Deanship of Graduate Studies

AL-Quds University

Association of Genetic Polymorphisms in Vitamin K Epoxide
Reductase and GAS6 haplotypes with Recurrent Pregnancy Loss
among Palestinian Women

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M.Sc Thesis

Jerusalem- Palestine

2010

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A thesis submitted in partial fulfilment of requirements for the degree of Master of Science in Biochemistry and Molecular Biology

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1431/2010

AL-Quds University
Deanship of Graduate Studies
Biochemistry and Molecular Biology/ Faculty of Medicine

Thesis Approval

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Declaration:

I certify that this thesis submitted for the degree Master of Science is the result of my own research, except where otherwise acknowledged, and that this thesis (or part of the same) has not been submitted for a higher degree to any other University or Institution

Signed

Anthar Saqer Mohammad Darwish

Date

Dedication

To those who taught me the beauty of life

        & the joy of science………

    Mom and Dad

Acknowledgements

Abstract

Understanding the relationship of Single Nucleotide Polymorphisms (SNPs) with the incidence of disease is a step towards individualized medicine. The main objective of this study is to explore the potential association of SNPs in Vitamin K epoxide reductase complex subunit 1 (*VKORC1)* gene and Growth Arrest- Specific 6 (*GAS6)* gene with the occurrence of unexplained Recurrent Pregnancy Loss (RPL) and Breast Cancer.

Variants in *VKORC1* gene have been found to affect the amount of reduced vitamin K (VK), a cofactor for γ-carboxylation of vitamin K–dependent proteins. The C allele of the *VKORC1* +2255 T/C SNP is associated with higher activity at this locus and has been linked to increased vascular events. VK is important for post-modification of clotting factors involved in the coagulation cascade and Vitamin K_2 (VK_2) exerts an apoptotic effect on cancer cells. Studying a polymorphism which affects the status of VK in the body may be significant for a thrombophilia approach to unexplained RPL and Breast Cancer incidence.

Gas6 (Growth Arrest- Specific 6) is a Vitamin K dependent protein that exerts an anti-apoptotic effect by interacting with receptor tyrosine kinase, TAM family; Tyro3, Axl and MerTK. GAS6 expression has been found to be up-regulated in several types of cancer; this protein is also involved in clot stability. The A allele of the *GAS6* polymorphism c.834+7G>A may have a protective role against thrombophilia. This allele is possibly linked with a decrease in expression. The *GAS6* c.834+7G>A SNP was explored for a potential protective role in cancer and RPL.

The same SNPs were screened in both Breast Cancer and RPL cases, but were considered for different prospects and each was approached as a separate case- control type study. Genotyping was preformed by using *Nco*I and Al*w*N I restriction enzymes for *VKORC1* and *GAS6* SNPs, respectively.

In the RPL study, 45 patients and 77 age matched controls were screened at the loci in question, no significant difference in haplotype distribution was observed for either *GAS6* c.834+7G>A (*P* = 0.83) or *VKORC1* +2255 T/C (*P* = 0.20) among the groups. This polymorphism maybe thrombophilia unrelated in the unexplained RPL cases. Further research is recommended to explore the significance of these SNPs in the Palestinian population.

In the Breast Cancer study, 81 patients and 84 controls were analysed for the indicative haplotypes. No significant difference was observed in the allele distribution for the *GAS6* c.834+7G>A SNP among all participants (*P* = 0.32). However, a significant difference in haplotype distribution for the *VKORC1* +2255T/C SNP was observed (*P* = 0.02). The TT haplotype was found in 32% of the Breast Cancer patients, and in only 16.7% of the control group. The CC and CT haplotypes were found in 83.3% of controls and 67.9% of patients. The T allele conferred a more than 2 fold increased risk for developing Breast Cancer OR 2.36, 95% CI (1.13 - 4.95).

Further work is needed to explain the association of the T allele of the *VKORC1* gene with Breast Cancer and other factors which may affect VK status in the body.

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Index of Abbreviations

A ....Adenine

BC..Breast Cancer

C...…Cytosine

CI...…Confidence Interval

df .... degrees of freedom

DNA...….……. Deoxyribonucleic acid

dNTPs.. Deoxynucleotide Triphosphates

G...…....Guanine

Gla.. γ-carboxyglutamic acid

Kb...….....Kilo base pairs

OR...………Odds Ratio

P...…..Probability

P_1........................…............... ....Primer 1

P_2..….......Primer2

PCR...….Polymerase Chain Reaction

RFLP..Restriction Fragment Length Polymorphism

mRNA...….Messenger ribonucleic acid

RPL... Recurrent Pregnancy Loss

RTKs..……… receptor tyrosine kinases

SNP..…..Single Nucleotide Polymorphisms

T...…Thymine

TAE... Tris-acetate-EDTA

VK_1...…..Vitamin K_1

VK_2..…..Vitamin K_2

X^2-test...Chi- Square Test

Chapter I

Introduction

Single nucleotide polymorphisms (SNPs) are the most common genetic variations among humans; it is a change in a single nucleotide at any location in the genome. Studying the pattern among SNPs in specific targeted genes and the incidence of a particular disease or response to a medical treatment, is one of the first steps towards a more individualized and prophylactic approach to medicine. Case-control SNP association studies are increasingly popular, populations may differ drastically in haplotype frequencies, therefore the findings of one study does not necessarily apply to all. It is important for research in this area to be conducted in the Palestinian population, in order to help transform our current health system to a more personalized approach. This thesis will explore the potential association between SNPs in the *VKORC1* and *GAS6* genes with the occurrences of Breast Cancer and Recurrent Pregnancy Loss (RPL). The background information for the rationale of this study is provided in the sections of this chapter.

1.1.1 Vitamin K

Vitamin K is a fat-soluble vitamin discovered in the 1930s, during cholesterol experiments in chickens (Dam and Schonheyder, 1934). Vitamin K (VK) is a collective term for several related chemical compounds, they all share a 2-methyl-1, 4 naphthoquinone backbone structure, but differ in the composition of the side chain at position C-3 (Oldenburg *et al.*, 2008). Vitamin K1 (VK_1), also known as phylloquinone, is present in cyanobacteria and plants; it possesses a mostly saturated C-20 phytyl side chain. Vitamin K2 (VK_2) is produced by microbial organisms and is characterized by a partly unsaturated, predominantly C-40 side chain (menaquinone). In organisms that produce VK, both types are involved in electron transport processes (Oldenburg *et al.*, 2008).
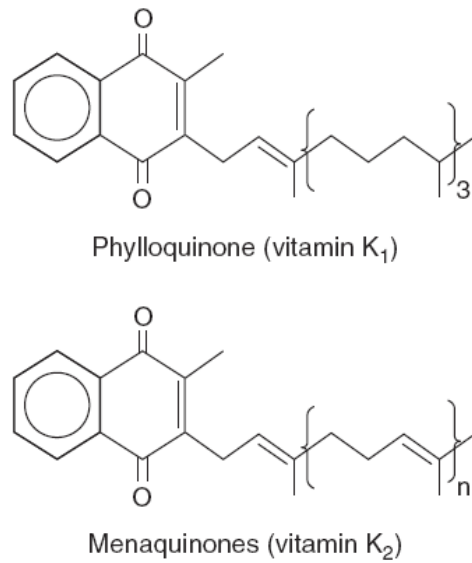
Fig 1.1 Vitamin K structures (Wallin R *et al.*, 2008)

1.1.2 Vitamin K dependent proteins

Vitamin K is an essential participant in posttranscriptional modification of proteins involved in coagulation, calcium metabolism, and other physiological processes (Oldenburg *et al.,* 2008). All of these proteins are modified by carboxylation of glutamic acid residues to form γ-carboxyglutamic acid (Gla), in the absence of vitamin K the carboxylation does not occur and the proteins are biologically inactive (Oldenburg *et al.*, 2008). VK dependent proteins in the coagulation cascade include the clotting factors II, VII, IX, X, and anticoagulant proteins C, S and Z which are integral to regulating hemostasis (Martinez and Barsigian, 1998). Each of these proteins is γ-carboxylated at several amino terminal glutamyl residues, which enables Ca^{+2} binding (Garcia and Reitsma, 2008 and Oldenburg *et al.*, 2008).

Osteocalcin and matrix Gla-protein are γ-carboxylated proteins involved in bone metabolism. Gas6 is another VK dependent protein; some of its functions include cell growth and survival. In addition, four presumed carboxylated transmembrane proteins (abbreviated as PRGP1, PRGP2, TmG3, and TmG4) are predicted to be VK dependent although their biological function remains to be identified (Kulman *et al.,* 2001 and Oldenburg *et al.*, 2006).

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1.1.3 The Vitamin K cycle

Vitamin K-dependent γ-carboxylation is carried out through a multi-component system of proteins located in the endoplasmic reticulum membrane (Furie and Furie, 1992). This system is composed of γ-carboxylase and vitamin K 2,3-epoxide reductase (VKOR). During γ-carboxylation, the hydroquinone cofactor is converted to the metabolite vitamin K 2,3-epoxide, which is subsequently returned to the reduced form by VKOR. This interconversion of vitamin K metabolites is referred to as the vitamin K cycle (Wallin *et al.*, 2008). Fig 1.2 summarizes γ-carboxylation and the VK cycle.

Fig 1.2 Vitamin K-dependent γ-carboxylation and VK cycle

(Vit. K_1H_2) reduced form of VK, (Vit. K1 > O) VK 2,3-epoxide , and (vitamin K 2,3-epoxide reductase) (Wallin *et al.*, 2008)

1.1.4 Vitamin K epoxide reductase complex subunit 1 (*VKORC1*) Gene

VKOR enzyme is a three- transmembrane structure in the rough endoplasmic reticulum, this protein has 163 amino acids and is coded for by the *VKORC1* gene (Oldenburg *et al.*, 2008 and Rost *et al.,* 2004). The *VKORC1* gene spans 5126 base pairs on human chromosome 16 and contains three exons (Rost *et al.,* 2004). *VKORC1* belongs to a large family of homologous genes found in vertebrates, insects, plants, protists, archea, and bacteria. All orthologs contain the same five completely conserved amino acids, including two cysteines found in a tetrapeptide motif that is apparently required for redox function (Garcia and Reitsma, 2008).

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1.1.5 *VKORC1* Polymorphisms

The anticoagulant warfarin targets the enzyme VKOR (Wallin *et al.*, 2008); a strong link between SNPs in the *VKORC1* gene and the requirement of warfarin dosage has been established. SNPs in *VKORC1* could reduce VKOR activity, like R98W, or result in warfarin resistance, like R58G, V29L, V45A, and L128R (Garcia and Reitsma, 2008).

Ten common non-coding SNPs in the *VKORC1* gene were identified, seven of which were significantly associated with warfarin dose maintenance. Five of these seven SNPs were strongly correlated with one another and can reflect the natural haplotype block of *VKORC1* (Siguret *et al.*, 2008). These 5 common non-coding polymorphisms are; "−1639A/G (rs9923231) in the promoter region, +1173T/C (rs9934438) in the first intron, +1542C/G (rs8050894) and +2255T/C (rs2359612) in the second intron, and +3730G/A (rs7294) in the 3' downstream region" (Wang *et al.,* 2006). These SNPs are located at positions 3673, 6484, 6853, 7566 and 9041 in the GenBank reference sequence (Wang *et al.,* 2006). *VKORC1* mRNA levels varied according to the haplotype combination, mRNA levels in the group with the G-C-G-C-A (3673-6484-6853-7566-9041) haplotype were ≈3 times as high as those in the wild-type group and the promoter with the G allele yielded a 44% increase of activity compared with the A allele of −1639 (Yuan *et al.,* 2005 and Rieder *et al.,* 2005).

The C allele of SNP +2255T/C conferred almost twice the probability of vascular disease in a Chinese study and the T allele of SNP +1173T/C was found to be lower among healthy individuals in comparison to their venous thromboembolism counterparts in a French study (Wang et al., 2006 and Lacut et al., 2007). The prevalence of *VKORC1* haplotypes differ among ethnic groups (Rieder *et al.,* 2005).

1.1.6 Vitamin K$_2$ and Apoptosis

VK$_2$ has been shown to suppress cancer growth; it induces apoptosis and differentiation in various cancer cells such as, leukaemia cells and hepatocellular carcinoma cells (Mizuta *et al.,* 2008). VK$_2$ is clinically used to suppress the onset of leukemia from

myelodysplastic syndrome (Mizuta *et al.*, 2008). The side chain of VK_2 appears to play an important role in its functions (Sakai *et al.*, 1994).

VK_2 has been shown to induce cell cycle arrest at G1/S, leading to growth inhibition of hepatoma cells both in vivo and in vitro. The expression levels of several growth-related genes, such as cyclin D1 and cyclin-dependent kinase 4 (Cdk4), and cyclin dependent kinase inhibitors, including p21 and/or p27, are altered in the growth-inhibitory process induced by VK_2 (Mizuta *et al.*, 2008).

Ovarian cancer TYK-nu cells treated with VK_2; undergo apoptosis by reduction in mitochondria membrane potential, activation of caspase 3, and release of cytochrome c into the cytosol, and DNA fragmentation (Shibayama-Imazu *et al.*, 2008). VK_2 induces apoptosis through one or more mitochondrial pathways (Green and Reed, 1998).

1.2 Growth Arrest Specific 6 (*GAS6*)

The *GAS6* gene spans 43.8Kb on chromosome 13 and constitutes 15 exons (Muñoz *et al.*, 2004). Expression of the *GAS6* was found to be upregulated 30 times when a cell enters the G_0 phase of the cell cycle (Bellido-Martı́n and de Frutos, 2008). Gas6 is a VK dependent multimodular protein; it requires several post-transcriptional γ-carboxyglutamic acid modifications (Hansson *et al.*, 2005). Gas6 serves as a ligand for the TAM family of receptor tyrosine kinases, (Tyro3, Axl and MerTK) and incomplete carboxylation of this protein results in loss of biological activity (Merli and Fink. 2008). Gas6 exhibits a broad range of regulatory functions associated with cell growth regulation, migration and proliferation, cell survival, apoptosis, recognition of dying cells, phagocytosis, and cell adhesion (Merli and Fink. 2008).

1.2.1 *GAS6* and Hemostasis

Studies focusing on the role of Gas6 in coagulation pathways have shown that Gas6 is a platelet response amplifier that plays a significant role in pathological thrombosis. Inhibition of Gas6 or the Axl receptor has been achieved in mouse models (Angelillo-

Scherrer *et al.*, 2005). "When the receptor is blocked initial platelet aggregation does occur, but stabilisation of platelet aggregates is impaired and mice are protected against life-threatening thrombosis (Mc Cormack, 2008)".

The SNP c.834+7 G >A, located on intron 8 of the *GAS6* gene was found to be protective against stroke (Muñoz, 2004). The AA haplotype was less prevalent among patients with stroke and acute coronary syndrome compared to their healthy counterparts (Muñoz, 2007 and Jiang, 2009). The effect of the different haplotypes on the function of this gene has not been elucidated. Therefore it is not clear if the genetic association with cardiovascular disease is related to *GAS6* or reflects a linkage to other genes in the same chromosomal region (Muñoz, 2007).

1.2.2 Anti-apoptotic effect of Gas6

Gas6 was identified as a growth factor and a rescue factor from apoptosis among different cell types, specifically in cultured cells deprived of serum (Bellido-Martı́n and de Frutos, 2008). The anti-apoptotic effect of Gas6 has been found in numerous experimental settings and among different species. Some of the cell types and lines tested include; NIH3T3 fibroblasts, vascular smooth muscle cells, endothelial cells, chondrocytes, neurons, oligodendrocytes, hepatocytic precursors, epithelial cells and different types of cancer cells (Bellido-Martı́n and de Frutos, 2008).The anti-apoptotic effect of Gas6 requires protein phosphorylation and in most cases the PI3K pathway has been demonstrated to be crucial for this effect (Bellosta *et al.*, 1997).

In certain tumor types, migration and invasiveness has been correlated with the expression of Gas6 receptors, in particular Axl and Gas6-dependent signaling through Axl has been shown to promote invasiveness of glioma cells in animal models (Bellido-Martı́n and de Frutos, 2008).

In breast cancer cell lines, *GAS6* is upregulated more than 23-fold by progesterone acting through the progesterone receptor B and membrane staining of the receptor for Gas6, Axl, has been reported to be higher in cancerous tissue than in the normal breast (Richer *et al.*, 2002 and Berclaz *et al.*, 2001). Furthermore, the *GAS6* locus has recently been described as a target for amplification in mouse models of breast cancer and in human breast cancer (Abba *et al.*, 2007).

1.3 Recurrent Pregnancy Loss

Miscarriage or spontaneous abortion occurs when a pregnancy ends naturally before a fetus is developed enough to survive outside of the uterus. Approximately 20% of all pregnancies end in miscarriage (American Medical Association, 2004). Spontaneous abortion is one of the most common obstetrical complications, if a women has a history of three or more consecutive miscarriages prior to 20 weeks of gestation, it is defined as Recurrent Pregnancy Loss (RPL) (Pabinger, 2005). RPL affects approximately 0.5 – 3 % of women, (Li *et al.*, 2002) usually causing emotional distress and depression.

 RPL is a very heterogeneous disease and the etiology for half of the cases remains unexplained (Kwak-Kim, 2009). The nature of this disease presents a great challenge to physicians. It requires each patient to have an individual treatment plan based on their special needs. Some of the identifiable causes for RPL include; Parental chromosomal anomalies , Uterine abnormalities, Endocrinological disorders, Immuniological factors, Cervical weakness, Infections, maternal diseases and Thrombophilia (Li *et al.*, 2002).

1.3.1   Thrombophilia

Thrombophilia is a term used to describe Hypercoagulability disorders that cause a predisposition to thrombosis (Martens and Emed, 2007). It is a multi- factorial defect, which can be inherited, acquired or influenced by environmental and life style factors (Walker, 2000). Some of the clinical manifestations of Thrombophilia include; Deep Vein Thrombosis (DVT), Pulmonary Embolism, Purpura fulminans, stroke, and acute myocardial infarction. Thrombophilia is also linked to several pregnancy complications such as, recurrent

pregnancy loss, intrauterine growth restriction, stillbirth, severe pre-eclampsia, and abruptio placentae (Heit, 2007).

The major heritable forms of thrombophilia consist of deficiencies in anti-coagulants; protein C, protein S and antithrombin. Furthermore, abnormalities in pro-coagulant factors have been correlated with thrombophilia, particularly, factor V Leiden and the prothrombin G20210A gene polymorphisms. In addition, homozygosity for methylenetetrahydrofolate reductase (*MTHFR*) C677T can be associated with hyperhomocysteinaemia, which is correlated with increased risk of vascular events (Robertson *et al.,* 2006).

Supportive data in the literature also suggests a role for increased concentration of plasma factors I (fibrinogen), II (prothrombin), VIII, IX, XI and polymorphisms in Factor XIII with thrombophilia (Heit, 2007). Dysfibrinogenemia and Reduced tissue factor pathway inhibitor also increases tendency for thrombosis (Heit, 2007).

Acquired thrombophilia is a result of; surgery and trauma, prolonged immobilization, older age, cancer, pregnancy and the puerperium, use of contraceptives or hormone-replacement therapy and antiphospholipid antibodies   (Seligsohn and Lubetsky, 2001).

1.3.2 Thrombophilia and Recurrent pregnancy loss

Normal pregnancy is accompanied by changes in coagulation that have likely evolved to protect women from the bleeding challenges of miscarriage and childbirth. Consequently, pregnant women are at an increased risk of thrombosis (James *et. al,* 2005). The possibility of thromboembolism increases 4- to 5-folds during pregnancy and the postpartum period; eighty percent of these events occur in veins with an incidence of approximately 2 per 1000 pregnancies (James, 2009[A]).

Usually pregnancy is associated with an increase in the concentrations of factors VII, VIII, X, von Willebrand factor and fibrinogen whereas; Factors II, V and IX are relatively unchanged (Bremme, 2003). In addition, Plasminogen activator inhibitor type 1 (PAI-1)

levels are increased fivefold and the levels of PAI-2, produced by the placenta, increase dramatically during the third trimester (James, 2009 [B]). PAI type 1 and 2 inhibit the activity of tissue Plasminogen activator (tPA) and Urokinase which consequently decreases the conversion of Plasminogen to Plasmin and inhibits fibrinolysis (Cesarman-Maus, 2005). As gestation progresses, there is also a significant fall in the activity of activated protein C and a decrease in protein S, both are important anticoagulants (Bremme, 2003). These changes, which may not completely return to baseline until more than 8 weeks after birth, begin with conception and result in the hypercoagulable state of pregnancy (James, 2009 [B]) .

Numerous case–control studies have investigated the impact of thrombophilia on pregnancy loss. In most of these studies factor V Leiden (FV 1691 G/A), prothrombin 20210 A/G and the methylene tetrahydrofolate reductase (MTHFR) gene 677 C/T variations were considered (Pabinger, 2005). The association of these genetic variants with RPL showed conflicting results in different populations.

Several studies have been conducted on Palestinian women suffering from RPL; in one report, a significant correlation was established between the occurrences of the Factor V Leiden mutation and poor pregnancy out come (Hussein, 2010).

In another study carried out on residents in the Gaza Strip, screening for polymorphisms in NOS3, ACE and PAI-1 genes failed to show any significant association (Al Sallout, 2010).

1.4 Breast Cancer

Abnormal cell growth that forms in the tissues of the breast, usually the ducts and lobules is defined as Breast Cancer. It can occur in both males and females, although male breast cancer is rare (American Medical Association, 2004). "Worldwide, it is estimated that more than one million women are diagnosed with breast cancer every year, and more than 410,000 will die from the disease (Coughlin and Ekwueme, 2009)." Life style, environmental factors and genetics all work together to predict the probability of developing breast cancer (Hartge, 2003).

Mutations in the *BRCA1* and *BRCA2* cancer predisposition genes are the principal cause of hereditary breast-ovarian cancer, however these mutations account for a small percentage of all breast cancer cases (Hall *et al.,* 2009).

Early detection is the best way to improve breast cancer prognosis and attempting to identify new genetic polymorphisms that may increase the susceptibility of developing the disease is a valuable contribution to the field of oncology.

1.5 Hypothesis and Objectives

VKORC1 haplotypes are indicative of warfarin resistance and thrombophilia related events, by effecting mRNA expression levels and hence the activity of VKOR. Variation in the expression levels of this gene maybe associated with the pathophyiology of other diseases such as breast cancer and unexplained RPL.

We hypothesize that the C allele of the VKORC1 +2255T/C SNP will be more prevalent among subjects with RPL, whereas the T allele will have a higher frequency within the breast cancer group. Furthermore, we predict a protective role for the AA haplotype of the *GAS6* SNP c.834+7 G >A, against breast cancer and RPL.

The objectives of this study are;

1) To test for potential new breast cancer and RPL biomarkers.
2) To explore the relationship between variation in VK cycle activity, with the occurrence of breast cancer and RPL.
3) To investigate a possible protective role for the *GAS6* SNP c.834+7 G >A and the development of Breast Cancer and RPL.
4) To contribute to the moving medical trend of individualized medicine.

Chapter II

Experimental Approach

Polymorphisms in the *VKORC1* and *GAS6* genes are potentially attractive Genetic Markers for Recurrent Pregnancy Loss and Breast Cancer. Case-control type studies were designed to investigate for possible associations. This project was conducted according to the standards of the Helsinki declaration, and approved by the research committee at Al-Quds University. Experimental work was conducted at the Medical Research Centre on Abu-Dies campus.

The testing of our hypotheses required the use of three very common and well established molecular biology techniques; Polymerase Chain Reaction (PCR), Gel Electrophoresis, and Restriction Fragment Length Polymorphism (RFLP).

The same SNPs are screened in both Breast Cancer and RPL cases, but are considered for different prospects; therefore each was approached as a separate study.   Participants were carefully genotyped at the loci in question, by amplifying the specified DNA fragments (PCR), then digested with restriction enzymes (RFLP), and documented by gel electrophoresis. Followed by a statistical analysis to test the extent with which a correlation can be established or denied. This chapter focuses on the techniques and procedure used for the experimental work.

2.1 Participant selection

2.1. 1 RPL - Control study

The DNA samples of participants in this investigation were available in the lab from a previous study on RPL and Factor V Leiden mutation among the Palestinian population, with full medical history of RPL patients and their age-matched controls. The inclusion criteria for the case group are women who endured three or more pregnancy losses in the first trimester or two more in the second trimester, without a known explanation. These women were otherwise healthy and had no anatomical abnormalities in the uterus. The control group was composed of age-matched women who had two or more successful pregnancies

and no medical problem. The number of participants that met the control criteria was 77, whereas 45 women classified as suitable RPL cases, all of whom had first trimester losses with the exception of one subject. The 122 subjects were from four major West Bank Cities; Bethlehem, Jericho, Ramallah, and Nablus.

2.1.2 Breast Cancer – Control study

81 breast cancer patients were included in this investigation, they were provided to us from a study that focused on risk assessment of breast cancer among Palestinians in the West Bank. The control group consisted of 84 post menopausal women with normal bone mass density and no history of clinical problems. They were personally called to verify the absence of breast cancer in their medical records and were considerably healthy women.

2.2 DNA Extraction

Isolation of Genomic DNA from fresh blood samples was previously done using commercially available Masterpure kits. DNA was qualified, quantified and stored at -30°C until use.

2.3 Polymerase Chain Reaction (PCR)

Development of the PCR technique in 1984 by Kary Mullis placed a milestone in the progress of Molecular Biology. This technique enabled scientists to amplify large quantities of selected segments of DNA in a short period of time. The principle behind this technique is based on mimicking the natural process of DNA amplification; by providing all the necessary constituents such as dNTPs, divalent cations (Mg^{+2}), buffer solution, and most importantly a heat stable DNA polymerase to synthesize new strands of DNA complementary to the designated template. To amplify the template strand, within the correct region two sets of primers (forward and reverse) are used, each annealing to the opposite end. A thermal cycler is utilized to provide the changes in temperature necessary for each step of the reaction to proceed. The first step of the reaction is separation of the double stranded DNA helix, denaturation of this structure requires a high temperature around (94- 96°C). The denaturation step described above is followed by the annealing step, in which the primers anneal to their complementary sequence on the template strand; the temperature at which this occurs varies with the sequences involved, usually between (50 – 65°C). After primer annealing, the temperature is raised to 72°C this marks the beginning of the elongation step,

new strand synthesis. At this temperature the enzyme Taq polymerase is at its optimal activity. The use of this heat stable polymerase, isolated from the thermophilic bacteria *Thermus aquaticus*, is the key behind the success of this technique (Nelson and Cox, 2005).

2.3.1 PCR amplification for *VKORC1* +2255 T/C

The required 198- bp sequence containing SNP +2255 T/C was amplified by using the following primers (Invitrogen);

Forward Primer (P$_1$)

$^{'}$5_-TCTGAACCATGTGTCAGCCAGGACC-_3$^{'}$

Reverse Primer (P$_2$)

$^{'}$5_-GAACAGAGAGAGGAACCAAGGGAGTGGA-_3$^{'}$

PCR-Ready ^{TM} *High Yield* tubes *Cat. # PCR-Y-192*, (Syntezza) were used for the amplification reactions. These tubes contain a ready mixture of dNTPs, heat stable DNA polymerase, divalent cations, PCR buffer and DNA loading dye.

The following reaction mix was added to each tube;

22 μL of distilled water, (Birzeit Pharmaceutical Company)

1 μL P$_1$ (1.5μg/μL)

1 μL P$_2$ (1.5μg/μL)

1 μL (0.2μg/μL) Genomic DNA

Total volume = 25μL

PCR reactions were preformed in *GeneAMP® PCR System 9700 PE* (Applied Biosystems). DNA was denatured at 95 ^{o}C for 5 min followed by 35 cycles of 95°C for 45 sec (denaturation), 58°C for 45 sec (annealing) and 72°C for 40 sec (elongation), and a concluding 7 min at 72°C for final extension.

To confirm the presence of PCR products, samples were run for 50 min on 2% Agarose Gel Electrophoresis at 120V. (Refer to section 2.5 for more details.)

2.3.2 PCR Amplification for *GAS6* SNP 843+7 G>A

The required 481- bp sequence containing SNP 843+7 G>A was amplified by using the following primers (Invitrogen);

Forward Primer (P$_1$)

$^{'}$5_- : TTCCCTCAACAAAGAGCCCG-_3$^{'}$

Reverse Primer (P$_2$)

$^{'}$5_- TCTCATCCCAAACCTCCACA-_3$^{'}$

PCR-Ready ^{TM} High Yield tubes *Cat. # PCR-Y-192*, (Syntezza) were used for amplification reactions. These tubes contain a ready mixture of dNTPs, heat stable DNA polymerase, divalent cations, PCR buffer and DNA loading dye.

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1 µL P$_1$ (1.5µg/µL)

1 µL P$_2$ (1.5µg/µL)

1 µL (0.2µg/µL) Genomic DNA

Total volume = 25µL

PCR reactions were preformed in *GeneAMP$^®$ PCR System 9700 PE* (Applied Biosystems). DNA was denatured at 95 $^{\circ}$C for 5 min followed by 35 cycles of 95°C for 45 sec (denaturation), 57°C for 45 sec (annealing) and 72°C for 60 sec (elongation), and a concluding 10 min at 72°C for final extension.

To confirm the presence of PCR products, samples were run for 50 min on 2% Agarose Gel Electrophoresis at 120V. (Refer to section 2.5 for more details.)

2.4 Restriction Fragment Length Polymorphisms (RFLP)

RFLP is a common laboratory technique for the genotyping of SNPs. This method utilizes restriction enzymes to discriminate between two sequences, based on cleavage site recognition for only one of the alleles. Digestion of the DNA sequence will result in fragments of varying lengths that can be separated by gel electrophoresis, to verify the genotype (Saiki *et al.,* 1985).

2.4.1 Genotyping *VKORC1* +2255 T/C polymorphism

PCR products were digested with the restriction enzyme *Nco*I (Invitrogen), which yields 2 DNA fragments of 26 and172 bp for the T allele, whereas the C allele is not cleaved and a single band of 198 bp appears on the gel (Wang *et al.,* 2006).

 The following digestion mix was added to10 µL of PCR product for each sample;

* 1.5 µL of 10x buffer (Invitrogen), for a final concentration of 1x

* 0.5U *Nco*I (10U/ 1 μL) ≈ 0.05 μL

* 3.45μL distilled water (Birzeit Pharmaceutical Company)

Total volume = 15 μL

Samples were incubated in a water bath at 37°C overnight, DNA fragments were then separated using 4% gel electrophoresis at 120 V for 75 min.

2.4.2 Genotyping *GAS6* 843+7 G>A polymorphism

PCR products were digested with the restriction enzyme Al*w*N I (Invitrogen), which yields 2 DNA fragments of 342 and 139 for the A allele, whereas the G allele is not cleaved and a single band of 481 bp appears on the gel (Muñoz, *et al.,* 2004).

 The following digestion mix was added to10 μL of PCR product for each sample;

* 1.5 μL of 10x buffer (Invitrogen), for a final concentration of 1x

* 0.5U Al*w*N I (10U/ 1 μL) ≈ 0.05 μL

* 3.45μL distilled water (Birzeit Pharmaceutical Company)

Total volume = 15 μL

Samples were incubated overnight in a water bath at 37°C, DNA fragments were then separated using 3% gel electrophoresis for 75 min. at 120V.

2.5 Gel electrophoresis

Gel electrophoresis was used for two main reasons, firstly to confirm the presence of PCR products and secondly to separate digested DNA fragments.

2%, 3% and 4% gels were prepared by mixing 2, 3 or 4 g of agarose powder with 100 ml of Tris-acetate-EDTA (TAE) buffer. This mixture was then stirred and heated over a hot plate until completely dissolved. 10 μL of Ethidium Bromide were added to the liquid gel before pouring in the casting tray. After solidification samples were loaded in the wells, 8 μL for PCR product and 12μL for digested samples. To measure the length of DNA bands on the gel, 2μL

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of 100 bp DNA ladder (135 ng / μL) (BIONEER) were loaded with each run. After 50-75 min. of electrophoresis at 120 V, the gel was viewed under UV-light and photographed.

2.6 Statistical analysis

Chi-square test was used to check the genotype distributions for Hardy-Weinberg equilibrium and to compare the observed allele and genotype frequencies in the patients with the controls. Significances level was established at $P < 0.05$.

SPSS Version 18 was also used to calculate odds ratio and 95% confidence interval.

Chapter III

Results

3.1 General Characteristics

Haplotypes for *VKORC1* and *GAS6* were determined for participants in two case-control type studies. One of the investigations undertaken, aimed towards studying the potential correlation between haplotype frequency and the incidence of Recurrent Pregnancy Loss in women that had three or more abortions of unknown etiology. The control group consisted of age matched healthy women with two or more successful pregnancies. The second study was conducted on Breast Cancer patients that had an aggressive prognosis and appeared at a relatively young age. The haplotype distribution for this group was compared to a cancer free control group of slightly older women. A summary of the number of participants in each group and their ages are described in Table 3.1.

Table 3.1 Participant Distribution

	Groups		Total
	Recurrent Pregnancy Loss		
	Cases	Control	
# of women	45	77	122
Mean age	30.5 ± 7.1	32.2 ± 4.3	
	Breast Cancer		
# of women	81	84	165
Mean age	48.5 ± 9.6	56 ± 6.6	

3.2 Genotyping of *VKORC1* SNP +2255 T/C

The polymorphism +2255 T/C was analyzed by amplifying a 198bp DNA fragment and confirmed by running on 2% gel agarose, stained with ethidium bromide and visualized under UV light as seen in Fig 3.1. This product was then digested with the restriction enzyme *NocI* and the digested products separated on 4% agarose gel as seen in Fig 3.2.

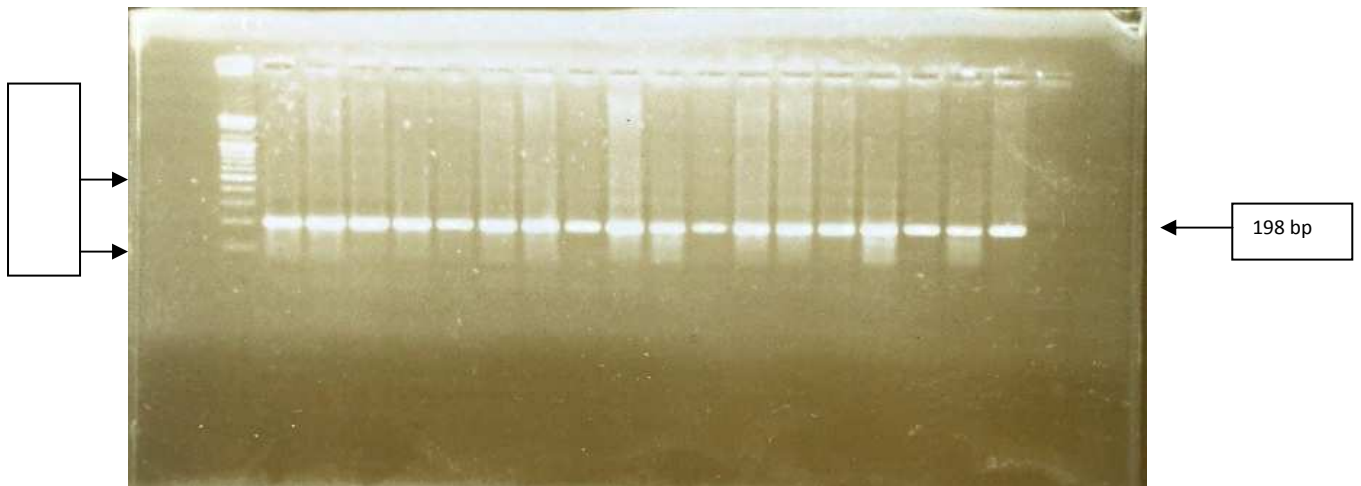1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

198 bp

Fig 3.1 Representative Agarose gel for *VKORC1* PCR product; the amplified 198 bp product is confirmed in lanes 2-19. The location of this segment is parallel to the 200bp band in the DNA ladder shown in lane 1.

Lane 20 displays the results of a negative control for the PCR reaction (exclusion of DNA template) which validate the absences of DNA contamination.

* 100 bp DNA marker ladder was employed in lane 1.

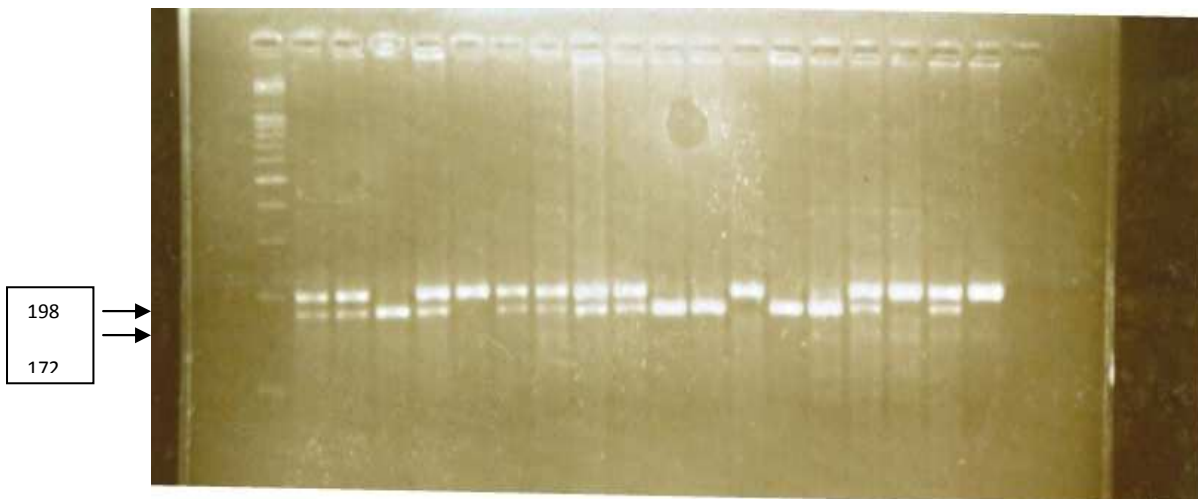1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

198

172

Fig 3.2 Representative Agarose Gel for *VKORC1* PCR product after digestion with the *Noc*I restriction enzyme; *Noc*I exclusively digests DNA fragments with the T allele resulting in 172 and 26 bp fragments whereas the length of the DNA fragments with the C allele remain unchanged. Samples in lanes 2, 3, 5, 7-10, 16, & 18 display two bands at 198 and 172bp, therefore indicate a heterozygous haplotype of (CT). Complete digestion of the PCR product can be observed in lanes 4, 11, 12, 14, & 15 indicating homozygous (TT) haplotype. The

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absence of digestion in lanes 6, 13, 17 & 19 is characteristic of the (CC) homozygous haplotype. Lane 20 contains a negative control sample, and lane 1 contains the 100bp DNA ladder.

* The remaining 26bp fragment after digestion with *NocI* for T alleles can not be seen due to small size.

3.3 Genotyping of *GAS6* SNP 843+7 G>A

The *GAS6* 843+7 G>A polymorphism was analyzed by amplifying a 481bp DNA fragment. The PCR product was then run on 2% agarose gel and visualized under UV light as shown in Fig 3.3. This product was then digested with the restriction enzyme *AlwN* I and the digested products were analyzed by running on 3% gel as seen in Fig 3.4.
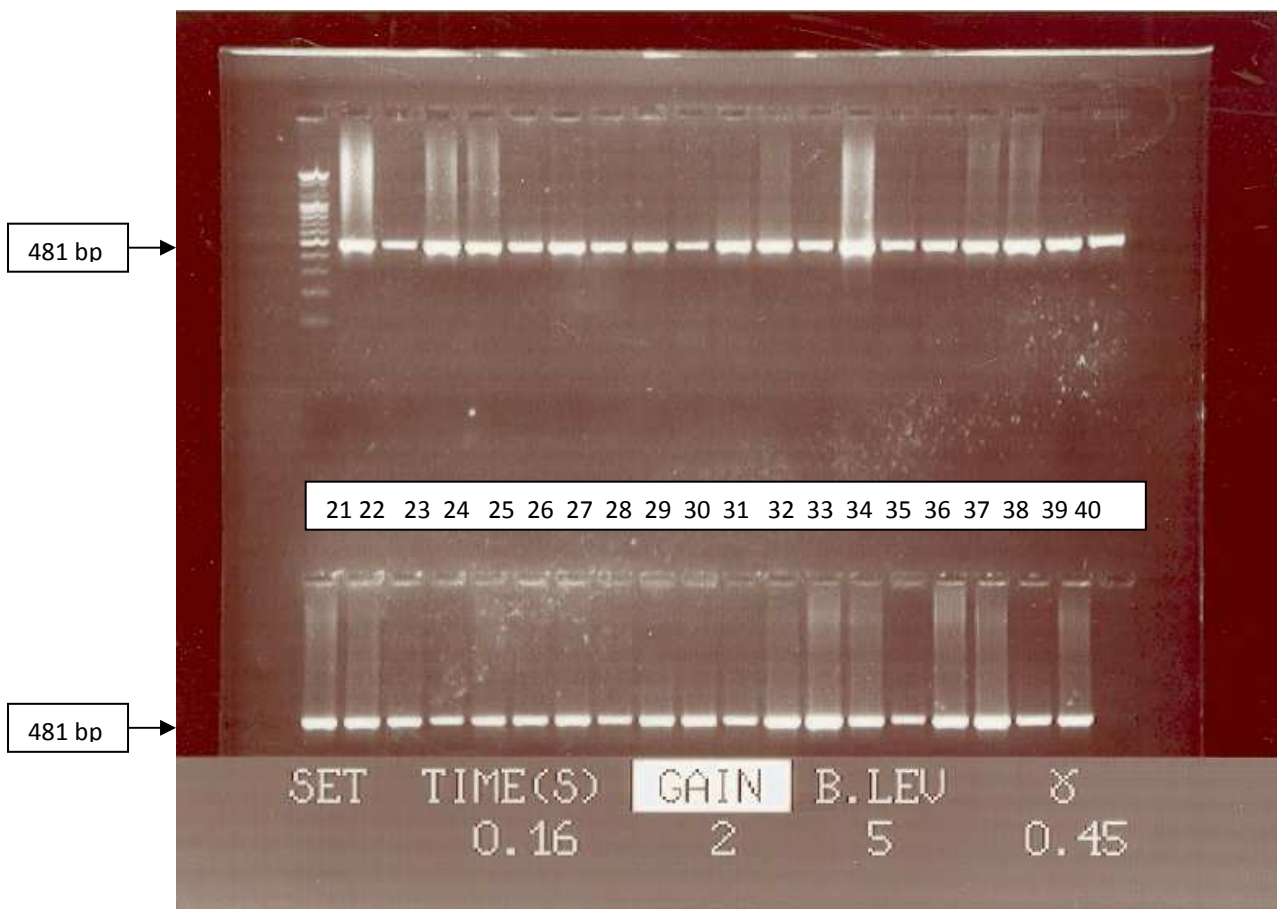
Fig 3.3 Representative Agarose Gel for *GAS6* PCR product; the specified 481 bp product is confirmed in lanes 2-39. Lane 40 is a negative control that confirms the absences of DNA contamination. Lane 1 contains a 100bp DNA ladder marker.

Fig 3.4 Representative Agarose Gel for *GAS6* PCR product after digestion with the *AlwN* I restriction enzyme; *AlwN* I exclusively digests DNA fragments with the A allele resulting in 342 and 139bp DNA fragments, whereas the G allele is undigested. Samples in lanes 4, 7, 11, 17, 19, 21, 22, 29, 30, 32, 34, 36, 37 & 38 display three DNA fragments at 481, 342, and 139bp indicating partial digestion; these individuals have a heterozygous haplotype of (GA). Complete digestion of the 481 bp fragment can be observed in lanes 3 & 39 indicating a homozygous (AA) haplotype. The absence of digestion in lanes 5, 6, 8-10, 12-16, 23-28, 31, 33, & 35 is characteristic of the (GG) homozygous haplotype. Lane 40 contains a negative control and lane 1 contains the 100bp DNA ladder marker.

3.4 Hardy-Weinberg equilibrium

It is useful to determine if the alleles being studied in these subjects are in Hardy-Weinberg equilibrium, in order to rule out changes in the allele frequency from one generation to the next as a result of natural selection, mutation, migration or genetic drift (Klug, Cummings and Spencer 2005). The chi-square test was used to check for fulfillment of the Hardy-Weinberg equilibrium in participants that were used as controls in the RPL (n=77) and Breast Cancer (n= 84) investigations. In addition, all individuals that did not meet the criteria for patient or control groups were also included (n=65).

Table 3.2 Hardy-Weinberg equilibrium for the *VKORC1* +2255 C/T allele

C allele frequency	T allele frequency		Total
0.562 (p) +	0.438 (q)		= 1
Expected Genotype frequencies $P^2 + 2pq + q^2$			= 1
CC	CT	TT	
71.380	111.263	43.357	226
Observed Genotype frequencies			
77	100	49	226
Chi-square test (df = 1) X^2= 2.308 $P > 0.05$ No significant difference. **Expectations of Hardy- Weinberg are fulfilled.**			

*$P > 0.05$ for X^2 less than 3.84 (df = 1)

Table 3.3 Hardy-Weinberg equilibrium for the *GAS6* SNP c.843+7 G>A

G allele frequency	A allele frequency		Total
0.6754 (p) +	0.3246 (q)		= 1
Expected Genotype frequencies $P^2 + 2pq + q^2$			= 1
GG	GA	AA	
87.13	83.75	20.12	191
Observed Genotype frequencies			
84	90	17	191
Chi-square test (df = 1) X^2= 1.063 $P > 0.05$ No significant difference. **Expectations of Hardy- Weinberg are fulfilled.**			

* $P > 0.05$ for X^2 less than 3.84 (df = 1)

Patient groups fulfilled expectations for the Hardy-Weinberg equilibrium for both *VKORC1* and *GAS6*. (Calculations not shown)

3.4.1 *VKORC1* +2255 T/C Haplotype frequencies in RPL patients and Controls

The haplotype distribution for the +2255T/C polymorphism among patients (n= 45) and controls (n= 77) is summarized in Fig 3.5. The CC, CT, and TT haplotype frequencies in patients with RPL were 15.6% (n= 7), 55.6% (n=25), and 28.9% (n=13), while the frequency in the control group was 29.9% (n= 23), 44.2% (n= 34) and 26% (n= 20), respectively. Chi-square test (df= 2) was used to determine if the differences in the two groups is considerable. The *P* value was found to be **0.20**, which indicates no significant difference in haplotype distribution.
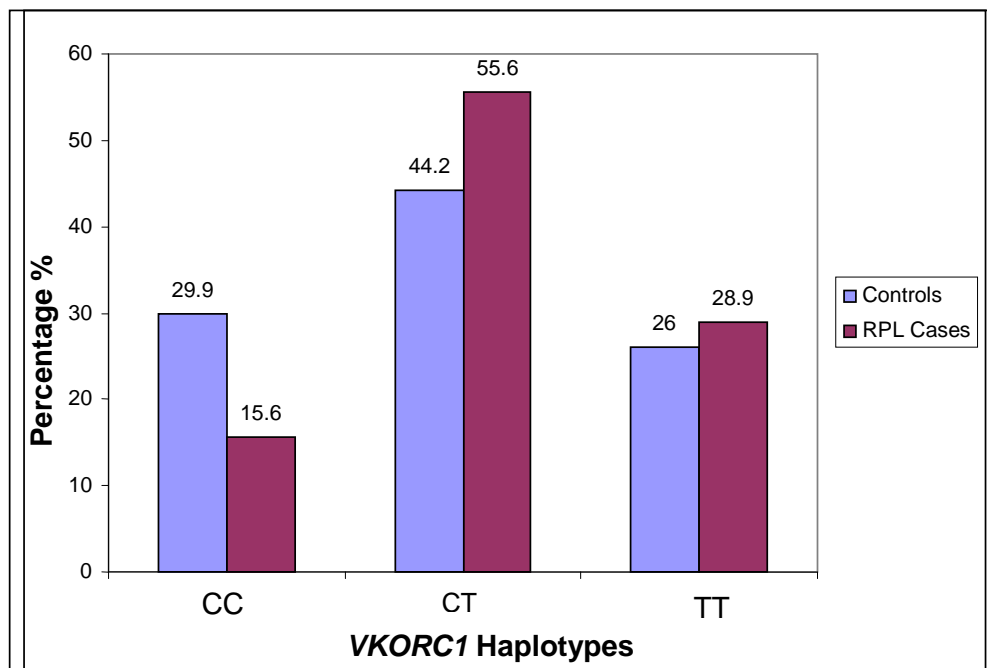

Fig. 3.5 *VKORC1* +2255 T/ C Haplotype distribution in RPL patients and Controls (*P* = 0.20)

To evaluate the association of the C allele with the occurrence of RPL, CC and CT haplotypes were joined in one group, the C allele exerts a dominant effect (Wang *et al.,* 2006), and the Odds ratio (OR) and 95% Confidence Interval (CI) were calculated. As shown in table 3.4

Table 3.4 Risk assessment of *VKORC1* SNP + 2255 with RPL

Haplotypes (%)		
	CC + CT	TT
Controls	74 (n = 57)	26 (n= 20)
RPL Cases	71.1(n= 32)	28.9 (n= 13)
X^2 test (df= 1) ; $P =$ 0.73		
OR (95% CI) = 1.16 (0.51 - 2.63)		

The results indicate no statistically significant association between the *VKORC1* haplotypes examined and RPL occurrence; although a slight difference is noticeable between the groups.

3.4.2 *GAS6* c.843+7 G>A Haplotype frequencies in RPL patients and Controls

The haplotype distribution for the *GAS6* c.843+7 G>A polymorphism among RPL patients (n= 45) and controls (n= 77) is summarized in Fig 3.6. The GG, GA, and AA frequencies in RPL patients were 42% (n= 19), 51% (n= 23), and 6.7% (n= 3), respectively, and in the controls were 37.7% (n= 29), 53.2% (n= 41), and 9.1% (n= 7). The *P* value **0.83** indicates an insignificant difference between the two groups.
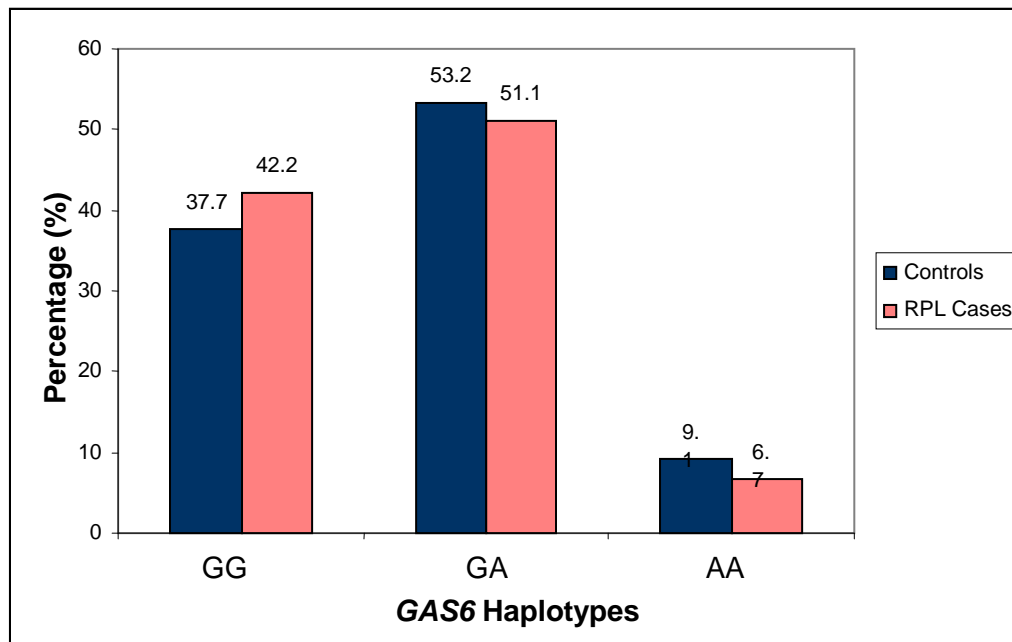

Fig. 3.6 *GAS6* c.843+7 G>A Haplotype distribution in RPL patients and Controls (*P*= 0.83)

To determine if the AA haplotype is associated with a decreased risk of RPL, the GG and GA haplotypes were placed in one group and compared to the AA haplotype. OR and 95% CI were calculated (table 3.5) It is not clearly established if either allele exerts a dominant effect, therefore allele frequencies in the examined groups were taken into consideration, OR and 95% CI were computed as well, as shown in table 3.5.

Table 3.5 Risk assessment of *GAS6* SNP c.843+7 G>A with RPL

	Haplotype Frequency (%)		Allele Frequency (%)	
	GG +GA	AA	G	A
Controls	90.9% (n= 70)	9.1% (n= 7)	63.6% (n= 98)	36.4% (n= 56)
RPL Cases	93.3% (n= 42)	6.7% (n= 3)	67.8% (n= 61)	32.2% (n= 29)
X^2 Test	Df= 2; *P*= 0.64		df= 1; *P*= 0.51	
OR (95% CI)	0.71 (0.18 – 2.91)		0.83 (0.48 – 1.44)	

These results show no significant association between the *GAS6* haplotypes and RPL occurrence.

3.5.1 *VKORC1* +2255 T/C Haplotype frequencies in Breast Cancer patients and Controls

The haplotype distribution for the +2255T/C polymorphism among breast cancer patients (n= 81) and controls (n= 84) is summarized in Fig 3.7. The CC, CT, and TT haplotype frequencies in breast cancer patients were 22.2% (n= 18), 45.7% (n= 37), and 32.1% (n= 26) and among controls were 38.1% (n= 32), 45.2% (n= 38) and 16.7% (n= 14), respectively. Chi-square test (df= 2) was used to determine if the differences in allele distribution between the two groups is considerable. *P* value was found to be **0.02**, which indicates a significant difference.
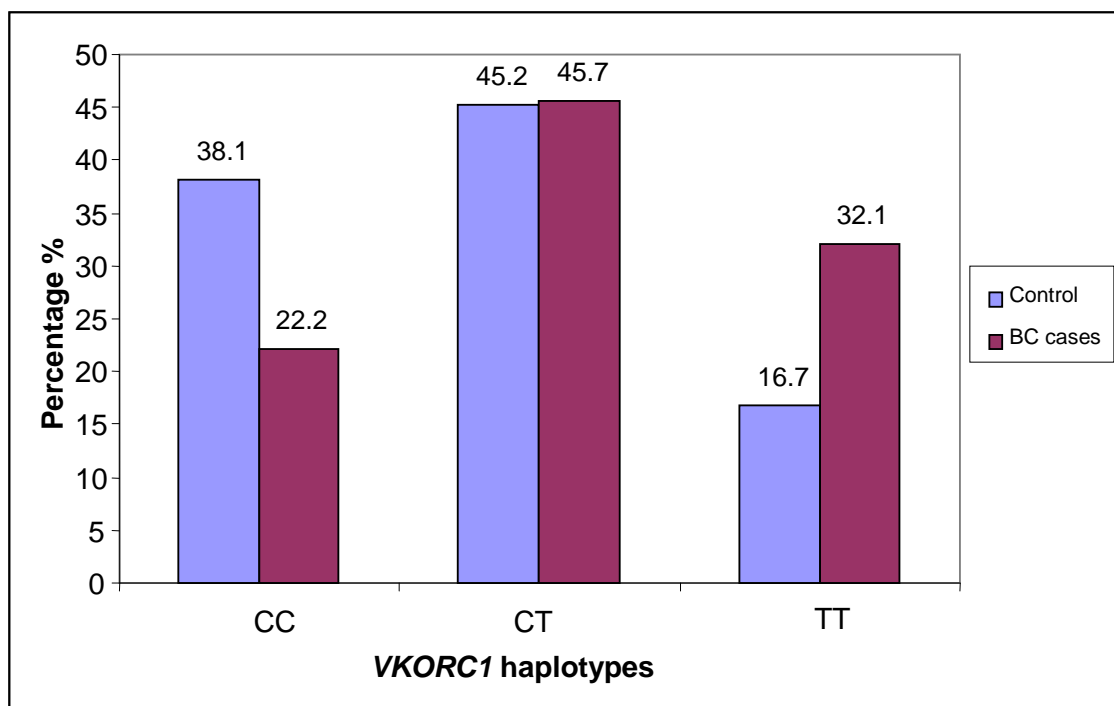
Fig. 3.7 *VKORC1* +2255 T/ C Haplotype distribution in Breast Cancer patients and Controls
(*P* = 0.02)

To evaluate the association of the C allele with the occurrence of Breast cancer, the CC and CT haplotypes were joined as one category and the TT haplotype in a separate category. OR and 95% CI, were calculated as shown in table 3.6.

Table 3.6 Risk assessment of *VKORC1* SNP + 2255 with breast cancer

	Haplotypes (%)	
	CC + CT	TT
Controls	83.3 (n = 70)	16.7 (n= 14)
BC Cases	67.9 (n = 55)	32.1 (n= 26)
X^2 test (df= 1) ; *P* = 0.02		
OR (95% CI) = 2.36 (1.13 - 4.95)		

The results clearly indicate that the TT haplotype is significantly associated with breast cancer occurrence.

3.5.2 *GAS6* c.843+7 G>A Haplotype frequencies in Breast Cancer patients and Controls

The haplotype distribution for the *GAS6* c.843+7 G>A polymorphism among breast cancer patients (n= 81) and controls (n= 84) is summarized in Fig 3.8. The GG, GA, and AA frequencies in patients were 38.3% (n= 31), 54.3% (n= 44), and 7.4 (n= 6) and the controls

were 46.4% (n= 39), 42.9% (n= 36), and 10.7% (n= 9), respectively. The *P* value **0.32** indicates an insignificant difference between the two groups.
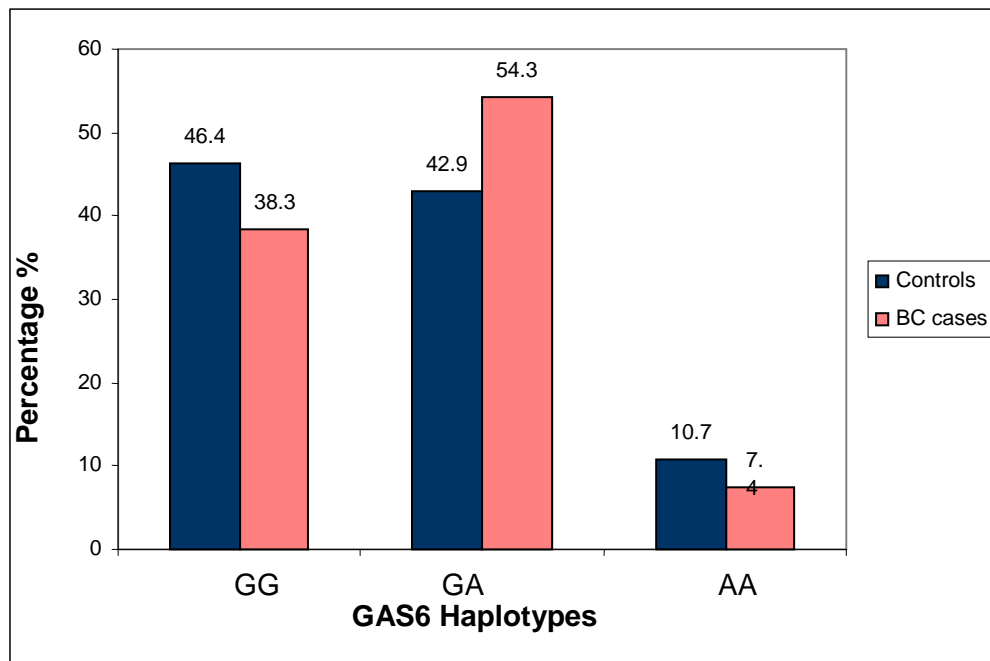

Fig. 3.8 *GAS6* c.843+7 G>A Haplotype distribution in BC patients and Controls (*P*= 0.32)

To determine if the AA haplotype is associated with a decreased risk of breast cancer, the GG and GA haplotypes were placed in one group and the AA haplotypes in a separate group. OR and 95% CI were calculated, as shown in table 3.7.

Table 3.7 Risk assessment of *GAS6* SNP c.843+7 G>A with Breast Cancer

	Haplotype Frequency (%)		Allele Frequency (%)	
	GG +GA	AA	G	A
Controls	89.3 (n= 75)	10.7 (n=9)	68.5 (n= 115)	31.5 (n= 53)
BC Cases	92.6 (n= 75)	7.4 (n= 6)	65.4 (n= 106)	34.6 (n= 56)
X^2 Test	df= 2; *P*= 0.46		df= 1; *P*= 0.56	
OR (95% CI)	0.67 (0.23 – 1.96)		1.15 (0.72 – 1.81)	

These results indicate no significant association between the *GAS6* Haplotypes and Breast cancer occurrence.

Chapter IV

Discussion

Breast Cancer and RPL are serious health concerns to an increasing portion of Palestinian women. Providing a better understanding of these complex diseases facilitates the medical trend towards a more individualized patient approach. In recent years, research efforts to understand genetic susceptibility to diseases and predicting treatment response based on genotype have been on the rise. Numerous studies have focused on investigating correlations between SNPs in specific target genes and the incidence of disease among various populations, which proves to be a valid risk assessment approach.

The present study is the first of its kind to investigate a correlation between *VKORC1* SNP +2255 T/C and *GAS6* SNP 843+7 G>A with the occurrence of RPL and Breast Cancer. Throughout the course of this research, each disease was approached as a separate study although the methodology was identical. In each case the haplotype frequency was compared between patients and control subjects to examine whether a correlation can be established.

RPL is a heterogeneous disease and the etiology of half the cases remains unexplained (Kwak-Kim, 2009), Thrombophilia may be a contributing factor in 40-60% of unexplained RPL cases (Brenner *et al.*, 1997). Thrombophilia risk factors are either acquired or inherited. Acquired factors include smoking, obesity, oral contraceptive pills, immobilization, and pregnancy (Cooper, 1994). Inherited factors include deficiencies in protein S or protein C, mutations in some of the proteolytic cascade proteins involved in hemostasis such as Factor II and Factor V, and Methylenetetrahydrofolate reductase (Cooper, 1994). A clear association has been established between fetal loss and certain thrombophilic states such as antiphospholipid syndromes, and antithrombin deficiency or a combination of these defects, although reports on the prevalence of inherited prothrombotic defects such as Factor V Leiden mutation and Methylenetetrahydrofolate reductase C677T polymorphism in fetal loss are contradictory (Biswas *et al.*, 2008).

Unexplained RPL among Palestinian women has attracted research efforts of several local groups. Two parallel studies were conducted on the association of Factor V Leiden with RPL among patients form the northern and southern area of the West Bank. The results from both studies were jointly analyzed and a significant association was observed (Hussein *et al*., 2010).

One of the main objectives of the current project is to test whether newly identified polymorphisms linked to thrombophilia could account for unexplained cases of RPL. Recently recognized polymorphisms in *VKORC1* +2255 T/C and *GAS6* 843+7 G>A have been linked to increased incidence of vascular disease and stroke, respectively (Wang *et al*., 2007 and Muñoz *et al.,* 2004).

Variants in *VKORC1* could serve as a common genetic risk factor for all vascular diseases. It's involved in γ-carboxylation of hemostatic and nonhemostatic proteins by controlling the vitamin K cycle. Polymorphisms of *VKORC1* have been shown to affect the expression and activity of VKOR and thus blood clotting (Yuan *et al.,* 2005 and Rieder *et al.,* 2005). The presence of the C allele at the +2255 locus conferred an almost 2-fold increase in vascular disease risk within the Chinese population (Wang *et al.,* 2006). This C allele reflects the G-C-G-C-A haplotype of the *VKORC1* gene (Wang *et al.,* 2006) and mRNA levels in the group with the G-C-G-C-A haplotype are approximately 3 times higher then the wild-type group (Rieder *et al,* 2005).

In our investigation, 45 women with unexplained RPL and 77 age- matched controls were genotyped at the *VKORC1* (+2255 T/C) locus. No significant difference was observed in haplotype distribution among the two groups (P = 0.20).

The lack of association observed could be due to; first, the possibility that RPL among the participants is not thrombophilia related. Second, since no prior studies regarding *VKORC1* polymorphisms or its haplotype block have been conducted in the Palestinian population it is difficult to assume compliance with other populations, regarding the link of this polymorphism with thrombophilia. Third, perhaps the number of participants in the study is not adequate to show a significant difference.

Some populations have shown no association between this polymorphism and vascular disease. A German case- control study on stroke patients found no link between *VKORC1* SNPs and increased risk for stroke (Arnold *et al.*, 2008). In addition, a USA study found no relationship with *VKORC1* variants and arterial or venous thrombosis (Hindorff *et al.*, 2007). One may speculate that the results observed in our study may be due to the lack of association of this SNP with thrombophilia in the Palestinian population. Therefore thrombophilia can not be dismissed as a cause in the unexplained RPL cases.

GAS6 represents another candidate gene with linkage to thrombophilia. The A allele in the *GAS6* c.834+7G>A polymorphism was shown to be associated with lower risk for stroke incidence (Muñoz *et al.,* 2007). Studies focusing on the role of Gas6 in coagulation pathways have shown that Gas6 is a platelet response amplifier that plays an important role in pathological thrombosis, and that Gas6-neutralising antibodies inhibit platelet aggregation *in vitro* (Angelillo-Scherrer *et al.*, 2001). Furthermore, when the Gas6 receptor is blocked, initial platelet aggregation does occur, but stabilisation of platelet aggregates is impaired and mice are protected against life-threatening thrombosis (Angelillo-Scherrer *et al.*, 2005). Recent publications have confirmed that all three RTKs (Axl, Tyro3 and Mer) are present on human platelets and that *GAS6* expression is high in blood (Gould *et al*, 2005 and Balogh *et al*, 2005). More studies are required to verify the role of variants in this gene with expression levels; Up- or down-regulation of *GAS6* expression from vascular cells appears to be important for its function (Muñoz *et al.*, 2007).

After screening of the *GAS6* polymorphism in our study, no significant difference in the frequency of the protective AA haplotype was observed between the control compared to the case group (P = 0.83). This could be due to similar reasons as explained for the *VKORC1* polymorphism.

Breast Cancer is one of the most devastating illnesses affecting the health of women today. Early detection is the key to better prognosis and research efforts that aim to identify genetic markers associated with an increased risk of occurrence constitute a valuable contribution to the field. Vitamin K2 (VK$_2$), menaquinones, can exert cell growth inhibitory effects in various human cancer cells and animal models (Li *et al.,* 2010 and Nimptsch *et al.*,

2010). Furthermore, the dietary consumption of VK_2 is associated with a reduced risk of incident and fatal cancer (Nimptsch K *et al.*, 2010). Considering the importance of *VKORC1* in the regulation of the vitamin K cycle, variants in this gene are attractive candidates to study their potential association with cancer. A case- control study on 81 Breast Cancer patients showed a significant difference in haplotype distribution for the *VKORC1* +2255T/C SNP compared to controls (P = 0.02). The TT haplotype was found in 32% of the Breast Cancer patients, compared to 16.7% of the control group. The CC and CT haplotypes were found in 83.3% of controls and 67.9% of patients. Therefore, the T allele conferred a more than 2 fold increased risk for developing Breast Cancer OR 2.36, 95% CI (1.13 - 4.95).

VK_2 induces apoptosis through one or more mitochondrial pathways (Green and Reed, 1998), and it is associated with the generation of superoxide, a reactive oxygen species (Shibayama-ImazuT *et al*, 2008). The production of superoxide and the induction of apoptosis by VK_2 were almost completely inhibited by cycloheximide, a protein synthesis inhibitor, suggesting de novo synthesis of enzymes involved in the production of superoxide may be required for apoptosis (Shibayama-Imazu *et al.,* 2006). One may speculate that VK_2 affects protein synthesis either by directly impacting gene regulation at the transcriptional level or through an unidentified intermediate factor that requires VK_2 for post transcriptional modification. In either case, if VK_2 involvement generates Vitamin K epoxide and the reduced form is required for continuation in activity, then the rate of reduction by VKOR will affect the progression of cells into apoptosis. The T allele of *VKORC1* is linked with a slower vitamin k cycle, this may account for the higher prevalence of the T allele with Breast Cancer patients. Interestingly, in another study *VKORC1* has been found to be over expressed in tumour tissues and may play a possible role in angiogenesis (Wang *et al.,* 2005).

Another possible explanation is that *VKORC1* haplotypes are only markers for Linkage disequilibrium and some genes in the linkage region independent of or in conjunction with VKORC1 confer susceptibility to Breast Cancer. At least 6 other genes are found in the natural haplotype block over the 68 000 bases around *VKORC1* (International HapMap Consortium, 2003); *ZNF668* (zinc finger protein), *ZNF646*, *BCKDK* (branched chain ketoacid dehydrogenase kinase), *MYST1* (histone acetyltransferase1), *PRSS8* (protease serine 8), and *PRSS36*. However, the function of these genes is still not clear (Wang *et al.,* 2006).

Gas6 exerts mitogenic activity in cell lines, and has been shown to commence coordinated entry into the S phase of the cell cycle, when bound to endogenous Axl (Goruppi *et al.*, 1996). Over expression of receptors for Gas6 belonging to the Axl/Tyro3 family had been reported in human mammary tumours, leukaemia and lung cancers (Mc Cormack *et al.*, 2008). Gas6 is up-regulated more than 23-folds by progesterone acting through the progesterone receptor B in breast cancer cell lines (Richer *et al*, 2002). Furthermore, the *GAS6* locus has recently been described as a target for amplification in mouse models and in human breast cancer (Abba *et al.*, 2007). Therefore, polymorphisms in the *GAS6* gene which may be associated with expression levels could be interesting potential biomarker for breast cancer incidence. Currently there are no studies that connect the *GAS6* c.834+7G>A polymorphism with changes in gene expression at this locus. Although, the A allele was linked with a decreased incidence of stroke (Muñoz *et al.*, 2007). This encouraged us to explore the possible relationship of a protective role for the A allele in breast cancer, based on the reasoning that Gas6 is involved in clot stability, and the A allele is linked with a decrease in clot stability presumably by lower *GAS6* expression levels. Then, perhaps the A allele could be protective in Breast Cancer as well by decreasing mitogenic activity of Gas6. However, our data indicates that no significance in haplotype distribution was observed between breast cancer patients compared to controls (P = 0.32).

In conclusion, a significant prevalence of the T allele of the *VKORC1* haplotype was established among breast cancer patients (P = 0.02) where as, the A allele of *GAS6* SNP showed no protective advantage (P = 0.32). In addition, no significant difference in haplotype distribution was observed for either *GAS6* c.834+7G>A (P=0.83) or *VKORC1* +2255 T/C (P = 0.20) among RPL cases compared to the control group.

Chapter V

Recommendations

RPL is a multi-factorial disease, it is highly unlikely that a single locus can predict pregnancy outcome alone. Multi-analysis of the association between numerous haplotypes involved with thrombophilia is recommended in these patients.

Further research to study the association of *VKORC1* variants with warfarin resistance in the Palestinian population, would be invaluable. One of the many potential medical treatments to benefit from modifying anti-coagulant dose would be RPL patients with thrombophilia etiology.

In addition, a clear elaboration of the *GAS6* c.834+7G>A polymorphism involvement in gene expression is needed. Also a search for other *GAS6* polymorphisms or mutations that can modify gene expression would benefit research efforts in RPL and Breast Cancer alike.

The association of the *VKORC1* T allele with Breast Cancer is a promising first step, although many other factors influence the VK status in the body such as, diet, absorption rate and transport (Booth and Al Rajabi, 2008). These factors should be taken into further account.

A recommended future project should focus on further elucidating the involvement of VK in apoptosis, perhaps searching for an unidentified VK- dependent factor in the signalling cascade or a direct role of VK in regulating gene expression.

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ملخص

يشكل فهم العلاقة بين المتغيرات الطبيعية في نيوكليتيدان محددة في العوامل الوراثية وحدوث المرض خطوة باتجاه تطوير التعامل الطبي على مستوى كل مريض بعينه . إن الهدف الأساسي لهذه الدراسة هو فحص احتمالية وجود علاقة بين المتغيرات الطبيعية في نيوكليتيدي محددة في العامل الوراثي للأنزيم المسئول عن اختزال فيتامين K (*VKORC1*) والعامل الوراثي للبروتين المحدد لنمو الخلايا رقم 6 (*GAS6*) وحالات الإجهاض المتكرر ومرض سرطان الثدي لدى النساء.

لقد تم سابقاً تحديد علاقة بين متغيرات (في نيكليتدان) محددة في العامل الوراثي لبروتين (*VKORC1*) وكمية فيتامين K المختزلة الناتجة والذي يعمل كعامل مساعداً في إضافة المجموعة الحامضين (̄COO) للبروتينات التي تعتمد فيعملها على ذلك وقد وجد أن نيوكليوتيد C في موقع 2255T/C + لهذا الجين مرتبط بفعالية عالية للأنزيم والذي يؤدي إلى متغيرات في الأوعية الدموية ويعتبر فيتامين K عاملاً أساسياً في إدخال هذا التعديل الكيميائي على العديد من عوامل التخثر إضافة إلى تأثيره على برمجة عملية النقل في الخلايا السرطانية لذلك فإن دراسة المتغيرات التي تؤثر على حالة فيتامين K في الجسم قد تكون مهمة في حدوث التخثر لحالات الإجهاض المتكرر واحتمالات الاصابة بسرطان الثدي.

إن بروتين *GAS6* يعتمد على فيتامين K في تأثيره على عملية برمجة الموت من خلال ارتباطه بأحد المتقبلات من عائلة TAM والتي تضم ثلاثة متغيرات Tyro3 ، AxI إضافة إلى MerTK ولقد وجد أن كمية بروتين *GAS6* ترتفع في عدة أنواع من السرطان مقارنة بالخلايا الطبيعية كما أنه يلعب دوراً في تثبيت خثرة الدم حيث أن المتغير في العامل الوراثي لهذا البروتين في موقع c.834+7 G>A قد يلعب دوراً ضد تخثر الدم حيث أن المتغير A في هذا الموقع مرتبط بانخفاض كمية بروتين *GAS6* في الخلايا.

هذا وقد تم في هذا البحث دراسة احتمالية دور المتغير المذكور في الحماية ضد السرطان والإجهاض المتكرر لقد تم إجراء هذه الدراسة في مجموعتين منفصلتين من حالات مرضية وأخرى حافظة من منظورين مختلفين، وتم فحص هذه المتغيرات باستخدام أنزيم Nco I وأنزيم AIwN I التي تقوم بقطع DNA في نقاط محددة في العاملين الوراثيين لبروتينات *VKORC1* و *GAS6* على التوالي.

وقد أظهرت النتائج في الجزء المتعلق بالإجهاض من المتكرر والذي ضم 45 حالة مرضية إضافة إلى 77 حالة حافظة عدم وجود علاقة ذات أهمية في توزيع النيوكليتدات في الموقعين المذكورين في العامل الوراثي *GAS6* c.834+7 G>A والعامل الوراثي *VKORC1* +2255 A/G بين المرضى والأصحاء في كافة المجموعات. ان هذه النتيجة قد تدل على أن هذه المتغيرات ليس لها علاقة في عملية التخثر الا أن الحاجة تدعو إلى إجراء مزيد من البحوث في هذا المجال في المجتمع الفلسطيني إضافة إلى ذلك فإن هناك حاجة إلى النظر في المتغيرات في هذه الجينات وعلاقتها بالتخثر لدى الأجنة في المستقبل أما في الجانب المتعلق بمرض سرطان الثدي والذي ضم 81 حالة مرضية و 84 حالة حافظة فإن نتيجة التحليل أظهرت عدم وجود علاقة ذات أهمية بين المتغير المذكور في العامل الوراثي لبروتين *GAS6* بينما أظهرت النتائج وجود علاقة ذات أهمية بين المتغيرات المذكورة في العامل الوراثي لبروتين *VKORC1* ومن هذا المرض تبين وجود ترتين TT لدى 32% من مرض سرطان الثدي مقابل 16.7% من المجموعة الحافظة بينما كان توزيع ترتين CC و CT في نفس الموقع بمقدار 83.3% في المجموعة الحافظة مقارنة مع 67.9% في مجموعة المرض، إن وجود نيوكليوتيد T في هذا الموقع يزيد من احتمالية الإصابة بسرطان الثدي بمقدار الضعف، وعليه فإن الحاجة تدعوا إلى إجراء مزيد من البحوث في المستقبل لدراسة طبيعة العلاقة بين نيوكليوتيد T في العامل الوراثي لبروتين *VKORC1* وسرطان الثدي إضافة إلى عوامل أخرى قد يكون لها علاقة في وضع فيتامين K في الجسم.