia of <10% due to deficiencies in the above mentioned anticoagulant proteins [21]. A major breakthrough in the genetics of Thrombophilia was the elucidation of activated protein C resistance (APCR) in 1993 [23].

Both RPL and Thrombophilia are multi-factorial conditions. Thrombophilia might be caused by inherited or acquired factors that disrupt the normal haemostasis of the coagulation pathway.

Several factors such as chromosomal anomalies, endocrinological defects, structural uterine anomalies, microbial infections and prothrombotic states might be the cause of RPL, where in 40% of the cases the reason remains unexplained [24]. However, severe complications during pregnancy have been reported for the first time in 1996, in a couple of studies on families who were identified for their history of VTE due to inherited or acquired thrombophilia [25].

Genetic risk factors of thrombophilia may influence the probability of RPL occurrence. The predisposition by gene mutations indicates that certain coagulation factors are modified. The most common are FVL, FII, MTHFR. Factor V is an essential component in the blood coagulation cascade. When activated, Factor Va (FVa) serves as the non-enzymatic, protein cofactor for the prothrombinase complex, which converts prothrombin to thrombin leading to formation of a hemostatic plug. To avoid excessive clotting and as a part of the negative feedback process, activated protein C (APC) inactivates the coagulant protein FVa by cleaving in an ordered sequence at specific sites. The first cleavage site is Arginine (Arg) 506, and the second is (Arg) 306 followed by (Arg) 679 [26].

Factor V Leiden (FVL) G1691A refers to a point mutation (guanine to adenine) in the gene at position 1691, leading to the substitution of arginine (Arg) by glutamine (Gln) at amino acid position 506 of the factor Va protein, making it less susceptible for cleavage by Activated Protein C (APC) [27], which is responsible for the inherited APCR [23]. Therefore, the presence of FVL mutation does not activate blood coagulation instead it impairs the normal inactivation of coagulation. This impairment of normal inactivation in coagulation can result in thrombosis [23, 27-29]. The mutant protein (FVL) is inactivated at a 10-fold slower rate than normal, and persists

longer in the circulation, resulting in increased thrombin generation and a prothrombotic state [30].

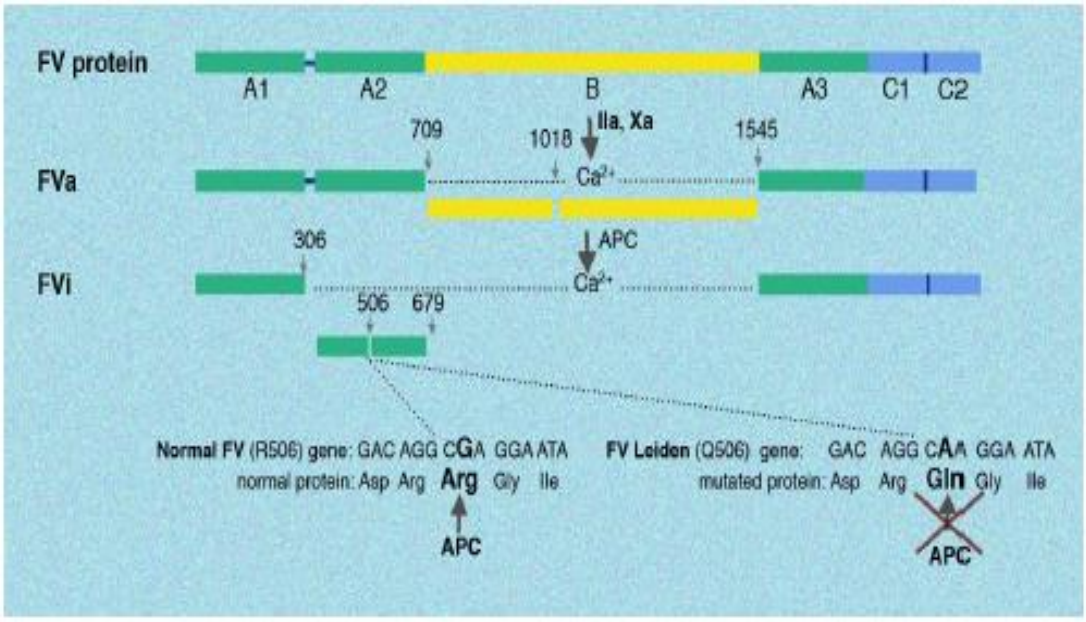


Fig.2: activation and degradation of normal FV and FV Leiden. FV circulates as a single-chain high molecular weight protein. Thrombin or (FXa) cleaves a number of peptide bonds, which results in the liberation of the B domain and generation of FVa. Three peptide bonds in FVa are cleaved by APC (Arg306, Arg506, and Arg679) resulting in inhibition of FVa activity. The FV Leiden mutation eliminates one of the APC cleavage sites, which impairs the degradation of FVa. *Illustration by Marie Dauenheimer, adopted from Dahlback, B., Advances in understanding pathogenic mechanisms of thrombophilic disorders. Blood, 2008.* ***112****(1): p. 19-27* [21].

Prothrombin or Factor II (FII) is the precursor of the serine protease thrombin, a key enzyme in the processes of haemostasis and thrombosis, which exhibits procoagulant, anticoagulant, and antifibrinolytic activity. The mutation on the prothrombin gene G20210A involves a single base-pair substitution (guanine to adenine) at position 20210 in the 3'-untranslated region of the gene which results in an accumulation of mRNA and increased prothrombin protein synthesis in the blood, leading to a 3-fold increased risk of thrombotic events [31, 32]. It is important to

emphasize that the prothrombin G20210A mutation plays a role in increasing the risk of thrombosis in pregnant women by approximately tenfold [10].

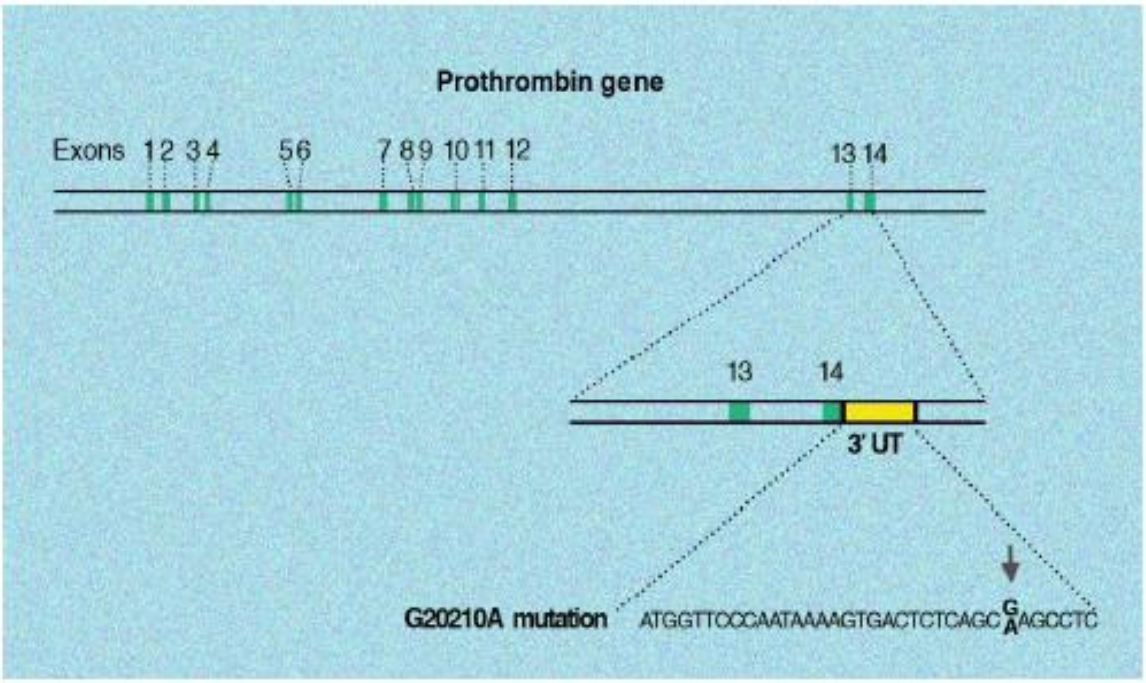


Fig.3: 20210G>A mutation in the prothrombin gene. The single point G to A mutation at position 20210 affects the 3' untranslated region of the prothrombin gene (F.II). Thus the protein-coding sequence of the prothrombin gene is not affected by this mutation. *Illustration by Marie Dauenheimer, adopted from Dahlback, B., Advances in understanding pathogenic mechanisms of thrombophilic disorders. Blood, 2008.* ***112****(1): p. 19-27* [21].

**Protein S deficiency**

Protein S (PS) is a vitamin K dependant protein, it is synthesized in hepatocytes and megakaryocytes. 50% circulates the blood free and 50% circulates bound to C4b binding protein. The deficiency of PS in 74% of patients leads to a deep vein thrombosis (DVT) incident. The deficiency is caused by an autosomal dominant trait that causes either quantitative or qualitative insufficiency [22]. PS is a natural anticoagulant protein and serves as the cofactor for protein C.

In haemostasis, APC together with PS rapidly inactivates the procoagulant cofactors FVa and FVIIIa by specific proteolysis, forming a negative feedback loop to avoid hypercoagulability state. In case of either the quantitative or qualitative deficiency, the negative feedback in the haemostasis will be altered with a higher frequency of blood to clot.

Acquired factors such as advancing age (>50), overweight, smoking, lack of exercise, use of contraceptives containing estrogen, pregnancy, immobilization caused by illness or after operations, can be a leading cause of hypercoagulability. However, an important acquired risk factor for thrombophilia is the antiphospholipid antibody syndrome (APS) characterized by the presence of antiphospholipid antibodies (directed against anticardiolipin and ß$_2$-glycoprotein I) and/or lupus anticoagulant [14].

APS due to a heterogeneous family of immunoglobulins (auto-antibodies) bind to plasma proteins such as prothrombin and activated protein C distressing the haemostasis [22]. pregnancy failure and pregnancy complications are clinical criteria for the diagnosis of antiphospholipid antibody syndrome [14] For early miscarriage, In vitro experiments have shown that antiphospholipid antibodies inhibit extravillous-trophoblast differentiation and subsequent placentation [33]. To detect Lupus antibodies (LA), their activity may cause prolongation of the aPTT, which is not corrected on 50:50 mix with normal plasma.

It has been reported that thrombophilia has direct effects on the coagulation system. There are genetic as well as acquired factors that may contribute to this effect. However, the strength of association and the specific role of each factor has not been well elucidated. There are very few published papers about thrombophilia and RPL among Palestinian women. Recently, two papers

were published about this topic. Hussein et al in 2010 were able to correlate significantly RPL (recurrent miscarriages & still birth) and FVL mutation, after week 10 of gestation. FVL mutation was detected in 28.2% of the studied cases as compared to 11.7% in control group [34]. However, a year later Abu-Asab et al reported the absence of significant association between FVL, FII, and MTHFR and RPL in the first and second trimester. The only significant association was between FVL and Still birth [35].

The scope of the problem related to pregnancy conditions has been extracted from reports of the ministry of health in Palestine for the years 2010, 2011 and mid 2013. The rate of still births per 1000 for these years was 3.6, 3.5 and 4 respectively. In 2010, the rate of pregnancy with obstetric conditions was 10/1000. It was noteworthy to report that recurrent pregnancy loss in women with three or more losses was about 10% of women with pregnancy loss in 2010, and 10.2% in 2011 [36-38]. These figures may not reflect the real scope of this problem but we can conclude that research must be conducted to determine the actual effects of the specific factors involved in RPL.

It is apparent from these reports that the problem of thrombophilia and its association with RPL and other related pregnancy complications has not been properly addressed. Therefore, a more comprehensive investigation should be carried out to further clarify this issue among Palestinian women.

## 1.3 Statement of the problem

The etiology of pregnancy loss is an important yet unresolved clinical problem. The complex nature and pathogenesis of thrombophilia-associated fetal loss is poorly understood and not well characterized. It has been reported the existence of possible association between thrombophilia and several complications during pregnancy, as a result of microthrombi in the placental circulation resulting in decreased utero-placental perfusion [8].

Few studies have been conducted in Palestine to address this problem with conflicting results. In this research we hypothesize a possible association between RPL and both the genetic factors of thrombophilia (factor V, factor II, and Protein S), and the acquired factors mainly APS (the lupus anticoagulant, the anticardiolipin antibodies and $\beta_2$-Glycoprotein 1 antibodies). Therefore, it is extremely important to determine the frequency of the various factors of thrombophilia (inherited and acquired) in women suffering from RPL.

# 2. Aims of the study

The effort of research aims to investigate the impact of maternal inherited and acquired thrombophilia on pregnancy outcome among Palestinian women in a case-control study.

**Specific objectives:**

1- To determine the relationship between FVL (G1691A) and Prothrombin/FII (G20210A) mutations and the risk of RPL among Palestinian women.

2- To evaluate the relationship between Palestinian women having RPL and Protein S deficiencies.

3- To determine the relationship between the acquired thrombophilia caused by APS and the risk of RPL among Palestinian women.

4- To assess the impact of having combined thrombophilia risk factors on Palestinian women suffering from RPL.

# 3. Methodology

## 3.1 Study population and sampling

A total of 116 samples were collected from Palestinian women at a child bearing age (18 – 45 years old), the sampling process was with the cooperation of physicians specialized in obstetrics and gynecology in the West Bank – Palestine, who advised to contact cases that suffer from RPL for unidentified reasons (excluding women with known hormonal, genetic, and anatomic anomalies). On which 50 out of 52 were included as cases by having unexplained RPL from different stages of pregnancy (3 times in the 1$^{st}$ trimester, twice in the 2$^{nd}$ trimester, and once in the 3$^{rd}$ trimester). Two women who had 1 pregnancy loss in the 1$^{st}$ trimester were excluded. Also, 50 out of 64 healthy women, with no chronic diseases, who have 2 or more successful pregnancies without complications, were included as controls. 14 samples that were collected as controls were excluded due to a current pregnancy or a previous history of thrombosis or a pregnancy loss.

## 3.2 Data collection methods

Blood samples were collected aseptically in EDTA, citrate and plain tubes between May and December 2014 from 50 women with RPL and 50 healthy controls. Samples of whole blood collected in EDTA tubes were stored at - 80°C until the beginning of DNA extraction process. Serum was separated from blood samples collected in plain tubes and stored at - 20°C for the ACA and AGPA testing. Fresh plasma was separated from citrated blood samples and tested for PT and aPTT within 2 hours from blood withdrawal then the excess plasma was stored at - 20°C for Total and Free PS testing later on.

The guidelines of the ethical review committee at Birzeit University were followed. The potential risks were explained to each participant, the consent (annex 7.1) of all participants was obtained prior to the enrollment in the study. All participants filled a questionnaire that was administered by the author (annex 7.2) including demographics as well as medical related questions.

### 3.3 Laboratory analysis methods

#### PT and aPTT tests

PT and aPTT tests were conducted on plasma obtained from citrated tubes. PT and aPTT kits were obtained from human company (Germany), the tests were conducted according to the recommendations of the manufacturer. In brief, platelet poor plasma (PPP) was separated from citrated blood samples at 1500 x g for 15 minutes. The clotting time of plasma was measured using Diateck-C4 instrument (Dialab, Austria).

For the PT test, the plasma was incubated at 37°C for 2 minutes and then a source of tissue factor (Thromboplastin) and calcium were added to the samples. Coagulation Time was recorded in seconds. International normalization ratio (INR) was automatically calculated by the Diateck-C4 instrument.

For the aPTT test, the plasma was incubated at 37°C for 2 minutes and then a plasma activator and phospholipid were added to the test specimen, the mixture was incubated (3-5 minutes) for activation. After recalcification with calcium chloride the clot formation was timed.

## Antiphospholipids antibodies Tests

**Anti-Cardiolipin Antibody Assay** for the patients and controls was conducted by the ELISA method (AESKU.DIAGNOSTICS). The test is a quantitative measurement for IgG and IgM class antibodies against Cardiolipin in human serum. The company's instructions were followed throughout the procedure. The presence of Anticardiolipin antibodies in patients characterizes the primary antiphospholipid syndrome (APS). For the interpretation of the results, the standard curve was plotted for the optical density (measured at 450 nm) of each calibrator against the corresponding concentration for IgG and IgM. This step was automatically done and interpreted by BioRad software (BioRad, USA).

**Anti-β2 glycoprotein 1 Antibody Assay** for the patients and controls was conducted by the ELISA method (DRG, Germany). The test is a quantitative measurement for IgG and IgM class antibodies against β2 glycoprotein-1 in human serum. The company's instructions were followed throughout the procedure. For the interpretation of the results, the standard curve was plotted for the optical density (measured at 450 nm) of each calibrator against the corresponding concentration for IgG and IgM. This step was automatically done and interpreted by BioRad software (BioRad, USA).

### Protein S levels in plasma

Protein S determination for the patients and controls was conducted by the ELISA method (AESKU.DIAGNOSTICS). The antigenic test is a quantitative measurement for total and free protein S concentrations in human plasma. The company's instructions were followed throughout the procedure. The low concentration of protein S in patients indicates a natural

anticoagulant deficiency. For the interpretation of the results, the standard curve was plotted for the optical density (measured at 450 nm) of each dilution of the reference plasma against the corresponding value of the reference level in %. This step was automatically done and interpreted by BioRad software (BioRad, USA).

**DNA extraction**

Genomic DNA was extracted from whole blood following the salting out method by Master pure DNA purification kit (Epicenter Biotechnologies, Wisconsin, USA). The purified DNA was solubilized in TE buffer and the concentration was determined using the NanoDrop Lite spectrophotometer (Thermo Scientific, Wilmington, USA). DNA was stored at -20$^{o}$ C until use. The DNA was analyzed using Allele-specific PCR to determine the mutations for Factor V and Factor II.

**Allele specific PCR**

The presence of mutations on Factor V Leiden (G1691A) and Factor II (G20210A) were detected by the allele specific PCR technique. Three primers were used for each factor; 2 forward primers, one normal (N) and another mutated (M) and common reverse primer (C). The sequences of the primers for FVL: Forward-N: 5'-gca gat ccc tgg aca gac g-3'and Forward-M: 5'-gca gat ccc tgg aca gac a-3'. Reverse C: 5'-gga cta ctt gac aat tac tgt tct ctt g-3', The primer sequences for FII: Forward-N: 5'-gca ctg gga gca ttg agg atc-3', Forward-M: 5'-gca ctg gga gca ttg agg att-3', Reverse C: 5'-tct aga aac agt tgc ctg gca g-3'

The PCR reaction was carried out in thermocycler C1000 (biorad, USA), 25 ul volumes consisted of 12.5 ul master mix 2x (Thermo Scientific, USA), 0.8 ul of each primer, 2 ul (50 ng) DNA templates and 8.9 ul sterile nuclease free distilled water. The PCR program was as follows: initial denaturation for 6 minutes at 95°C; followed by 30 cycles of denaturation for 30 seconds at 95°C; annealing for 35 seconds at 56 °C; and extension for 45 seconds at 72°C ,followed by a final extension of 5 minutes at 72°C. The PCR products for the samples were electrophoresed on 2% agarose containing 1uM ethidium bromide and the size determined against 50 bp DNA ladder (gene direx 100 bp ladder RTU).

### 3.4 Statistical Analysis methods

The different qualitative variables (FVL, FII, PS, and APS) for cases and controls were reported as percentage and tested for significance using the Chi square test. The risk was reported as odds ratio at 95% confidence interval. Furthermore, the interaction between FVL and PS deficiency in cases and controls was also investigated using the Chi square test. As for the quantitative variables such as age, marital age and BMI for both cases and controls were reported as mean ± standard deviation and the significance was measured by independent samples T-test. Significance was set at $P < 0.05$ and all tests were two-tailed.

The data was analyzed using SPSS software version 18 (Chicago, IL).

# 4. Results

A total of 100 samples, 50 cases and 50 controls, were included in the study and analyzed to determine the interaction of genetic as well as acquired factors of thrombophilia in RPL. Table 1 contains the results of the questionnaire for the cases and controls. It includes the demographic data, BMI, history of smoking as well as hormonal intake.

**Table 1:** Clinical factors of the study women.

| **Characteristics** | **Cases (N=50)** | **Controls (N=50)** | **P value** | **OR** | **95% CI** |
|---|---|---|---|---|---|
| Age (yr) | 29.7 ± 5.8 | 35.9 ± 4.6 | 0.000* | --- | --- |
| Marrital age (yr) | 22.9 ± 4.7 | 23.0 ± 3.9 | 0.963 | --- | --- |
| BMI | 25.4 ± 5.3 | 25.7 ± 4.4 | 0.717 | --- | --- |
| History of cardiovascular disease within family (%) | 26 | 28 | 0.822 | 1.107 | 0.458-2.678 |
| History of RPL within family (%) | 36 | 8 | 0.001* | 0.155 | 0.048-0.500 |
| Consanguinity (%) | 26 | 18 | 0.334 | 0.625 | 0.239-1.630 |
| Smoker (%) | 14 | 14 | 1.000 | 1.000 | 0.323-3.095 |
| Surgery (%) | 34 | 26 | 0.383 | 0.682 | 0.288-1.614 |
| Estrogen therapy (%) | 12 | 2 | 0.056** | 0.150 | 0.017-1.292 |
| Thyroid problem (%) | 4 | 2 | 0.500 | 0.490 | 0.043-5.582 |

* Significant difference
** Borderline

The results of the screening tests that may affect thromophilia and RPL include Coagulatin factors. The results for the PT, INR and aPTT for the cases are elevated in 46% (23/50), 10% (5/50), and 2% (1/50) respectively. as for the controls elevated results are observed in 30%

(15/50), 10% (5/50) respectively for PT and INR. While none was elevated for the aPTT test in control samples.

Lupus anticoagulants (LA) are usually present in samples with prolonged aPTT. Since all aPTT results among cases and controls were not prolonged (98%), LA are not suspected. There is no need to do 50:50 mixing method to detect LA as a major factor of APS.

The mutation in FV gene at position 1691 was detected in 16 cases and 5 controls. The G20210A mutation in the prothrombin gene was found in 3 cases and 2 controls. Results of the genetic analysis are shown in Table 2.

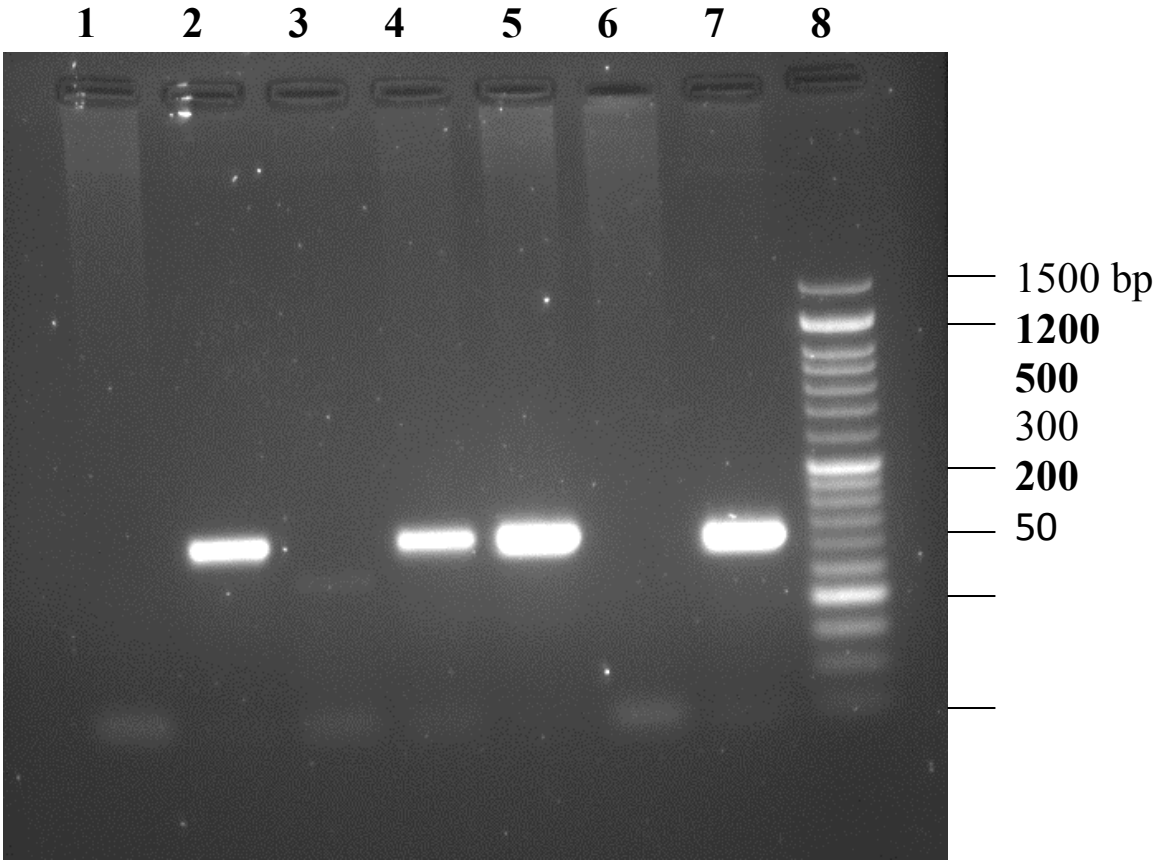


Figure 4: Gel electrophoresis for FII G20210A mutation.
1 Blank, 2 and 3 normal sample, 4 and 5 a heterzygous sample, 6 and 7 a homozygous sample, 8 50bp DNA ladder

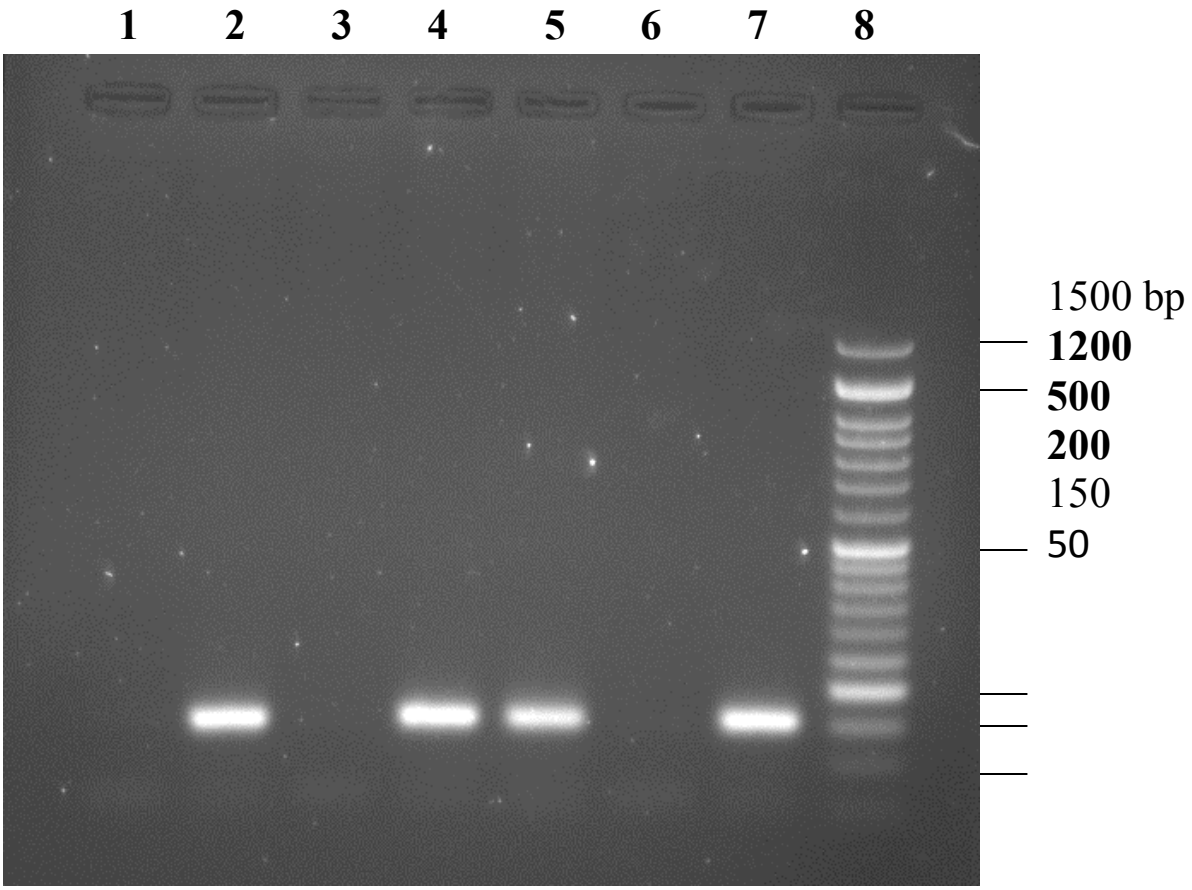


Figure 5: Gel electrophoresis for FVL G1691A mutation.
1 Blank, 2 and 3 normal sample, 4 and 5 a heterzygous sample, 6 and 7 a homozygous sample, 8 50bp DNA ladder

**Table 2:** Results of the genetic analysis for FVL and FII in cases and controls.

| | Cases (N=50) | | Controls (N=50) | | P value | OR | 95%CI |
|---|---|---|---|---|---|---|---|
| **Condition** | **Normal** | **Mutated** | **Normal** | **Mutated** | | | |
| FVL | 34 (68%) | 16 (32%) | 45 (90%) | 5 (10%) | 0.007* | 4.235 | 1.412-12.705 |
| FII | 47 (94%) | 3 (6%) | 48 (96%) | 2 (4%) | 0.500 | 1.532 | 0.245-9.587 |

* Significant difference

Enzyme Linked Immunosorbent Assay (ELISA) was conducted to determine the antibody levels of anticardiolipins (IgG, IgM) and anti $\beta_2$-glycoprotein I (IgG, IgM) in both cases and controls.

The results are summarized in Table 3 below. Total and free Protein S levels in plasma were quantitatively determined by the ELISA method and the results are also included in Table 3.

**Table 3:** Results of the analysis performed by ELISA for Protein S and the acquired factors of APS in cases and controls.

| Condition | Cases (n=50) | | | Controls (n=50) | | | P value | OR | 95% CI |
|---|---|---|---|---|---|---|---|---|---|
| | P % | N % | E % | P% | N% | El% | | | |
| **ACA** | | | | | | | | | |
| IgM | 0 | 100 | - | 0 | 100 | - | NC | NC | NC |
| IgG | 4 | 96 | - | 0 | 100 | - | 0.247 | NC | NC |
| **AGPA** | | | | | | | | | |
| IgM | 10 | 78 | 12 | 6 | 80 | 14 | 0.878 | 1.143 | 0.380-3.436 |
| IgG | 0 | 84 | 16 | 0 | 86 | 14 | 0.812 | NC | NC |
| **Protein S** | | | | | | | | | |
| Total | 38 | 62 | - | 20 | 80 | - | 0.047* | 0.408 | 0.166-1.001 |
| Free | 34 | 66 | - | 16 | 84 | - | 0.038* | 0.370 | 0.142-0.962 |

ACA: Anti-cardiolipin antibodies, AGPA: Anti $\beta_2$-glycoprotein I antibodies
NC: not calculated since there is zero cases or controls
P: positive, N: negative, E: equivocal.
* significant difference

FVL mutation by itself had significant association with RPL as well as total and free PS deficiency. Testing the combination of the two factors (FVL & PS) were observed in 7 (14 %) of patients, P=0.012 (OR 1.163 [1.040-1.300]). The risk of having RPL with these two factors combined is 16%.

# 5. Discussion

The presence of both acquired and genetic thrombophilia factors was assessed in this case control study, conducted on women with unexplained RPL. Data collected from 50 women with RPL and 50 matched controls have focused on the role of abnormal procoagulant activity in the pathogenesis of RPL. The genetic factors that are commonly involved in thrombophilia and subsequent RPL include FVL, FII and PS have been evaluated in this study. In addition, the acquired factors that may contribute to this condition including APS (LA, ACA and AGPA) were also evaluated.

In this study group, mutation of the FVL was 32%, 10% (p=0.007, OR= 4.235) for cases and controls respectively. The rate of FII 20210 was 6%, 4% (p=0.500, OR= 1.532) for cases and controls respectively. Deficiency of total and free Protein S was detected in 38% and 34% of cases as compared to 20% and 16% controls respectively (P=0.047, P=0.038). As for The anticardiolipin antibodies were not detected in both cases and controls. However, the anti β2-glycoprotrein I antibodies for IgM only were positive at a rate of 10% of the cases and 6% of controls with no statistical significance.

In this project we evaluated different parameters through a questionnaire as shown in Table 1. Our results reflected the absence of significant difference between cases and controls with the history of cardiovascular disease within family, BMI, marital age and smoking. Regarding the history of cardiovascular disease within family, it didn't show an association with RPL because

not all thrombotic events can lead to thrombophilia. Considering the absence of significance between cases and controls with marital age and BMI, it is clear that cases and controls were matching. As for the age of the women tested, there was significant difference because the control group was older. In this case, it will not affect our results because the older the age of the woman the less likely to have children. Considering the association of other parameters evaluated in the questionnaire, the estrogen therapy for example, was on the border of being significant which may point to test a larger sample to elucidate its effect. Although there was no significant association between having abnormalities in estrogen and thyroid hormones levels, the cases (8 women) were included in the study. Such cases must be excluded when testing a larger sample for RPL that may show a significant association with thrombophilia.

There were significant association between the genetic factors tested; FVL (P=0.007), total PS (P=0.047) and free PS (P=0.038). It has been suggested that FVL is the most significant genetic factor responsible for RPL. However, several reports indicated that FVL is not signicant in early pregnancies and the effect has been found to be more pronounced in later stages of pregnancies in causing pregnancy loss [39, 40]. Our study indicated that the rate of FVL among women with RPL was 32% (30% heterozygous and 2% homozygous) as compared to 10% in controls. Few studies have been conducted in Palestine, regarding thrombophilia and RPL. One study reported the presence of significant association between FVL and RPL which is similar to our finding [34]. Their results indicated a rate of 28.2% FVL mutation in patients and 11.7% in controls. Contrary to our results and those of Ayman Hussein et al. study, a study conducted in Al-Quds University, did not find significant association between FVL and early RPL (< 24 weeks of gestation). The rate of FVL in their study was 23.4% in cases and 18.2% in controls [35].

However, these two studies neglected to evaluate the interaction of acquired and other genetic factors in causing RPL. The rate of FVL in our study is much more elevated than those reported in Iran (2.5%), India (4.7%), a USA hospital (20%), (7.9%) in Turkey, and 19.4% in Tunisia [39-43].

Mutations on FVL and FII have been extensively studied to determine their association with RPL. Studies in different populations have shown varied results concerning FII. On one hand, FII has not been found to have any significance in RPL [43]. However, other studies found the rate of FII is 10.9% in cases while 2.04% in controls ($p<0.05$) indicating the possibility of an association with RPL [44]. Yet, another study reported the presence of an association with increasing age (>29 years old) [45].

Among Palestinian women, one study reported a rate of 3.3% in cases and 4.2% in controls with non-significant association with RPL [35]. In our study the rate of FII was 6% in cases and 4% in controls (P=0.500, OR=1.532 [0.245-9.587]). Although the sample size was relatively small but our results were on the borderline. Since, the mean age of the women tested was 29.7 ± 5.8, suggesting that our results may have significant association in women older than 29 years as reported previously [45].

The APS is an acquired autoimmune condition with clinical manifestation of thrombosis and/or pregnancy complications and loss [46]. The clinical criteria of APS requires the presence IgG and/or IgM for both ACA and AGA [47]. The prevalence of APS in women with RPL varies. The effect of APS is not significant on women characterized with RPL only and have no

additional autoimmune disease [48]. The positive results of APS antibodies (ACA and AGPA) in this work as shown in Table had no significant association between APS and RPL (P=0.247 and 0.812).

It has been known that mutations in protein S gene predispose the development of venous thromboembolic disorders in humans [49]. Anticoagulant treatment with low molecular weight heparin in women with hereditary thrombophilia is safe and reduces the risk of fetal loss [50]. Protein S deficiency is a rare inherited condition that may lead to fetal loss. Since the homozygous genotype in neonates is fatal, women with protein S deficiency are usually heterozygote [51]. In this study we tested for total and free protein S deficiencies. The rate of total protein S deficiency was 38% in cases and 20% in controls while the rate of free protein S was 34% in cases and 16% in controls. The results were significant (P=0.047 for total PS and 0.038 in free PS) and may lead to pregnancy loss if improperly treated. Such results indicate an association between protein S deficiency (total and/or free) and pregnancy loss.

Our results strongly suggest that deficiencies in the hereditary factors (FVL and Protein S) are significantly associated with recurrent pregnancy loss. The association of Factor II another hereditary factor with RPL was of borderline significance (P=0.05). The reason can be due primarily to the sample size and possibly to the age of women with RPL. Since the contribution of FII has its manifestation in pregnancy loss at later stages of pregnancies and in women older than 29 years, it is hard to observe its effects in this relatively small sample. Acquired factors had no significant association with RPL in this study. These acquired factors are commonly associated with deep vein thrombosis and to a certain extent with RPL. The small sample size

may be the reason for the shortcomings of this project to clarify the role of acquired factors in RPL.

Significantly more pregnancy terminations had been observed in women with FVL mutation or PS deficiency than in controls. The possibility of increased risk of RPL in patients with combined defects was 16%. This elevation in the rate of the combined effect (FVL and PS) may be noticeable in certain cases only, without an amplified risk more than of each factor alone. When FVL mutation was evaluated alone the risk of RPL was found to be 30% and for PS alone was 6%. This may indicate the absence of association of RPL and thrombophilia when considering the effect of FVL and PS together.

Our results show an important association between certain thrombophilia factors with the incidence of RPL. The strength of this work was in evaluating most of the factors presumably contributing to thrombophilia that result in RPL. The weakness of this work was mainly in the small sample size, ruling out women with microbial infections, and some of the questions in the questionnaire were not specific regarding if hormonal administration and surgery were before or after RPL. In order to verify the presence or absence of the effect of the different factors studied, we need to perform a larger research with an increased sample size.

# 6. Recommendations

- To conduct a research with an increased sample size on a national scale, including Palestinian women from the West-Bank and the Gaza strip which is essential to expand on the current knowledge base.

- To cooperate with the ministry of health To develop a checklist as a diagnostic criteria were physicians specialized in gynecology and obstetrics can follow to help in excluding patients with anatomic, hormonal or other known defects that may cause RPL before ordering the tests of hypercoagulability.

- Based on the results of a wider research project, a scheme for routine testing can be developed in order to investigate the associated factors of thrombophilia with RPL. This will help in early diagnosis and treatment options to minimize the devastating psychological trauma among women with RPL.

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# 8. Annexes

## 8.1 Consent form

**برنامج الماجستير في العلوم الطبية المخبرية**
**موافقة على المشاركة في البحث**

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**عنوان البحث:** الأسباب الوراثية والمكتسبة لعملية فرط التخثر في النساء الفلسطينيات ذوات فقدان الحمل المتكرر.

**الباحثون:** د. تامر العيساوي و د. محمد فراج والطالبة لورا بطماني/ جامعة بيرزيت

**حيثيات البحث**: نرغب بمشاركتكم في هذا البحث.من خلال الموافقة على أخذ كمية قليلة من الدم لعمل الفحوصات المتعلقة بعملية تخثر الدم. ولمساعدتكم في اتخاذ القرار نود أن نوضح ما يلي:

1. الغرض والإجراءات المتبعة في البحث

• تحديد بعض الأسباب وراء عملية التخثر في الدم للنساء ذوات فقدان الحمل المتكرر.

2. أية مخاطر يمكن نوقعها لا توجد مخاطر عدا عن الحد الأدنى من الراحة أثناء سحب الدم.

3. كيف سيتم الحفاظ على السرية: لن يتم استخدام اسم المشارك ، سيتم ترميز العينة.

4. أي علاج طبي في حالة وجود نقص في أي من الفحوصات : يتم إبلاغ الشخص بالنتيجة والمتابعة مسؤوليته الشخصية.

**5.**عدد ألمشاركين 160

ان مشاركتك في هذا البحث سوف تسهم في زيادة المعرفة. ونتائج هذه الدراسة سيكون لها تأثير ايجابي على الوضع الصحي في المجتمع الفلسطيني ولهذا نشكرك جدا على المشاركة.

اسم المشاركة: _____ رقم الهاتف/الجوال: _____

توقيع المشاركة: _____ التاريخ: _____

## 8.2 Questionnaire

## استمارة البحث

**عنوان البحث: الأسباب الوراثية والمكتسبة لعملية فرط التخثر في النساء الفلسطينيات ذوات فقدان الحمل المتكرر**

اسم المشتركة:_____

تاريخ الميلاد: _____ العمر عند الزواج: _____

الوزن:_____ الطول: _____

**ضع إشارة صح في المكان المناسب**

**تاريخ العائلة**

هل هناك بالعائلة مصابين بأمراض القلب والشرايين؟ نعم _____ لا _____

هل هناك تاريخ فرط حمل عند الأم أو الخالات أو الأخوات؟ نعم _____ لا _____

هل الزواج زواج أقارب؟ نعم _____ لا _____

**طريقة المعيشة والتاريخ الطبي للمشتركة:**

1) إمراة عاملة _____ ربة منزل _____
2) هل أنت مدخنة (سجائر أو أرجيلة)؟ نعم ____ لا _____
3) هل تمارسين الرياضة بشكل منتظم؟ نعم _____ لا _____
4) هل خضعت لعملية جراحية في الماضي: نعم _____ (نوع العملية _____) لا _____
5) هل تعانين من مرض السكري؟ نعم _____ لا _____
6) هل تعانين من ضغط دم مرتفع؟ نعم _____ لا _____
7) هل تناولت سابقاً حبوب منع الحمل؟ نعم _____ لا _____
8) هل حصلت على علاج هرموني بلإستروجين؟ نعم _____ لا _____
9) هل حصلت على علاج هرموني للغدة الدرقية؟ نعم _____ لا _____
10) ما هي عدد المرات التي حصل فيها فرط حمل؟ _____
11) حددي الشهر الذي حصل فيه فرط الحمل

المرة الأولى: _____ المرة الثانية: _____ المرة الثالثة: _____

12) هل تناولت مكملات غذائية تحتوي على فيتامين B12 خلال فترات الحمل غير المكتمل؟ نعم _____ لا _____
13) هل تناولت مكملات غذائية تحتوي على حمض الفوليك خلال فترة الحمل غير المكتمل؟ نعم _____ لا _____
14) هل تتناولي أدوية مميعة للدم: نعم _____ (نوع الدواء _____) لا _____

## 8.3 Results of the cases and controls included in the study.

| Controls ID | PT | INR | aPTT | PROTS Tot | PROTS Free | FV | FII |
|---|---|---|---|---|---|---|---|
| 1585 | 13.4 | 0.95 | 23.5 | 147.1 | 69.7 | N | N |
| 1902 | 12.2 | 0.86 | 25.5 | 157.9 | 56.5 | N | N |
| 2568 | 12 | 0.84 | 24.6 | 89.8 | 59 | N | N |
| 2682 | 12.8 | 0.91 | 24.7 | 213.9 | 51.7 | Hetero | N |
| 2687 | 13.4 | 0.95 | 29.7 | 42.5 | 67 | N | N |
| 2798 | 14.1 | 1.01 | 24.7 | 90.1 | 61.6 | N | N |
| 2808 | 12.6 | 0.89 | 29.1 | 45.5 | 67.6 | N | N |
| 2902 | 12.8 | 0.91 | 25.3 | 77.1 | 74.2 | N | N |
| 2927 | 13.4 | 0.95 | 24.5 | 86.7 | 61.8 | Hetero | N |
| 2950 | 11.7 | 0.82 | 25.1 | 86.3 | 48.2 | N | N |
| 2965 | 13.7 | 0.98 | 32.5 | 91.6 | 54.5 | N | Hetero |
| 2976 | 14.2 | 1.02 | 29 | 51.4 | 75.3 | N | N |
| 2978 | 13.7 | 0.98 | 22.1 | 125 | 65.8 | N | N |
| 2996 | 13.5 | 0.96 | 0 | 70.6 | 100 | N | N |
| 3017.1 | 12.7 | 0.9 | 25.8 | 114.1 | 77 | Hetero | N |
| 3037 | 11.7 | 0.82 | 24.5 | 78.8 | 76.9 | N | N |
| 3072 | 12.5 | 0.88 | 28.1 | 74.8 | 58.6 | N | N |
| 3106 | 11.9 | 0.83 | 30.8 | 62.5 | 40.7 | N | N |
| 3109 | 13.1 | 0.93 | 28.2 | 96.8 | 54.2 | N | N |
| 3187 | 14.1 | 1.01 | 25.8 | > 150 | 91.6 | N | N |
| 3188 | 11.8 | 0.83 | 27.6 | 177.3 | 76.9 | N | N |
| 3215 | 13.2 | 0.94 | 28 | 70.5 | 45.2 | N | N |
| 3253 | 14.3 | 1.02 | 29.1 | 74.8 | 50.2 | N | N |
| 3284 | 12.3 | 0.87 | 27.3 | 188.3 | 70.6 | N | N |
| 3352 | 13.7 | 0.98 | 28.6 | 48.2 | 52.2 | N | N |
| 3452 | 11.9 | 0.88 | 27 | 124 | 114 | N | N |
| 3478 | 14.3 | 1.02 | 27.7 | 29.2 | 41.5 | N | N |
| 3556 | 14.5 | 1.04 | 23.8 | 306.2 | 76.9 | N | N |
| 3576 | 14.8 | 1.06 | 22.1 | 129.2 | 71.7 | N | N |
| 3609 | 12.9 | 0.91 | 26.1 | 161.1 | 54.4 | N | N |
| 3616 | 12.1 | 0.85 | 26.5 | 101.8 | 70.8 | N | N |
| 3649 | 11.4 | 0.8 | 33.2 | 183.7 | 56.4 | N | N |
| 3739 | 15.2 | 1.1 | 25.7 | 51.9 | 86 | N | N |
| 3853 | 14.3 | 1.02 | 26.7 | 83.2 | 41.7 | N | N |
| 4010 | 14.5 | 1.04 | 19.7 | 81.3 | 52.7 | N | N |
| 4080 | 13.6 | 0.97 | 22.4 | 72.4 | 58.9 | Hetero | N |
| 4087 | 15 | 1.08 | 33.1 | 39.3 | 63.3 | N | N |
| 4116 | 13.4 | 0.95 | 33 | 72.1 | 58.5 | N | N |
| 4364 | 12.5 | 0.88 | 24.3 | 158.1 | 69.5 | Hetero | N |
| 4585 | 13.9 | 0.99 | 27.8 | 56.9 | 96 | N | N |
| 4726 | 14.1 | 1.01 | 29.8 | 152.3 | 59.5 | N | N |
| 4739 | 14.3 | 1.02 | 26.3 | 57.4 | 65.4 | N | N |
| 4810 | 14.9 | 1.07 | 30.2 | 39.6 | 57.4 | N | N |
| 5055 | 12.8 | 0.91 | 28.1 | 68.4 | 39.4 | N | N |
| 5236 | 11.9 | 0.83 | 24.4 | 85.7 | 36.2 | N | N |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 5341 | 12.6 | 0.89 | 26.1 | 98.5 | 51.5 | N | N |
| 5655 | 15 | 1.08 | 23.7 | 142.3 | 61.2 | N | Homo |
| 5704 | 13.1 | 0.93 | 30.1 | 73.7 | 62.6 | N | N |
| 921 | 13.3 | 0.94 | 28 | 62.8 | 39.5 | N | N |
| 923 | 12.8 | 0.91 | 34.6 | 131.1 | 60 | N | N |

| **Cases ID** | **PT** | **INR** | **aPTT** | **PROTS Tot** | **PROTS Free** | **FV** | **FII** |
|---|---|---|---|---|---|---|---|
| 3514 | 14.2 | 1.02 | 28.4 | 65.5 | 76.5 | N | N |
| 55000800 | 13 | 0.92 | 26.5 | 55.9 | 92.5 | Heter | N |
| 55000822 | 14.4 | 1.03 | 31.3 | 54.7 | 87.6 | N | N |
| 55000823 | 13.7 | 0.98 | 30.4 | 49.1 | 74.1 | Heter | Heter |
| 55000825 | 14.6 | 1.05 | 32.5 | 60.3 | 88.9 | N | N |
| 55000826 | 14.4 | 1.03 | 34.2 | 48 | 80.2 | N | N |
| 55000827 | 14.4 | 1.03 | 34.5 | 52 | 80.4 | N | N |
| 55000845 | 13.6 | 0.97 | 26.4 | 76.3 | 160.6 | N | Heter |
| 55000846 | 12.6 | 0.89 | 28.7 | 43.3 | 58.6 | N | N |
| 55000847 | 9.7 | 0.67 | 29.1 | 50 | 78.7 | N | N |
| 55000848 | 14.2 | 1.02 | 25.9 | 37.3 | 47.2 | Homo | N |
| 55000849 | 13.1 | 0.93 | 32.4 | 42.2 | 52.3 | N | N |
| 55000850 | 13.8 | 0.98 | 28.3 | 44 | 72.5 | Heter | N |
| 55000852 | 13 | 0.92 | 21.3 | 127.6 | 112.5 | N | N |
| 55000853 | 14.2 | 1.02 | 29.1 | 37.3 | 58.4 | N | N |
| 55000854 | 14.2 | 1.02 | 27.2 | 70.8 | 118.7 | N | N |
| 55000855 | 14.2 | 1.02 | 29.8 | 138.5 | 137.6 | N | N |
| 55000856 | 14.3 | 1.02 | 26.9 | 46.8 | 65.9 | N | N |
| 55000857 | 13.9 | 0.99 | 27.5 | 49.3 | 68.4 | Heter | N |
| 55000858 | 14.7 | 1.06 | 28.3 | 70.6 | 98.8 | Heter | N |
| 55000859 | 15.7 | 1.14 | 23.9 | 56.5 | 105.1 | N | N |
| 55000860 | 14.5 | 1.04 | 28.8 | 51.1 | 86.4 | N | N |
| 55000861 | 13.7 | 0.98 | 25.2 | 76.8 | 65.9 | Heter | N |
| 55000862 | 14.2 | 1.02 | 25.1 | 50.1 | 73.7 | N | N |
| 55000867 | 12.2 | 0.86 | 23.4 | 140.1 | 91.9 | N | N |
| 55000868 | 12.5 | 0.88 | 27.7 | 105.5 | 59.9 | N | N |
| 55000869 | 11.7 | 0.82 | 24.5 | 135.7 | 74.3 | N | N |
| 55000870 | 12.5 | 0.88 | 28.6 | 62.3 | 114.4 | N | N |
| 55000871 | 12.7 | 0.9 | 32.1 | 63.5 | 103.9 | Heter | N |
| 55000872 | 12 | 0.84 | 26.1 | 131.5 | 79.2 | N | N |
| 55000873 | 13.8 | 0.98 | 31.5 | 67.1 | 46.8 | Heter | N |
| 55000874 | 14.2 | 1.02 | 30.1 | 48.5 | 21.7 | Heter | N |
| 55000875 | 13.5 | 0.96 | 28.3 | 99.8 | 49.8 | N | N |
| 55000876 | 14.6 | 1.05 | 29.8 | 63.7 | 34 | N | N |
| 55000877 | 13.1 | 0.93 | 27.5 | 61.7 | 31.4 | N | Heter |
| 55000878 | 14.5 | 1.04 | 26.4 | 50.2 | 23.4 | Heter | N |
| 55000879 | 14.4 | 1.03 | 28.6 | 77.8 | 46.7 | Heter | N |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 55000880 | 13.6 | 0.97 | 28.1 | 71.1 | 41 | Heter | N |
| 55000881 | 11.7 | 0.81 | 29.6 | 61.8 | 27.6 | N | N |
| 55000882 | 13.7 | 0.98 | 29 | 88.5 | 45.4 | Heter | N |
| 55000883 | 14.2 | 1.02 | 32 | 77.5 | 46.7 | N | N |
| 55000884 | 15.5 | 1.12 | 26.4 | 72.3 | 50.7 | N | N |
| 55000885 | 13.5 | 0.96 | 27.9 | 89.8 | 60 | Heter | N |
| 55000886 | 14.4 | 1.03 | 28.7 | 85.5 | 54.6 | N | N |
| 55000887 | 13 | 0.92 | 28.5 | 93.3 | 43.4 | N | N |
| 55000888 | 13.1 | 0.93 | 26 | 77.9 | 39.3 | N | N |
| 55000889 | 15.1 | 1.09 | 27.2 | 59.4 | 29.2 | N | N |
| 55000890 | 13.6 | 0.97 | 20 | 67.5 | 28.8 | N | N |
| 55000891 | 14.9 | 1.07 | 24 | 90.6 | 238.2 | N | N |
| 55000892 | 12.4 | 0.87 | 42.5 | 71.9 | 49.1 | Heter | N |

N: NORMAL

Heter: HETEROZYGOUS

Homo: HOMOZYGOUS