NEAR EAST UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

# The Allelic Distributions of SNPs from Thrombosis Associated Genes in Patients with Miscarriages in Erbil, Iraq

By

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Master of Science in Medical Biology and Genetics

Supervisor:

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APPROVAL

Thesis submitted to the Institute of Health Sciences of Near East University in partial fulfillment of the requirement for the degree of Master of Science in Medical Biology and Genetics.

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## DEDICATION

This thesis is lovingly dedicated to my respective parent who have been our constant source of inspiration. They have given me the drive and discipline to tackle any task with enthusiasm and determination. Without their love and support this project would not have been made possible.

## ACKNOWLEDGEMENTS

I would like to express my special appreciation and thanks to my advisors Assoc. Prof. Pinar Tulay and Prof. Dr. Hazha Jamal Hidayat. I would like to thanks they are for encouraging my research and for allowing me to grow as research scientist. They advise on research as well as on my careers have been magnitude. A special thanks to my family, Words cannot express how grateful I'm to my parent and siblings of all the sacrifices that they have made on my behalf. Their prayer for me was what sustained me thus far. A great thanks to my department and head of my department Prof. Nedime Serakinci to encourage and support me in all times.

Thank a lot for Immunogene center for genetic disease diagnosis and Maternity hospital Erbil-Iraq for allowing me to do my research there.

**ABSTRACT**

# The Allelic Distributions of SNPs from Thrombosis Associated Genes in Patients with Miscarriages in Erbil, Iraq

REBIN SALAH ALI

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**ABSTRACT:** Abortion is the most common complication during pregnancy. The phenomenon of thrombophilia is important in pregnant women and it has been associated with recurrent pregnancy loss. Genotyping of single nucleotide polymorphisms (SNPs) of the genes involved in thrombophilia may be useful.

There are many factors that affect abortion and recurrent miscarriage such as female age and embryonic aneuploidy, antiphospholipid syndrome, parental structural chromosomal abnormalities, uterine structural abnormalities, endocrine factors, including thyroid function, polycystic ovary syndrome (PCOS) and prolactin, immunological factors and progesterone supplementation, infection, lifestyle and sperm DNA damage. One of the other factors that has been associated with recurrent pregnancy loss (RPL) is inherited thrombophilia. The specific genotypes of several SNPs have been associated with increased risk of recurrent miscarriage. In this project, we aimed to evaluate the allelic frequencies of SNPs within seven genes that are associated with thrombophilia in the Iraq-Erbil population. Thus, it was aimed to determine the relationship between the genotypes of these SNPs and the number of abortions, age and family history.

Blood samples were obtained from 50 women with recurrent miscarriages and 30 women who did not have any spontaneous abortions as control cases. Using PCR-RFLP, SNPs within the Methylene Tetrahydrofolate Reductase *(MTHFR) C677T* and *A1298C, Plasminogen Activator Inhibitor-1 (PAI-1), Factor V Leiden, Prothrombin G20210A, Factor XIII* and *Fibrinogen beta chain* were genotyped.

The results of this study showed that there was no association between recurrent miscarriage with age however the two genes *MTHFR C677 and MTHFR A1298C* were related to family history. On the other hand, *MTHFR C677T and PAI-1* (4G/5G) were shown a significant association with recurrent pregnancy loss. More importantly, the allelic frequencies in RPL and control cases showed statistically significant differences for all the SNPs investigated. Thus, the results of this study may show the importance of genetic testing in RPL patients. This study forms the basis of future studies and further analyses including more number of patients will be designed.

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

## TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

## LIST OF ABBREVIATION

| | |
|---|---|
| ACOG: | American College of Obstetricians and Gynecologists |
| RPL: | Recurrent Pregnancy Loss |
| RSA: | Recurrent Spontaneous Abortion |
| WHO: | World Health Organization |
| VTE: | Venous Thromboembolism Event |
| PT: | Prothrombin |
| RM: | Recurrent Miscarriage |
| APS: | Antiphospholipid Syndrome |
| PGT: | Pre-implantation Genetic Testing |
| SCH: | Subclinical Hypothyroidism |
| TSH: | Thyroid Stimulant Hormone |
| PCOS: | Polycystic Ovarian Syndrome |
| TLC: | Tender Loving Care |
| BV: | Bacterial Vaginosis |
| APC: | Actuated Protein C |
| DVT: | Deep Vein Thrombosis |
| FBG: | Fibrinogen |
| t-PA: | tissue Plasminogen |
| u-PA: | urokinase Plasminogen |
| PCR: | Polymerase Chain Reaction |
| RFLP: | Restriction Fragment Length Polymorphism |
| NTD: | Neural Tube Defects |
| SGA: | Small of Gestational Age |
| MTHFR: | Methylene Tetrahydrofolate Reductase |
| PAI-1: | Plasminogen Activator Inhibitor-1 |
| FVL: | Factor V Leiden |

# CHAPTER ONE

## 1.Introduction

The spontaneous abortion refers to the miscarriage phenomena taking place within 20 weeks of the gestation (Dugas & Slane, 2019). While 15% of the clinical pregnancies end in unconstrained premature birth, the rate of recurrent pregnancy loss (RPL) was determined to be approximately 5%, and is identified as one of the most prominent female infertility cause (Adler et al., 2018; D'Uva, Micco, Strina, & Placido, 2010). Every woman might have a period of unnatural birth cycle during her conceptive ages, but the rate at which this becomes RPL is believed to vary between 1 and 5% of expecting couples (Stefanski et al., 2018). World Health Organization (WHO) has determined that three or more consecutive premature deliveries before $20^{th}$ week of gestation is defined as RPL (Stefanski et al., 2018).

The underlying mechanisms of spontaneous abortions is complicated and a number of causes have been proposed. These include endocrinological abnormalities and many genetic factors. This thesis project aimed to investigate the allelic distribution of SNPs that are associated with thrombosis, including factor V Leiden (R506Q), prothrombin (G20210A), factor XIII (V34L), fibrinogen beta chain, plasminogen activator inhibitor-1 and the methylene tetrahydrofolate reductase (MTHFR, C677T) and (MTHFR A1298C), in women with miscarriages in Erbil, Iraq.

### 1.1. Spontaneous abortions and the possible underlying mechanisms

It has been estimated that approximately 10 to 15% clinically-monitored pregnancies end in spontaneous miscarriage (Homer, 2019; Nybo Andersen, Wohlfahrt, Christens, Olsen, & Melbye, 2000; Saravelos & Li, 2012). Embryonic aneuploidy is established as the primary cause of these miscarriages (Greaney, Wei, & Homer, 2017). The main reason of embryonic aneuploidies is the

meiotic chromosome segregation errors that take place within the oocytes, and this phenomenon is known to increase in frequency with advancing maternal age (Greaney et al., 2017; Nybo Andersen et al., 2000). Furthermore, translocation carriers are known to have an increased risk of producing aneuploid gametes and therefore aneuploid embryos. General population is known to have 0.7% frequency of chromosomal anomalies, but it has been shown that they are present in 5% of the couples experiencing recurrent miscarriages (Franssen et al., 2006; Jaslow, Carney, & Kutteh, 2010; Popescu, Jaslow, & Kutteh, 2018; Stephenson & Sierra, 2006).

There are many factors that affect recurrent pregnancy loss, such as uterine structural abnormalities, endocrine factors like (thyroid function, polycystic ovary syndrome and prolactin anomalies), immunological factors and progesterone supplementation, infection, lifestyle, sperm DNA damage and inherited thrombophilia (Homer, 2019). Certain high-fever sicknesses like lupus have also been associated with RPL (Carp et al., 2002; Di Micco et al., 2007; Eldor, 2001; Prandoni, Tormene, Simioni, & Girolami, 2001). There are a number of reports pointing to the thrombophilia phenomena as one of the possible causes for RPL cases, particularly in situations where other RPL associated abnormalities (i.e. endocrine disorders including anovulation and diabetes, and uterine distortions and hereditary factors, etc.) are eliminated. Another potential cause is the presence of a series of markers for a hypercoagulable state, such as D-dimer and prothrombin markers (de Boer, ten Cate, Sturk, Borm, & Treffers, 1989). These cases may lead to development of venous thromboembolism (VTE) during pregnancy (Colman-Brochu, 2004). The risks associated with such cases also increase in cases of thrombotic hazard factors, like the acquired thrombophilia cases (Robertson et al., 2006).

### 1.2 Inherited Thrombophilia and Pregnancy Loss

Thrombophilia has been associated with RPL in many studies showing that it is one of the factors causing miscarriages, making up for 40% of the cases, particularly during the early periods of the pregnancy (Barut et al., 2018; Bigdeli et al., 2018). On the other hand, a number of studies have opposing results showing that RPL is not associated with inherited thrombophilia (Abu-Heija, 2014; Parand et al., 2013). The normal physiological changes taking place during the pregnancy elevate the venous thromboembolic event (VTE) risks. Combined with other potential factors like acquired or inherited thrombophilia, the risks accumulate high enough to make it a serious concern (Arachchillage & Makris, 2019; Battinelli, Marshall, & Connors, 2013; Heit et al., 2005). That being said, the exact frequency of thrombophilia is difficult to determine due to different criteria set forth for diagnosis, and the diversity of the baseline values based on ethnicity amongst the patient groups (D'Uva et al., 2008). The distinction between acquired, inherited, and consolidated thrombophilia is well established in clinical practice (Franchini & Veneri, 2005).

Acquired thrombophilia cases are usually due to insufficient clotting inhibitor presence, or their variations in their functionality, both cases which lead to tendency for hypercoagulation (Pickering, Marriott, & Regan, 2001). For inherited thrombophilia, on the other hand, number of single nucleotide polymorphisms (SNPs) have been associated with inherited thrombophilia as the risk factors for VTE cases (Stefanski et al., 2018). The three most SNPs are Factor *V* Leiden *(FVL*), Prothrombin *G20210A (PT G20210A*), and methylene tetrahydrofolate reductase *(MTHFR*) (Bigdeli et al., 2018; Lopez-Jimenez et al., 2016). Amongst these, Factor *V* Leiden corresponds to two-third of actuated protein C obstruction cases, which in turn is one of the risk factors associated with RPL (Nasibeh Roozbeh et al., 2017; Rai et al., 2001). This has been established in the end of the last millennia, where lack of various clotting inhibitors (like protein-S and protein-C) was

strongly associated with RPL cases (Dugas & Slane, 2019; ten Kate & van der Meer, 2008). In the last decade, *PAI-1* 4G/5G gene variations have also been pointed out as a potential cause of RPL, as it was discovered that they could potentially cause hypofibrinolysis, which in turn leads to hypercoagulation tendency (Adler et al., 2018). Some studies also showed a relationship between 4G/4G gene variation and RPL, particularly in cases where the anamnesis contains VTE (Regina Komsa-Penkova et al., 2013; Coulam et al., 2008; D'Uva et al., 2010). Overall, inherited thrombophilia refers to hereditary causes leading to blood coagulation led by hypercoagulation state. This situation may lead to abnormal fetal implantation, and therefore, unpredictable pregnancy losses (Ebrahimzadeh-Vesal et al., 2014; Younis, Ohel, Brenner, & Ben-Ami, 1997).

Even though there are many studies reporting that acquired thrombophilia is associated with repeated fetal loss, others failed to confirm this idea (Dizon-Townson et al., 2005; Silver et al., 2010). These studies suggested that RPL is not associated with FVL and Prothrombin *G20210A* (Bigdeli et al., 2018; Parand et al., 2013), *MTHFR C677T* (Isaoglu et al., 2014; Sah et al., 2018), *MTHFR C677T* and *A1298C* (Hwang et al., 2017), *Fibrinogen beta chain* (Karami, Askari, & Modarressi, 2018) and *Plasminogen Activator Inhibitor Type 1* (Bertinato et al., 2013). The *Factor XIII* polymorphism is related with RPL in Asian population but there is no relationship between *Factor XIII* and RPL in European and the South American population (Jung, Kim, Song, & Choi, 2017). Although RPL patients are not being strictly monitored for thrombophilia, with the exceptions of cases where other VTE present (Arachchillage & Makris, 2019; El Hachem et al., 2017; Practice Committee of the American Society for Reproductive, 2012; Toth et al., 2018), these patients are routinely screened in some countries, such as in Iraq. Furthermore, the RPL patients are sometimes prescribed antithrombotic medication without scanning for thrombophilic anomalies (Arachchillage & Makris, 2019).

### 1.2.1. Factor *V* Leiden

Factor *V* Leiden thrombophilia cases occur when the initiated protein C (APC) cycle is encountered with an insufficient anticoagulant response, leading to increased risk of VTE, which is the most commonly encountered as deep VTE (DVT) (Kujovich, 2011). An alteration of the nucleotide at 1961 position of factor *V* gene from guanine to adenine has been associated with RPL (Albagoush & Schmidt, 2020; Federici & Al-Mondhiry, 2019; Jusic, Balic, Avdic, Podanin, & Balic, 2018; Kardi, Yousefian, Allahveisi, & Alaee, 2018; Mierla et al., 2012). This SNP in inherited thromboembolism have been associated with RPL (Barut et al., 2018). This polymorphism causes the substitution of Arg50Gln amine-corroder enzyme, which in turn causes lack of Protein C and reduced clotting prevention capability (Federici & Al-Mondhiry, 2019; Rosendaal, Koster, Vandenbroucke, & Reitsma, 1995). APC is a special anticoagulant protein structure that inhibits factor *V* and *VIII*, helping prevent further clot formations (Dahlback, 2008; Segers, Dahlback, & Nicolaes, 2007). This type of thrombophilia is usually accompanied by a VTE history and/or aspiratory embolism. This is particularly a concern for pregnant women with VTE history or family history of intermittent thrombosis (Duhl et al., 2007; Ebrahimzadeh-Vesal et al., 2014; Grody et al., 2001).

### 1.2.2. Prothrombin (G20210A)

An SNP, G20210A (rs1799963), within the prothrombin gene also known as coagulation factor *II (FII)* is located at the nucleotide position of 20210 within the 3' untranslated, non-coding region on chromosome 11 (Jiang et al., 2014). In the European population, frequency of A allele was reported to be (2%) for this polymorphism which is rarer than the Factor *V* Leiden polymorphism (Jiang et al., 2014). On the other hand, it is almost non-existent in individuals of African or Asian ancestry (3.5%) (Jiang et al., 2014). In the Iraqi population, Prothrombin (G20210A) and RPL

have not been associated (Shaima S.  et al., 2018). The nucleotide position of 20210 Prothrombin gene from guanine to adenine has been associated with RPL (Federici & Al-Mondhiry, 2019; Kardi et al., 2018). A number of studies reported that there is an increased risk of recurrent pregnancy loss in woman with inherited factor *V* Leiden and *prothrombin G20210A* polymorphisms (Jusic et al., 2018; MahdiehKamali et al., 2018). On the other hand, a number of studies have opposing results showing that RPL is not associated with *FVL and Prothrombin G20210A* (Bigdeli et al., 2018; Parand et al., 2013). Since prothrombin is the antecedent of thrombin, which in turn controls the blood coagulation events, the increased plasma protein levels are observed in those with G20210A substitution with an hypercoagulation risk, which in turn translates to two to four times higher VTE risk (Jiang et al., 2014; Rosendaal, 2005).

### 1.2.3. Factor *XIII*

Fibrin stabilizing factor, also known as factor *XIII (FXIII),* plays a key role in coagulation events and fibrinolysis processes. *FXIII* is normally found idly in the blood circulation in two configurations called subunits A and B. The latter version is encoded in chromosome 1q31-32, while the former is encoded in 6p24-25. SNP (rs5958) at (chr6:6318562) position of factor *XIII* gene (C>A / C>T) has been associated with RPL (Ariens et al., 2002; Kreutz et al., 2014). *FXIII*-A functions with the transglutaminase action, which inhibits the thrombin-initiated fibrinolysis events. This process takes place to ensure that the fibrin coagulation is safe in terms of lysis risk (Wells, Anderson, Scarvelis, Doucette, & Gagnon, 2006). Most commonly type associated with thrombotic cases is Val34Leu gene variant in *FXIII* (V34L) (IvanaJoksic et al., 2019). *FXIII* Val34Leu polymorphism may damage fibrinolysis and it has been reported that there is a general risk of recurrent pregnancy loss in females homozygous for *FXIII* 34Leu also in compound

heterozygotes of *FXIII* 34Leu and hypofibrinolytic type 4G of current 4G/5G *(PAI-1)* polymorphism (Dossenbach-Glaninger et al., 2013).

### 1.2.4. Fibrinogen beta chain

Fibrinogen is a dimer of three polypeptides known as alpha, beta, and gamma. There are a small number of polymorphisms that may lead to increased fibrinogen levels in circulation, and most of them are located within the uncoded regions. Most of these polymorphisms are within the Bcl-1 allele in the 3'UTR section of the beta chain where gamma and alpha polymorphism takes place (at 455 location of the 5' promoter of the beta-gene) (Manal G. Al-Astal, Fadel A. Sharif, 2014; Lane & Grant, 2000).

Fibrinogen beta is another key element of the coagulation pathway and it functions in mediating the endothelial movement and platelet aggregation (Voetsch & Loscalzo, 2004). It has been shown that variations of *FGB* gene (rs5918 T > C and rs1800790 G > A) has been associated with RPL (Karami et al., 2018). *FGB* gene leads to increase in fibrinogen levels and platelet coagulation is performed excessively, leading to different thromboembolic risks in coronary supply path (Karami et al., 2018; Leander et al., 2002).

Another variation of G to A taking place at the 448 nucleotide causes an arginine to lysine alteration at the $13^{th}$ amino acid related to the C-terminal of the beta chains. Another common polymorphism is the C to T variation taking place at the 148 location of the 5' promoter region of the beta gene (Kottke-Marchant, 2002).

Investigations on the prevalence of beta-fibrinogen G/A polymorphism in patients with RPL in different populations have inconsistent results, while some researchers have linked this polymorphism and RPL (Jeddi-Tehrani et al., 2011; Karami et al., 2018; Ticconi et al., 2011;

Torabi et al., 2012), others reported no significant relationship (Maziri P et al., 2017; Ozdemir et al., 2012).

### 1.2.5. Plasminogen activator inhibitor-1

Plasminogen activator inhibitor-I *(PAI-1)* is a key glycoprotein with a molecular weight of 50 kDa and acts as a fibrinolysis inhibitor. Plasminogen's natural transformation to plasmin is an important step of the fibrinolytic cycle, which is mediated by agents like tissue plasminogen (t-PA) and urokinase plasminogen (u-PA) (Kuhli et al., 2005). *PAI-1* acts by performing its t-PA inhibitory effects rather quickly, and this fact is used by the metabolism to manage the clot formation processes. The plasma and placental levels of *PAI-1* in women with preeclampsia was higher than that of infertile women altogether (Shakarami, Akbari, & Zare Karizi, 2015). There have been controversial studies reporting the association of *PAI-1* variations rs2227631(-844G>A), rs1799889 (-675 4G/5G), rs6092 (43G>A), rs2227694 (9785G>A), and rs7242 (11053T>G) (Jeon et al., 2013). The association between PAI-1 gene polymorphisms (PAI-1-844G/A and PAI-1-675G/A) and the (RPL) is significant and controversial (Barut et al., 2018; Bigdeli et al., 2018; Chen, Nie, & Lu, 2015).

### 1.2.6. *MTHFR*

*MTHFR* enzyme is one of the important agents in amino-acid cycles, particularly in transformation of homocysteine into methionine. Hereditary polymorphisms in *MTHFR* gene can cause reduced activity or complete absence of its activity, which in turn would result in increased homocysteine activity. This particularly holds for patients with lower folate levels (Holmes et al., 2011). It has been suggested that use of folic acid every day may normalize the homocysteine levels, however this is yet to be confirmed (Shiran et al., 2015).

A common alteration in the *MTHFR* gene is C to T variation of the 677 polymorphisms (c.665C>T, rs1801133). This polymorphism causes the catalyst enzyme to become thermolabile, meaning it functions with reduced activity in higher temperatures. If this variation occurs as a TT homozygous alteration, it usually causes increased homocysteine levels accompanied by lowered serum folate levels (DhatriMadduru et al., 2019; Dean, 2012). Another *MTHFR* alteration is the A to C mutation at position 1298 (c.1286A>C, rs1801131) which doesn't cause increased homocysteine levels (DhatriMadduru et al., 2019).

The most common genetic polymorphisms of *MTHFR* are at position 677 (rs1801133 (C>T) and 1298 rs1801131 (A>C) which have been widely addressed in RPL cases (DhatriMadduru et al., 2019).

### 1.3. Aim of study:

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

## CHAPTER TWO

## 2. METHODOLOGY, RESEARCH DESING

Ethical approval was granted by the Near East University Institutional Review Board (YDU/2019/71-865).

### 2.1Materials

### 2-1.1 Instruments:

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

**Table 2.1** Utilized instruments, brands and manufactures

| NO. | Equipment | Brand | Origin |
|---|---|---|---|
| 1 | Laminar air flow hood | Bioquel | UK |
| 2 | Water bath | Thermostatic | Taiwan |
| 3 | Sensitive balance | Sartorius | Germany |
| 4 | Nano Drop Spectrophotometer | Thermo Scientific | USA |
| 5 | Micropipettes | Eppendorf | Germany |
| 6 | Eppendorf tube | Eppendorf | Germany |
| 7 | Centrifuge 13500 | Datthan Scientific | Korea |
| 8 | Autoclave | Binder | Germany |
| 9 | UV-Documentation | IVB | Germany |
| 10 | Gel electrophoresis instrument | GFL | Germany |
| 11 | PCR system (Polymerase Chain Reaction) Thermo cycler | Eppendorf | Germany |

**Table 2.2** Table listing the chemicals including kits, brands and manufactures.

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

## 2.2 Methods:

### 2.2.1. Sample collection:

The blood samples were collected from the Maternity Teaching Hospital. The study included 50 females with recurrent abortion and 30 healthy females in Erbil city-Iraq. The patients were included in the study if they were experiencing more than two miscarriages in the first trimester of pregnancy and patients were excluded if they had just one abortion or none in the sample group. In the control group, only the patients who did not have any spontaneous abortions were included in the study. In both groups, no endometrial anomalies were reported for the patients. A questionnaire form was completed by each patient at the time of blood withdrawal (Appendix).

### 2.2.2. DNA extraction from whole blood

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

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

### 2.2.3. Primer Design

A primer is strand of nucleic acid that serves as a starting point for DNA synthesis. Selection of primers was depending on the target region in the sequence. Seven pairs of primers were designed by Online primer program (http://www.nlm.nih.gov/tool/primer-blast) targeting the SNPs of interest.

**Table 2.3** The sequences of the forward and reverse primers.

| | |
|---|---|
| MTHFR-C677T- Forward | 5′- TGAAGGAGAAGGTGTCTGCGGGA-3′ |
| MTHFR-C677T-Reverse | 5′- AGGACGGTGCGGTGAGAGTG-3′ |
| MTHFR-1298-A-Forward | 5′- GGAGCTGACCAGTGAAGA-3′ |
| MTHFR-1298-A- Reverse | 5′- TGTGACCATTCCGGTTTG-3′ |
| MTHFR-1298-C- Forward | 5′- CTTTGGGGAGCTGAAGGA-3′ |
| MTHFR-1298-C- Reverse | 5′- ACAAAGACTTCAAAGACACTTG-3′ |
| PAI-4G- Forward | 5′- AGAGTCTGGACACGTGGGGA-3′ |
| PAI-5G- Forward | 5′- AGAGTCTGGACACGTGGGGG-3′ |
| PAI-4G&5G- Reverse | 5′- TGCAGCCAGCCACGTGATTGTCTAG-3′ |
| Internal control 4G&5G-Forward | 5′- AAGCTTTTACCATGGTAACCCCTGGT-3′ |
| FXIII-V-L- Forward | 5′- CATGCCTTTTCTGTTGTCTTC-3′ |
| FXIII-V-L- Reverse | 5′- TACCTTGCAGGTTGACGCCCCGGGGCACTA-3′ |
| Fibrinogen beta chain-G-A-Forward | 5′- AGGGTCTTTCTGATGTGT-3′ |
| Fibrinogen beta chain-G-A-Reverse | 5′- AAGTTAGGGCACTCCTCA-3′ |
| FV-G-A Forward | 5′- TCAGGCAGGAACAACACCAT-3′ |
| FV-G-A- Reverse | 5′- TACTTCAAGGACAAAATACCTGTAAAGCT-3′ |
| FII-G20210A- Forward | 5′- TCTAGAAACAGTTGCCTGGC-3′ |
| FII-G20210A-Reverse | 5′- ATAGCACTGGGAGCATTGAAGC-3′ |

### 2.2.4. PCR (polymerase chain reaction) for DNA amplification

PCR was used to amplify the regions of interest for each gene. The PCR amplification of the genes was performed with the designed pairs of primers spanning the target region. Each PCR was performed in a final volume of 25µl and the final concentrations of the primers were 0.5 µM. The reaction mixture of each PCR is shown in table 2-4 and the PCR conditions are shown in table 2-5, respectively.

**Table 2.4** The table showing the reaction mixture of PCR. Each PCR using different sets of primers followed the same components of the reaction.

| **Reaction mixture** | **Vol./reaction** |
|---|---|
| Taq 2x master mix Red | 18 µl |
| Forward primer (10µM) | 1 µl |
| Reverse primer (10µM) | 1 µl |
| Template DNA | 5µl |
| Total volume | 25 µl |

**Table 2.5** PCR Profile for all the polymorphisms

| | Temp. And time | Temp. And time | Temp. And time | Temp. And time | Temp. And time | Temp. And time | Temp. And time | # of cycle |
|---|---|---|---|---|---|---|---|---|
| PCR steps / SNP | MTHFR 677 C/T | MTHFR 1298 A/T | Factor V Leiden G/A | Prothrombin 20210 G/A | Factor XIII V/L | Fibrinogen beta chain A/G | PAI-1 G/G | 1 |
| Denaturing | 94 °C/30 second | 94 °C/30 second | 94 °C/30 second | 94 °C/30 second | 94 °C/30 second | 94 °C/30 second | 94 °C/30 second | 1 |
| Annealing | 58°C/30 second | 60°C/30 second | 60°C/30 second | 58°C/30 second | 63°C/30 second | 58°C/30 second | 58°C/30 second | 35 |
| Extension | 72°C/30 second | 72°C/30 second | 72°C/30 second | 72°C/30 second | 72°C/30 second | 56°C/30 second | 60°C/30 second | |

### 2.2.5.1 Restriction Fragment Length Polymorphism (RFLP) by using restriction enzyme for DNA cutting at specific site

RFLP is a molecular method of genetic analysis that allows identification based on unique patterns of restriction enzyme that cuts in specific regions of DNA with known variability. It can be used to genotype polymorphisms to distinguish between individuals. Hinf I (10 u/µl.2000 unit) restriction enzyme was used to genotype the *MTHFR C677T* genes. The reaction mixture of RFLP was prepared as shown in figure 2.6. The mixture was incubated at 37$^{o}$C for 16 hours or overnight. The product was analyzed using 2% agarose gel electrophoresis as described in section 2.2.6. Following the gel electrophoresis analysis, a product of 190bp corresponds to the CC genotype representing the wildtype, 190bp,160bp represent the CT heterozygote and 160bp represent the homozygotes TT genotype, respectively.

Hind III (10µ/ µl.5000unit) restriction enzyme was used to genotype the *Factor V Leiden* genes. The reaction mixture of RFLP was prepared as shown in figure 2.6. The mixture was incubated at 37$^{o}$C for 16 hours or overnight. The product was analyzed using 2% agarose gel electrophoresis as described in section 2.2.6. Following the gel electrophoresis analysis, a product of 240bp corresponds to the GG genotype representing the wildtype, 240bp,210bp representing the GA heterozygote homozygotes and 210bp representing the homozygotes AA genotypes, respectively.

Hind III (10 µ/ µl.5000 unit) restriction enzyme was used to genotype the *Prothrombin G20210A* genes. The reaction mixture of RFLP was prepared as shown in figure 2.6. The mixture was incubated at 37$^{o}$C for 16 hours or overnight. The product was analyzed using 2% agarose gel electrophoresis as described in section 2.2.6. Following the gel electrophoresis analysis, a product of 350bp corresponds to the GG genotype representing the wildtype, 350bp and 100bp

representing the GA heterozygote and 100bp representing the homozygotes AA genotypes, respectively.

HpyfI (10 µ/ µl.500 unit) restriction enzyme was used to genotype the *Factor XIII* genes. The reaction mixture of RFLP was prepared as shown in figure 2.6. The mixture was incubated at 37$^{o}$C for 16 hours or overnight. The product was analyzed using 2% agarose gel electrophoresis as described in section 2.2.6. Following the gel electrophoresis analysis, a product of 190bp corresponds to the V/V genotype representing the wildtype, 190bp and 160bp representing the V/L heterozygote and 160bp representing the homozygotes L/L genotypes, respectively.

BsuRI (10 µ/ µl.3000 unit) restriction enzyme was used to genotype the *Fibrinogen beta chain* genes. The reaction mixture of RFLP was prepared as shown in figure 2.6. The mixture was incubated at 37$^{o}$C for 16 hours or overnight. The product was analyzed using 2% agarose gel electrophoresis as described in section 2.2.6. Following the gel electrophoresis analysis, a product of 225bp,120bp corresponds to the GG genotype representing the wildtype, 335bp, 225pb and 120bp representing the AG heterozygote and 335bp representing the homozygotes AA genotypes, respectively.

**Table 2.6** The reaction mixtures of RFLP profile for *MTHFR C677T, factor V Leiden (R506Q), prothrombin (G20210A), factor XIII (V34L) and fibrinogen beta chain*.

| **Component** | **Vol./reaction** |
|---|---|
| Volume of each enzyme | 1 µl |
| PCR product | 10 µl |
| RFLP Buffer | 3 µl |
| Sterile water | 16 µl |
| Total volume | 30 µl |

### 2.2.5.2 Touchdown polymerase chain reaction (TD PCR)

A touchdown is the method of PCR that requires starting by high annealing temperature and progressively decreasing the annealing temperature for each PCR cycle. The higher annealing temperatures within the primary cycles of a touchdown confirm that only much specific base pairing occurs between the DNA and thus the primer, hence the primary sequence to be amplified is presumably to be the sequence of interest. Amplification of *MTHFR A1298C* gene was performed with this method.

The 10 µl of PCR product was analyzed using 2% agarose gel electrophoresis as described in section 2.2.6. Following the gel electrophoresis analysis, a product of 70bp corresponds to the AA genotype representing the wildtype, 70bp representing the AC homozygotes and 110bp representing the heterozygote CC genotypes, respectively.

### 2.2.5.3 Amplification Refractory Mutation System (ARMS)

ARMS is a method of PCR during which DNA is amplified by specific allele primers. It is an acutely useful method for recognition of point polymorphisms. ARMS was used for analysis of the *PAI-1* polymorphism.

The 10 µl of PCR product was analyzed using 2% agarose gel electrophoresis as described in section 2.2.6. Following the gel electrophoresis analysis, a product of two-band represents the 4G wild type allele (225bp, 140bp), one-band represent the 5G allele (225bp) and two-band represent the 5G alleles (225bp, 140bp), respectively.

### 2.2.6. Agarose Gel Electrophoresis

Two grams of agarose gel (Cinna Gencompanies) in 1X1000 TBE (Tris- Borate EDTA) buffer was prepared to analyses the RPLF products. One in ten dilution with distilled water and 5µg/ml ethidium bromide was used to visualize the PCR products. Five µl of PCR product and six µl of 100 bp molecular weight DNA Ladder were added to the wells. The gels were visualized by UV gel visualization systems.

### 2.3. Statistical analysis

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

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

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

## CHAPTER THREE

### 3.Result

This study included 50 women who were experiencing more than two miscarriages in the first trimester of pregnancy such as patient and 30 women who did not have any spontaneous abortions as control cases. All RPL women were in the age range of 19 to 43 years and number of miscarriages ranged from 2 to 10. The clinical pregnancy outcomes were investigated according to seven genotypes of thrombophilia polymorphisms, including *MTHFR C677T, MTHFR A1298C, Prothrombin G20210A, factor XIII, fibrinogen beta chain, PAI-1* and *factor V.*

The homozygote CC wildtype genotype of *MTHFR C677T* polymorphism was represented by the product band at 190bp (Figure 3.1a). The homozygote TT genotype of *MTHFR C677T* polymorphism was represented by the band at 160bp, whereas the heterozygote genotypes were represented by two bands at 190bp and 160bp, respectively (Figure 3.1b). The patient's allelic frequencies for *MTHFR C677T* gene were 64.0% for C/C wild type, 24.0% for heterozygote C/T and 12.0% for homozygote T/T, respectively (Table 3.1). The control group allelic frequencies for *MTHFR C677T* gene were 100.0% for C/C wild type, 0.0% for heterozygote C/T and 0.0% for homozygote T/T, respectively (Table 3.2).

Table 3.1: Results representing the frequency analysis of thrombophilia associated gene polymorphisms in patients with RPL.

| | | type | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | MTHFR C677T | | MTHFR A1298C | | Prothrombin G20210A | | Factor XIII | | Fibrinogen beta chain | | PAI-1 | | Factor V Leiden | |
| | | Count | % | count | % | Count | % | Count | % | Count | % | Count | % | Count | % |
| genes | Wild type (Normal) | 32 | 64.0% | 18 | 36.0% | 50 | 100.0% | 39 | 78.0% | 34 | 68.0% | 0 | 0.0% | 50 | 100.0% |
| | Heterozygote | 12 | 24.0% | 27 | 54.0% | 0 | 0.0% | 11 | 22.0% | 16 | 32.0% | 42 | 84.0% | 0 | 0.0% |
| | Homozygote | 6 | 12.0% | 5 | 10.0% | 0 | 0.0% | 0 | 0.0% | 0 | 0.0% | 8 | 16.0% | 0 | 0.0% |

Table 3.2: Results representing the frequency analysis of thrombophilia associated gene polymorphisms in control cases.

| | | type | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | MTHFR C677T | | MTHFR A1298C | | Prothrombin G20210A | | Factor XIII | | Fibrinogen beta chain | | PAI-1 | | Factor V Leiden | |
| | | Count | % | count | % | Count | % | Count | % | Count | % | Count | % | Count | % |
| genes | Wild type (Normal) | 30 | 100.0% | 30 | 100.0% | 30 | 100.0% | 30 | 100.0% | 30 | 100.0% | 30 | 100.0% | 30 | 100.0% |
| | Heterozygote | 0 | 0.0% | 0 | 0.0% | 0 | 0.0% | 0 | 0.0% | 0 | 0.0% | 0 | 0.0% | 0 | 0.0% |
| | Homozygote | 0 | 0.0% | 0 | 0.0% | 0 | 0.0% | 0 | 0.0% | 0 | 0.0% | 0 | 0.0% | 0 | 0.0% |

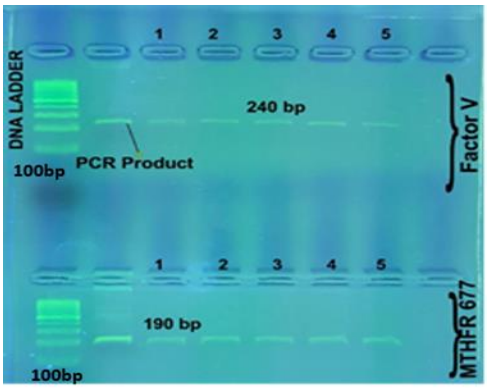



Figure 3-1a

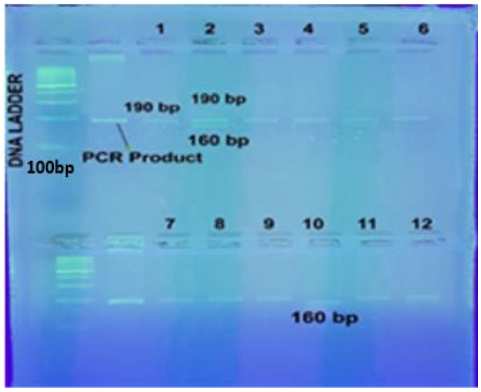



Figure 3-1b

Figure 3-1a The gel electrophoresis of the *MTHFR (C677T)* polymorphism showing the control cases. The first lane represents the DNA ladder, second lane PCR product prior of RFLP and from the third until seventh lanes are the number of control cases. Figure 3-1b the gel electrophoresis of the *MTHFR (C677T)* polymorphism showing the RPL patients' cases. The first lane represents DNA ladder, second lane PCR product prior of RFLP and from the third until last lanes represent number of RPL patients' cases. For all the analysis, 100bp ladder was used.

The homozygote AA wild type genotype of *MTHFR A1298C* polymorphism was represented by the product band at 70bp (Figure 3.2a). The homozygote CC genotype of *MTHFR A1298C* polymorphism was represented by the band at 110bp (Figure 3.2c), whereas the heterozygote AC genotypes were represented by two bands at 70bp and 110bp, respectively (Figure 3.2b and Figure 3.2c). The patients wild type allele for *MTHFR A1298C* was detected at a lower frequency compared to the wild type *MTHFR C677T* gene, in such A/A wild type was observed in 36.0% of the females with RPL, whereas the heterozygote A/C was 54.0% and the homozygote C/C was 10.0%, respectively (Table 3.1). The control group allelic frequencies for *MTHFR A1298C* gene were 100.0% for A/A wild type, 0.0% for heterozygote A/C and 0.0% for homozygote C/C, respectively (Table 3.2).

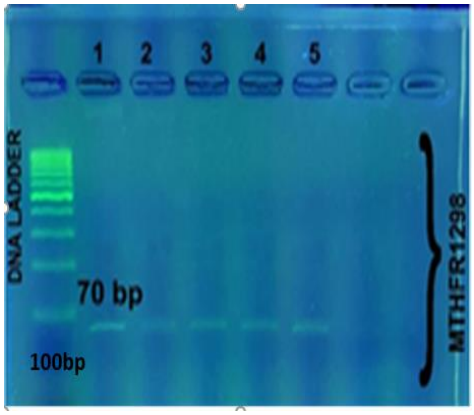



Figure 3-2a The gel electrophoresis of the *MTHFR A1298C* polymorphism showing the control cases A and C. The first lane represents DNA ladder, second lane until sixth lanes represent number of RPL control cases. For all the analysis, 100bp ladder was used.

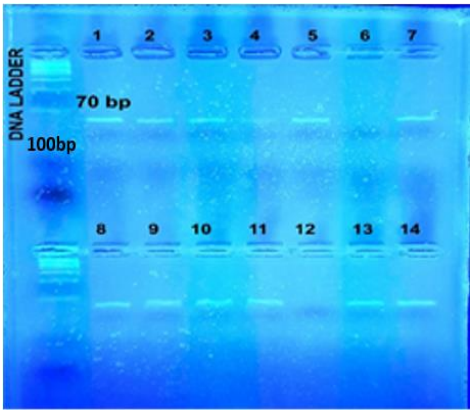



Figure 3-2b

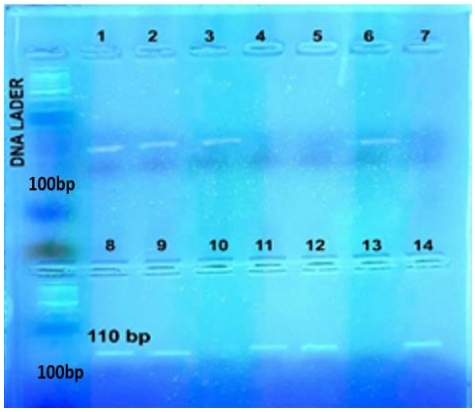



Figure 3-2c

Figure 3-2b The gel electrophoresis of the *MTHFR (A1298C)* polymorphism showing the RPL patients' cases (A). Figure (3-2c) the gel electrophoresis of the *(MTHFR A1298C)* polymorphism showing RPL patients' cases (C). The first lane represents DNA ladder, second lane until last lanes represent number of RPL patients' cases. For all the analysis, 100bp ladder was used.

The homozygote G/G wildtype genotype of *Prothrombin (G20210A)* polymorphism was represented by the product band at 350bp (Figure 3.3a). The wild type G/G genotype of *Prothrombin G20210A* were observed for all the patients. All of the patients for *Prothrombin (G20210A)* polymorphism had the genotype of G/G without any case with heterozygote GA and

homozygote AA genotype, respectively (Figure 3.3b). All of the control group for *Prothrombin (G20210A)* polymorphism had the genotype of G/G.

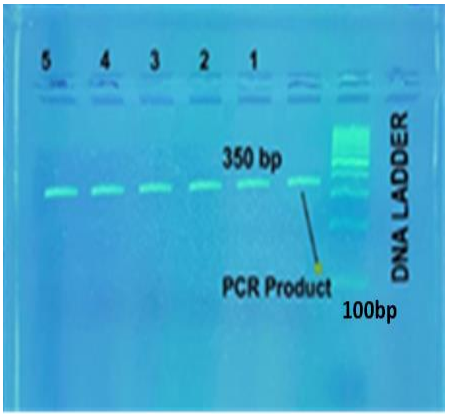



Figure 3-3a

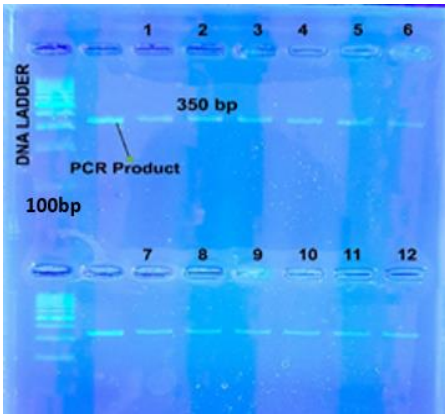



Figure 3-3b

Figure 3-3a The gel electrophoresis of the *Prothrombin G20210A*) polymorphism showing the control cases. The second lane represents DNA ladder, third lane PCR Product prior of RFLP and from the fourth until eighth lane is number of control cases. Figure 3-3b the gel electrophoresis of the *Prothrombin (G20210A*) polymorphism showing the RPL patients' cases. The first lane DNA ladder, second lane PCR product prior of RFLP and from the third until last lanes represent number of RPL patients' cases. For all the analysis, 100bp ladder was used.

The homozygote V/V wild type genotype of *Factor (XIII)* polymorphism was represented by the product band at 190bp (Figure 3.4a). The patient's allelic frequencies of *Factor XIII* ratio for V/V wild type was 78.0% and for heterozygote V/L was 32.0% with no homozygote L/L genotype, respectively (Table 3.1). The control group allelic frequencies of Factor XIII ratio for V/V wild type was 100.0%, for heterozygote V/L was 0.0% and for homozygote L/L was 0.0%, respectively (Table 3.2). The heterozygote genotypes of *Factor (XIII)* polymorphism were represented by two bands at 190bp and 160bp (Figure 3.4b).

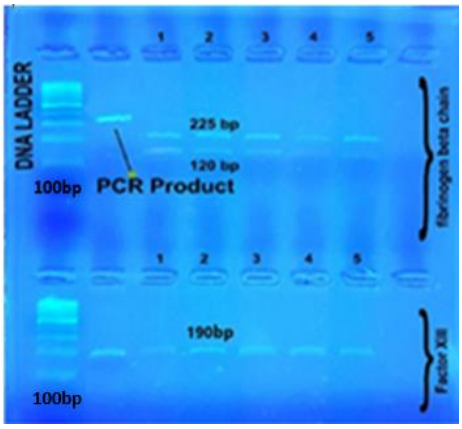



Figure 3-4a

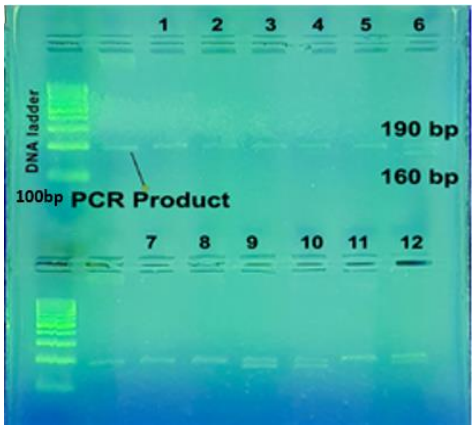



Figure 3-4b

Figure 3-4a The gel electrophoresis of the *Factor XIII* polymorphism showing the control cases. The first lane represents DNA ladder, second lane PCR Product prior of RFLP and from the third until seventh lane is number of control cases. Figure 3-4b the gel electrophoresis of the *Factor XIII* polymorphism showing the RPL patients' cases. The first lane DNA ladder, second lane PCR product prior of RFLP and from the third until the last lanes represent number of RPL patients' cases. For all the analysis, 100bp ladder was used.

The homozygote G/G wildtype genotype of *Fibrinogen Beta Chain* polymorphism was represented by two product band at 225bp and 120bp, respectively (Figure 3.5a). The heterozygote A/G genotypes of *Fibrinogen Beta Chain* polymorphism were represented by three bands at 335bp, 225bp and 120bp, respectively (Figure 3.5b). The patents allelic frequency of *Fibrinogen beta chain* was higher for the wild type G/G with 68.0% followed by the heterozygote A/G genotype with 22.0%, respectively. All of the control group for *Fibrinogen Beta Chain* polymorphism had the genotype of G/G wildtype, respectively (Table 3.1). Similarly, homozygote A/A genotype was not observed for these patients either.

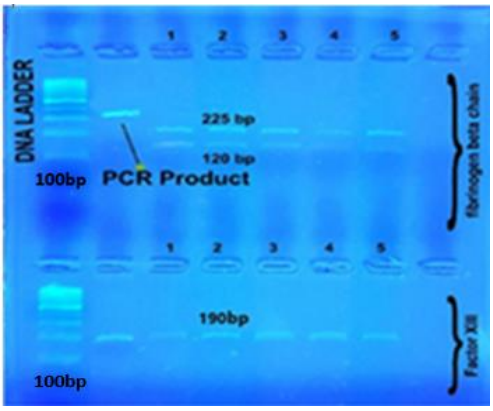



Figure 3-5a

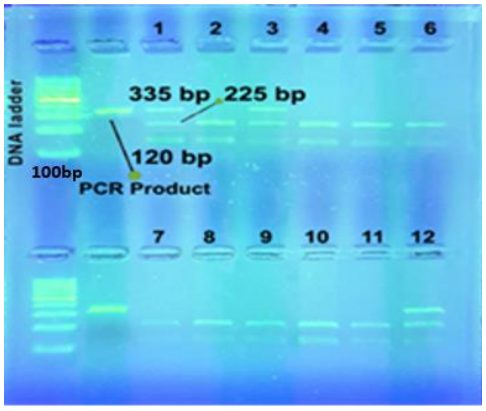



Figure 3-5b

Figure 3-5a The gel electrophoresis of the *Fibrinogen Beta Chai*n polymorphism showing the control cases. The first lane represents DNA ladder, second line PCR product prior of RFLP and from the third until seventh lane is number of control cases. Figure 3-5b the gel electrophoresis of the *Fibrinogen Beta Chain* polymorphism showing the RPL patients' cases. The first lane represents DNA ladder, second lane PCR product prior of RFLP and from the third until last lanes represent number of RPL patients' cases. For all the analysis, 100bp ladder was used.

The homozygote 5G/5G genotype of PAI-1 polymorphism was represented by three bands. One band represents the 4G allele (225bp) and two bands represent the 5G alleles (225bp, 140bp), respectively (Figure 3.6a and 3.6b). The heterozygote 4G/5G genotypes were represented by four bands, two bands for 4G allele (225bp, 140bp) and another two bands for 5G (225bp, 140bp) (Figure 3.6a and 3.6b). The homozygote 4G/4G wildtype genotype of PAI-1 polymorphism was represented by three bands. Two bands represent the 4G alleles (225bp, 140bp) and one bands represent the 5G allele (225bp). There was no patents wild type genotype of 4G/4G detected for *PAI-1,* however the heterozygote 4G/5G was detected to be 84.0%, and the homozygote 5G/5G was 16.0% respectively (Table 3.1). All of the control group for *PAI*-1 polymorphism had the genotype of 4G/4G wildtype (Figure 3.6c and 3.6d).

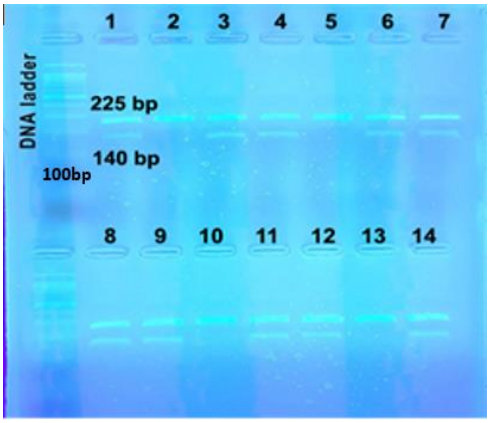



Figure 3-6a

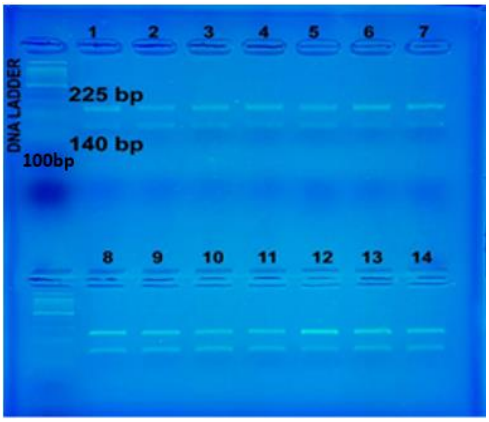



Figure 3-6b

Figure 3-6a The gel electrophoresis of the *PAI-1 (4G)* polymorphism showing the RPL patients' cases. Figure 3-6b the gel electrophoresis of the *PAI-1 (5G)* polymorphism showing the RPL patients' cases. The first lane represents DNA ladder, and the second until last lanes represent number of RPL patients. For all the analysis, 100bp ladder was used.

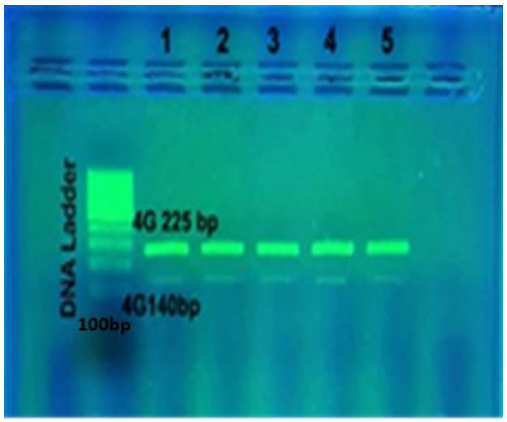



Figure 3-6c

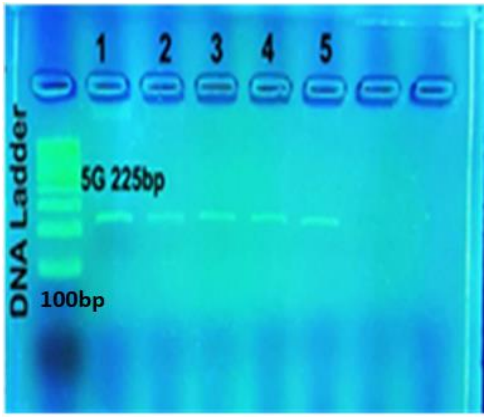



Figure 3-6d

Figure 3-6c The gel electrophoresis of the *PAI-1 (4G)* polymorphism showing the control cases. Figure 3-6d the gel electrophoresis of the *PAI-1 (5G*) polymorphism showing the control cases. The first lane represents DNA ladder, second lane until sixth lanes represent number of control cases. For all the analysis, 100bp ladder was used.

The homozygote G/G wildtype genotype of *Factor V* polymorphism was represented by the product band at 240bp (Figure 3.7a). All of patients had wild type G/G genotype of *Factor V* polymorphism without any case of heterozygote G/A and homozygote A/A genotype, respectively (Figure 3.7b). Thus, all the patients with the *Factor V Leiden* were detected to have the wild type

G/G genotype. all the control group with the Factor V Leiden were detected to have the wild type G/G genotype respectively (Table 3.2).

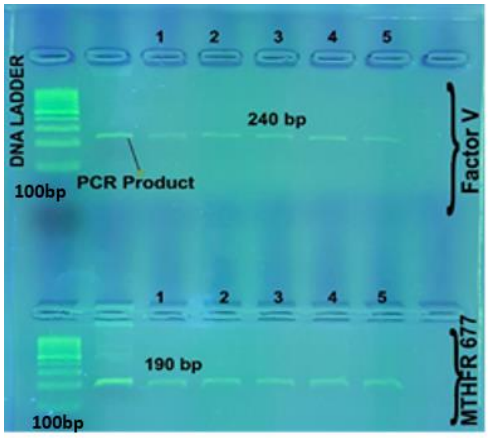



Figure 3-7a

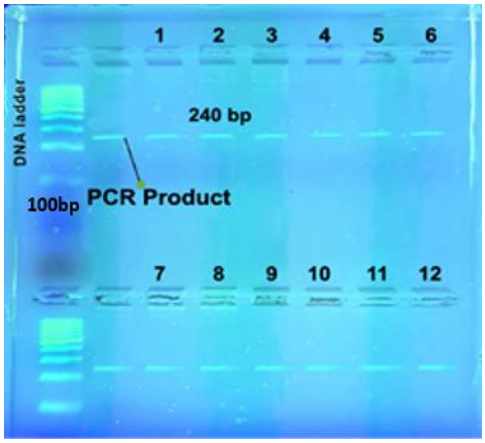



Figure 3-7b

Figure 3-7a The gel electrophoresis of the *Factor V* polymorphism showing the control cases the first lane represents DNA ladder, second lane PCR Product prior of RFLP and from the third until seventh lane represent the number of control cases. Figure 3-7b the gel electrophoresis of the *Factor V* polymorphism showing the RPL patients' cases. The first lane represents DNA ladder, second lane PCR product prior of RFLP and from the third until last lane is number of RPL patients' cases. For all the analysis, 100bp ladder was used.

Table 3.3 shows the relationship between each gene and the family history (mothers or siblings) of miscarriage. There was a statistical significance between the allelic frequencies of heterozygote and homozygote alleles of the *MTHFR C677T (*p=0.039) and *MTHFR A1298C* (p= 0.049). There was no statistical significance between the family history of miscarriage and the genotypes of the *Factor XIII, Fibrinogen beta chin* and *PAI-1* (p>0.05). No statistical analysis was performed for the *prothrombin G20210A and Factor V Leiden* since all the genotypes were the wild type. Generally, the results demonstrate that only *MTHFR C677T* and *MTHFR A1298C* have a

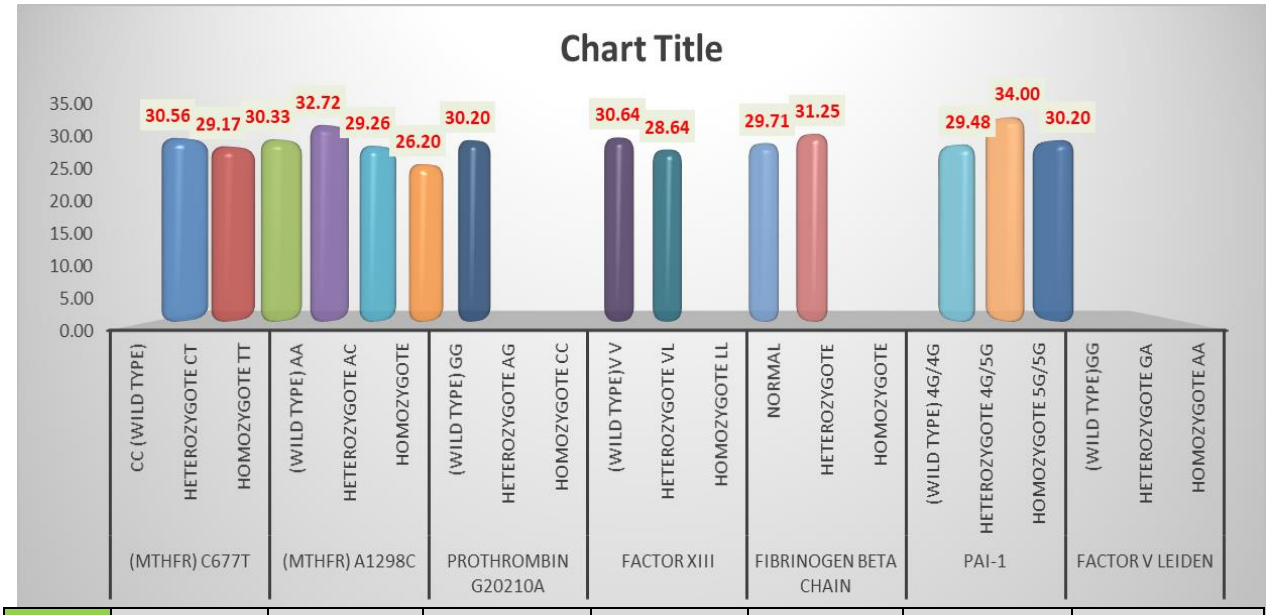
relationship with miscarriage in the family history and the other genes did not have a relationship with miscarriage in the family history.

Table 3.3 The results showing the frequency of each allele of the genes investigated according to the family history of miscarriages.

| type | genes | Family history (mothers or siblings) of miscarriage | | | | ch-squre (p-value) |
|---|---|---|---|---|---|---|
| | | Yes | | No | | |
| | | Count | % | Count | % | |
| MTHFR C677T | CC (Wild type) | 16 | 84.2% | 16 | 51.6% | 6.494 (0.039) * |
| | Heterozygote CT | 1 | 5.3% | 11 | 35.5% | |
| | Homozygote TT | 2 | 10.5% | 4 | 12.9% | |
| MTHFR A1298C | AA (Wild type) | 10 | 52.6% | 8 | 25.8% | 6.158 (0.046) * |
| | Heterozygote AC | 9 | 47.4% | 17 | 54.8% | |
| | Homozygote CC | 0 | 0.0% | 6 | 19.4% | |
| Prothrombin G20210A | GG (Wild type) | 19 | 100.0% | 31 | 100.0% | N/A |
| | Heterozygote GA | 0 | 0.0% | 0 | 0.0% | |
| | Homozygote AA | 0 | 0.0% | 0 | 0.0% | |
| Factor XIII | V V (Wild type) | 15 | 78.9% | 24 | 77.4% | 0.016 (0.899) |
| | Heterozygote VL | 4 | 21.1% | 7 | 22.6% | |
| | Homozygote LL | 0 | 0.0% | 0 | 0.0% | |
| Fibrinogen beta chain | GG (Wild type) | 10 | 52.6% | 24 | 77.4% | 3.326 (0.068) |
| | Heterozygote AG | 9 | 47.4% | 7 | 22.6% | |
| | Homozygote AA | 0 | 0.0% | 0 | 0.0% | |
| PAI-1 | 4G/4G (Wild type) | 0 | 0.0% | 0 | 0.0% | 0.683 (0.409) |
| | Heterozygote 4G/5G | 17 | 89.5% | 25 | 80.6% | |
| | Homozygote5G/5G | 2 | 10.5% | 6 | 19.4% | |
| Factor V Leiden | GG (Wild type) | 19 | 100.0% | 31 | 100.0% | N/A |
| | Heterozygote GA | 0 | 0.0% | 0 | 0.0% | |
| | Homozygote AA | 0 | 0.0% | 0 | 0.0% | |

Further investigation involved analysis of each SNP genotype and the maternal age (Figure 3.8). There was no statistical significance between the maternal age and the genotypes of the *MTHFR C677T, MTHFRA1298C, Factor XIII, Fibrinogen beta chain* and *PAI-1* ($p>0.05$). No statistical analysis was performed for the *prothrombin G20210A* and *Factor V Leiden*, since all the genotypes were the wild type. The results showed that there is no relationship between each gene and the age of patients.

**Figure 3.8** relationship between each gene and the age of patients.




| | | | | | | |
|---|---|---|---|---|---|---|
| F-test (p-value) | **0.193 (0.825)** | **2.728 (0.076)** | **N/A** | **0.801 (0.375)** | **0.600 (0.442)** | **3.364 (0.073)** | **N/A** |

Further investigation involved analysis of genotypes of each SNP and the number of abortions (Table 3.4). There was statistical significance between the number abortions and the genotype of the *MTHFR C677T* ($p = 0.005$) and *PAI-1* ($p = 0.018$) and there was no statistical significance between the number abortions and the genotypes of the *MTHFRA1298C, Factor XIII* and *Fibrinogen beta chin*a ($p>0.05$). No statistical analysis was performed for the for the *prothrombin*

*G20210A and Factor V Leiden*, since all the genotypes were the wild type. The results showed that only the *MTHFR C677T* and *PAI-1* had a relationship with number of abortions.

**Table 3.4** The association between each SNP genotype and the number of abortions is shown.

| Type | Genes | NO.Abortion | | | |
|---|---|---|---|---|---|
| | | Count | Mean | Standard Deviation | F-test (p-value) |
| MTHFR 677 C>T | CC (wild type) | 32 | 2.50 | 0.80 | 6.058 (0.005)* |
| | Heterozygote CT | 12 | 4.58 | 3.42 | |
| | Homozygote TT | 6 | 2.67 | 0.52 | |
| MTHFR 1298 A>C | AA (wild type) | 18 | 3.11 | 2.03 | 0.327 (0.723) |
| | Heterozygote AC | 27 | 2.85 | 1.59 | |
| | Homozygote CC | 5 | 3.60 | 3.58 | |
| FII (Prothrombin) 20210 G>A | GG (wild type) | 50 | 3.02 | 1.96 | N/A |
| | Heterozygote GA | 0 | | | |
| | Homozygote AA | 0 | | | |
| Factor XIII | V V (Wild type) | 39 | 2.90 | 1.87 | 0.686 (0.412) |
| | Heterozygote VL | 11 | 3.45 | 2.30 | |
| | Homozygote LL | 0 | | | |
| Fibrinogen beta chain | GG (wild type) | 34 | 3.12 | 2.25 | 0.256 (0.613) |
| | Heterozygote AG | 16 | 2.81 | 1.17 | |
| | Homozygote AA | 0 | | | |
| PAI-1G/G | 4G/4G (Wild type) | 0 | | | 5.956 (0.018)* |
| | Heterozygote 4G/5G | 42 | 2.74 | 1.75 | |
| | Homozygote 5G/5G | 8 | 4.50 | 2.45 | |
| Factor V Leiden | GG (wild type) | 50 | 3.02 | 1.96 | N/A |
| | Heterozygote GA | 0 | | | |
| | Homozygote AA | 0 | | | |

Statistical analysis was performed to investigate each genotype of the SNPs within the genes in RPL and the control group (Appendix tables 3.5, 3.6, 3.7, 3.8, 3.9, 3.10 and 3.11). The results showed that the wild type genotype was observed significantly more in the control group compared to the RPL group (p=0.000; *MTHFR C677T, MTHFRA1298C, Factor XIII, Fibrinogen beta chain, PAI-1, prothrombin G20210A and Factor V Leiden*). The outcome of all of the genotypes indicate an association between SNPs in RPL compared to the control group.

## CHAPER FOUR

### 4.1 Discussion

Many factors are believed to contribute to the spontaneous abortion cases, such as embryo aneuploidies, hormonal problems, or uterine anomalies (Brown, 2008; Rouse et al., 2017). The aim of this project was to investigate the polymorphisms within seven genes which had effective connection with number of abortion when it has been compared with control cases (Bigdeli et al., 2018; Barut et al., 2018; Kamali et al., 2018) . In addition, the significance of the thrombophilia cases in women with recurrent miscarriages were analyzed with regards to the polymorphisms of the genes. Association of genotypes of these polymorphisms in RPL patients can help develop strategies for early diagnosis and treatment of recurrent pregnancy loss cases (Bigdeli et al., 2018). Even though some studies suggest a strong association, others failed to show this in different populations (Caroline et al., 2014; Zonouzi, A. P. et al; 2013). In clinical basis, the SNPs associated with thrombophilia are routinely genotyped in females with miscarriages in Iraq. Since the association between these polymorphisms and the RPL has been reported to be population specific (Kamali et al., 2018), the aim of this study was to investigate the frequency and correlation of seven polymorphisms associated with thrombophilia and RPL in aged matched Iraqi population.

In the present study, the analysis of data collected regarding the gene known as the *MTHFR* C677T has shown that the maternal polymorphisms of the gene have a statistically significant relationship with recurrent pregnancy loss (RPL) cases, as also previously reported (Yang et al., 2016). Spontaneous abortion cases in humans occur as a result of sophisticated interactions between a range of genetic factors and certain environmental conditions (Zetterberg, 2004). One of the most prominent examples of such interactions is the clear correlation between the heightened homocysteine content and deficiencies in the neural tubes. This phenomenon has led the

researchers to believe that increased homocysteine levels are potentially embryotoxic and could have negative effect on fetal viability. The researchers also claimed that polymorphic TC genotypes of rs1801133 of *MTHFR* structures are amongst the risk factors for a series of developmental diseases like neural tube defects, orofacial clefts, and even Down syndrome. The results of this study showed that CT genotype of *MTHFR C677T* had a significant impact on the number of abortions among the patients. Studies have shown that out of 50 women, 32 had the CC (wild type) genotype, while the number of patients with heterozygote and homozygote was 12 and 6 respectively, which had a great relationship with the family history of miscarriage. In concordance with the results of this study investigation of 50 patients in Duhok-Iraq showed that there is a relationship between (CT and TT) genotype *MTHFR C677T* and RPL (Shaima S. et al., 2018).

In this study, AC and CC genotype of *MTHFR A1298C* did not show any association with recurrent pregnancy loss when compared with non-control cases, our results was coincident with the previously published studies showing the effects of *MTHFR A1298C* on recurrent abortion (Cao et al., 2013). The researchers used a total of 1163 RPL cases and a control group of 1061 individuals, and analyzed the random-effect rates for CC versus total genotypes ($p = 0.3456$), for CC + AC versus total genotypes ($P = 0.0833$) and for C versus total alleles ($p = 0.7112$). As such, the researchers claimed no correlation between *MTHFR A1298C* polymorphisms and RPL cases. This finding is in line with the results of this study regarding this specific gene polymorphism. On the other hand, in this study when *MTHFR A1298C* was compared with control cases, the result showed that *MTHFR A1298C* was associated with RPL. Previously published studies investigating 100 Syrian women with recurrent abortion reported that CC genotype for *MTHFR A1298C* either

alone or both CT genotypes have a high risk of RPL in Syrian women (Al-Achkar et al., 2017), that is consistent with our result.

In this study, the patients had the GG (wild type) allele for the *Prothrombin G20210A* and *Factor V Leiden*. The results of this study are consistent with the results of the study that total of 4,167 cases were analyzed for the *prothrombin G20210A* polymorphisms. They found only of 157 (3.8%) women in their study group had the *Prothrombin* gene polymorphism (156 heterozygous and one homozygous) (Silver et al., 2010). The researchers have reported that the rate of pregnancy loss was similar whether the women had the *Prothrombin G20210A* mutation or not (Silver et al., 2010). This result was cross analyzed with multivariance analysis where numerous factors like patient age, race, RPL history, small of gestational age (SGA) neonate events, and thromboembolism history. None of these parameters showed and association (Silver et al., 2010). The researchers therefore concluded that *Prothrombin G20210A* polymorphism had no relationship with RPL, preeclampsia, or abruptions. In this study, a very small variance has occurred for women with heterozygotes and homozygotes of this polymorphism (Silver et al., 2010; Cardona, H. et al., 2012). This data indicates that it might be irrelevant to monitor women in terms of these polymorphism, particularly if they don't have a history of thrombosis. The association of *Prothrombin G20210A* polymorphism with RPL was also investigated in 50 Duhok-Iraqi patients (Shaima S. et al., 2018) and 70 Baghdad-Iraqi patients, 40 of which were patient and 30 were in control (Dhuha Salim Namaa et al., 2016). In both Iraqi populations, there was no statistical connection between *Prothrombin G20210A* polymorphism and RPL cases.

In this study *Factor XIII* did not show any association with recurrent pregnancy loss when compared with non-control cases, but when dealing with control cases it has great association with RPL. This finding is also supported by previously published study investigating 140 women in

Iran with recurrent abortion, showing a relationship between Factor XIII and recurrent pregnancy losses (Seyed Mehdi Sajjadi et al., 2016).

The results of this study showed that the *factor V Leiden* polymorphisms had not been associated with pregnancy loss when compared with control and RPL cases. Previously published studies in the Iraqi population supports these findings (Shaima S. et al., 2018, Dhuha Salim Namaa et al., 2016). Furthermore, similar results were also observed in Indian population (Reddy et al., 2019).

According to the result of this study plasminogen activator inhibitor-1 *(PAI-1)* −675 4G/5G, *beta fibrinogen (BF)* −455G/A have an association with recurrent pregnancy loss when compared with the control cases. In concordance with these results, the correlation of the plasminogen activator inhibitor-1 *(PAI-1)* 675 4G/5G, *beta fibrinogen (BF*;−455G/A, integrin beta 3 (ITGB3; 1565T/C & 1298A/C) polymorphisms with RPL were performed previously showing an association between *BF* −455G/A genotype and RPL (Jeddi-Tehrani et al., 2011). Consequently, they concluded that it is important to check thrombophilia screening in patients with RPL.

Moreover, in this study *beta fibrinogen (BF)* −455G/A did not show a relation with recurrent pregnancy loss when compared with non-control cases. These results were also supported in Iranian population in 110 RPL and 110 control patients, respectively (Karami et al., 2018).

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

patients in Iraq. Thus, if it is shown that there is no association between these polymorphisms with RPL, there would be significant labor and cost deduction in the healthcare systems.

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

## 4.2. Conclusions

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

The study also investigated a series of other variables like age, family history and number of previous miscarriages, with the above-mentioned polymorphisms and RPL occurrence. The results of this analysis showed that there were no statistical correlations between patient age and polymorphisms of genes but a relationship was detected between two *MTHFR* polymorphisms (namely the C677T and A1298C) and family history of miscarriages. Inter-relationships between the genes were also examined, revealing that the presence of *MTHFR C677T* and *Plasminogen activator inhibitor-1(PAI-1)* was in fact correlated with RPL. The results of this analysis showed that there were no statistical correlations between patient age, family history and number of previous miscarriages (non-control cases) with the *Prothrombin (G20210A), Factor XIII, Fibrinogen beta chain* and *Factor V Leiden.* Comparison of all the genes individually with the control group, on the other hand, revealed that all the genes are correlated with RPL cases in a statistically meaningful manner.

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## Appendix

## Questioner form

- Serial Number of samples:
- Date of collection:
- Patient Name:

- Age:
- Address:

- No of aborted:
- Fetal age at abortion:
- Drug used during pregnancy:

- Source of sample:
- Family history:

Table (3.5) comparable *(MTHFER C677T)* and number of abortions with control cases.

NO.Abortion

| | N | Mean | Std. Deviation | Std. Error | F(p-value) |
|---|---|---|---|---|---|
| CC (wild type) | 32 | 3.00 | 2.000 | 0.354 | 7.702 (0.000)** |
| Heterozygote CT | 12 | 3.25 | 2.379 | 0.687 | |
| Homozygote TT | 6 | 2.67 | 0.516 | 0.211 | |
| Control | 30 | 0.00 | 0.000 | 0.000 | |
| Total | 80 | 2.52 | 2.119 | 0.274 | |

Table (3.6) comparable *(MTHFER A1298C)* and number of abortions with control cases.

NO.Abortion

| | N | Mean | Std. Deviation | Std. Error | F(p-value) |
|---|---|---|---|---|---|
| AA (wild type) | 18 | 4.00 | 2.951 | 0.695 | 12.181 (0.000)** |
| Heterozygote AC | 27 | 2.58 | 0.703 | 0.138 | |
| Homozygote CC | 5 | 2.00 | 0.000 | 0.000 | |
| Control | 30 | 0.00 | 0.000 | 0.000 | |
| Total | 80 | 2.52 | 2.119 | 0.274 | |

Table (3.7) comparable *(Prothrombin G20210A)* and number of abortions with control cases.

NO.Abortion

| | N | Mean | Std. Deviation | Std. Error | F(p-value) |
|---|---|---|---|---|---|
| GG (wild type) | 50 | 3.02 | 1.964 | 0.278 | 23.326 (0.000)** |
| Control | 30 | 0.00 | 0.000 | 0.000 | |
| Total | 80 | 2.52 | 2.119 | 0.274 | |

Table (3.8) comparable *(Factor XIII)* and number of abortions with control cases.

| NO.Abortion | N | Mean | Std. Deviation | Std. Error | F(p-value) |
|---|---|---|---|---|---|
| V V (wild type) | 39 | 2.90 | 1.875 | 0.300 | 12.033 (0.000)** |
| Heterozygote VL | 11 | 3.45 | 2.296 | 0.692 | |
| Control | 30 | 0.00 | 0.000 | 0.000 | |
| Total | 80 | 2.52 | 2.119 | 0.274 | |

Table (3.9) comparable *(Fibrinogen beta chain)* and number of abortions with control cases.

| NO.Abortion | N | Mean | Std. Deviation | Std. Error | F(p-value) |
|---|---|---|---|---|---|
| GG (wild type) | 34 | 3.118 | 2.253 | 0.386 | 11.667 (0.000)** |
| Heterozygote AG | 16 | 2.813 | 1.167 | 0.292 | |
| Control | 30 | 0.000 | 0.000 | 0.000 | |
| Total | 80 | 2.517 | 2.119 | 0.274 | |

Table (3.10) comparable *(PAI-1)* and number of abortions with control cases.

| NO. Abortion | N | Mean | Std. Deviation | Std. Error | F(p-value) |
|---|---|---|---|---|---|
| Heterozygote 4G/5G | 42 | 2.74 | 1.754 | 0.271 | 16.421 (0.000)** |
| Homozygote 5G/5G | 8 | 4.50 | 2.449 | 0.866 | |
| Control | 30 | 0.00 | 0.000 | 0.000 | |
| Total | 80 | 2.52 | 2.119 | 0.274 | |

Table (3.11) comparable *(Factor V Leiden)* and number of abortions with control cases.

**NO.Abortion**

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean |
|---|---|---|---|---|---|
| **GG (wild type)** | 50 | 3.02 | 1.964 | 0.278 | **23.326 (0.000)**** |
| **Control** | 30 | 0.00 | 0.000 | 0.000 | |
| **Total** | 80 | 2.52 | 2.119 | 0.274 | |