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Correlation between Protease activated receptor 1 (*Par1*) polymorphism and recurrent abortion in Palestine

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Correlation between Protease activated receptor 1 (*Par1*) polymorphism and recurrent abortion in Palestine

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Dedication

To my mother and father…
to my husband, Hussein…
to my son Ibrahim, and my daughters Ala' and Aya…
to my sisters and brothers…
to my teachers.

Mirvat Mahmoud Said Abu-Ghazaleh

Declaration:

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

Signed

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Abstract

Correlation between Protease activated receptor 1 (*Par1*) polymorphism and recurrent abortion in Palestine

Recurrent abortion (RA), affects 5% of women in their reproductive age in the first trimester of pregnancy. Although there are many causes for RA, a definite reason for the repeated miscarriage is still unknown in half of the cases. During placental invasion period, the expression of human protease activated receptor 1 (PAR1) peaks *in vivo* between the 7th -10th weeks of gestation and completely shuts off thereafter. Also, PAR1 is crucial to many important processes involved in placentation, and its polymorphisms were found to correlate with different diseases and unfavorable pregnancy outcomes. For that we aimed to find out the correlation between *Par1* polymorphisms and RA.

Our case-control study included 175 females from the north and the center areas of Palestine with two or more consecutive first-trimester miscarriages as cases and 241 females with two or more normal ended deliveries from the same ethnic background as controls. The research was carried out at Al - Quds Nutrition and Health Research Institute, Al - Quds University, Abu- Dies (2009-2010). DNA was extracted from blood samples of participants and analyzed for polymorphisms in the 5′ regulatory region of *Par1*, namely -506 Insertion/ Deletion (I/D) (rs11267092) and -1426 C/T (rs32934). The -506 I/D was analyzed by size polymorphic Polymerase chain reaction (PCR). The -1426 C/T polymorphism was analyzed by Restriction Fragment Length Polymorphism (RFLP), High resolution melting (HRM) and by direct sequencing for quality control purposes. To make it technically easier we made major modifications on published techniques used to genotype these polymorphisms. Indeed, our results showed that our modified techniques are reliable and easier to implement than published ones, especially the HRM which is an inexpensive, time saving, and high throughput method of analysis.

The polymorphisms inspected in this study showed no deviation from Hardy – Weinberg equilibrium. The minor allele frequency I for the -506 I/D polymorphism was 0.328 and 0.376 for cases and controls respectively (allelic OR 0.8; 95% CI= 0.6 -1.1, p 0.155). The minor allele frequency T for the –1426 C/T polymorphism was 0.012 and 0.009 for cases

and controls respectively (allelic OR 1.3; 95% CI= 0.8 -8.0, p 1.0). Our results showed no association between *Par1* polymorphisms (rs11267092 and rs32934) and RA.

Although our results showed no association between the polymorphisms we tested and RA, we think our study is valuable since we developed easier techniques that can be used to screen for these polymorphisms where their presence is associated with other diseases. Moreover, we ruled out the possibility that these polymorphisms could have association with RA in our population, although association was found between them and different unfavorable pregnancy out comes, in addition to different diseases in different ethnic groups. Nonetheless, there is still an open question whether these or other polymorphisms in *Par 1* gene could have association with unfavorable pregnancy out comes, in our population, if analyzed on the fetus tissue and not maternal tissue only.

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List of abbreviation

APC	Activated Protein C
ADP	Adenosine diphosphate
ASRM	American Society of Reproductive Medicine
APAs	Antiphospholipid antibodies
APS	Antiphospholipid Syndrome
ARMS	Amplification –Refractory Mutation System
Bp	Base pair
CTB	Cytotrophoblasts
CI	Confidence interval
DASH	Dynamic Allele Specific Hybridization
DEPC	Diethylpyrocarbonate
E	Embryo
EA	Esophageal adenocarcinoma
ECM	Extracellular matrix
FVL	Factor V leiden
F2R	Factor 2 receptor
GPCR	G-protein coupled receptors
GEFs	guanine nucleotide exchanging factors
HRM	High resolution Melting
HWE	Hardy–Weinberg equilibrium
IP3	Inositol (1,4,5) triphosphate
I/D	Insertion/ Deletion
LH	Luteinizing hormone
MAP	Mitogen activated protein
MMPs	Matrix metalloproteases
MMP-9	Matrix metalloproteinase-9
MTTHFR	Methyltetrafolate reductase
MOH	Ministry of health
OR	Odds Ratio
PAR	Protease-Activated receptor
PCOS	Polycystic ovary syndrome
PE	Preeclampsia
PTD	Preterm Delivery
RA	Recurrent abortion
RF	Rate of Liver fibrosis
RPL	Recurrent pregnancy loss
RT-PCR	Real-Time PCR

RFLP	Restriction Fragment Length Polymorphism
SGA	Small for gestational age
SNP	Single nucleotide polymorphism
SFLLRN	Serine- phenylalanine- leucine- leucine – arginine – aspargine
TAE	Tris acetate- EDTA
Tm	Melting Temperature
TxA2	Thromboxane A2
TRAPs	thrombin receptor-activating peptides
VTE	Venous thromboembolism

Chapter one

1.1 Introduction

Recurrent abortion (RA), defined as two or more consecutive spontaneous abortion, affects 5% of women in their reproductive age in the first trimester of pregnancy (Allison and Schust, 2009; Kovalevsky et al., 2004). While RA was found to correlate with chromosomal abnormalities, thrombophilia, metabolic disorders, anatomical causes, infectious diseases, and immune factors (Horne and Alexander, 2005), a definite reason for the repeated miscarriage is still unknown in up to 50% of cases (Allison and Schust, 2009).

Placentation, a normal physiological invasion process, in which cytotrophoblasts (CTB), specialized epithelial cells, differentiate, proliferate, and invade the decidua and the spiral arteries (Cross et al., 1994; Even-Ram et al., 2003). Proper placentation and maintenance of adequate placental circulation is important for successful pregnancy (Dudding and Attia, 2004). The CTB in their invasion process share many features with tumor cells in term of metastasis, growth, search for blood supply, and extracellular matrix (ECM) degradation (Cross et al., 1994; Damsky and Fisher, 1998). These properties are critical for appropriate feto-maternal interaction and should be tightly controlled (Damsky and Fisher, 1998; Even-Ram et al., 2003; Flamigni et al., 1991; Yin et al., 2003a). It has been reported that Protease Activated Receptor 1 (PAR1) is exclusively and transiently expressed in CTB during their invasion (Even-Ram et al., 1998). Moreover, it has been shown that PAR1 plays an important role in pathologic and physiologic invasion processes (Even-Ram et al., 1998).

PAR1, a seven transmembrane G-protein coupled receptor (GPCR), belongs to the PAR family (Grand et al., 1996). PAR1 is the main thrombin receptor on vascular cells and plays an important role in haemostasis, inflammation and vascular development (Coughlin,

2000; Traynelis and Trejo, 2007). Recent studies have advanced our knowledge about the important role that PAR1 plays in tumor development, metastasis, and angiogenesis (Even-Ram et al., 1998; Yin et al., 2003a; Yin et al., 2003b). It has been noticed that PAR1 is exclusively expressed between the 7th- 10th weeks of gestation and completely shut off thereafter (undetectable by the 12th week), suggesting a temporal and critical relationship between the expression of PAR-1 and early placentation (Even-Ram et al., 2003). Polymorphisms of *Par1* gene have been reported to have physiologic relevance (Altarescu et al., 2010; Arnaud et al., 2000; Dupont et al., 2003; Grisaru-Granovsky et al., 2007; Martinelli et al., 2008). The temporal expression of PAR1 during placental formation should be tightly controlled for appropriate feto-maternal interaction, for that, any polymorphism in *Par1* may influence critical processes involved in placentation resulting in an improper feto-maternal interaction and consequently adverse pregnancy outcome (Grisaru-Granovsky et al., 2007).

1.2 Justification

Since its discovery in 1991, extensive research studies have identified PAR1 as an important player in different pathologic and physiologic situations. It has been shown that PAR1 plays an important role in blood coagulation and homeostasis (Coughlin, 2000). It has been shown that 50% of *Par1* Knockout mouse embryos die as a result of multiple bleeding and cardiovascular failures at embryonic days 9-10 (E9-10), suggesting that PAR1 and coagulation factors may contribute to normal blood vessel development (Griffin et al., 2001). Moreover, PAR1 has been shown to be involved in the modulation of the extracellular matrix during placentation (Even-Ram et al., 2003). Needless to say, that all these processes are critical for successful placentation. In normal placentation the expression of PAR1 on CTB peaks *in vivo* between the 7th -10th weeks of gestation and completely shuts off after that (Even-Ram et al., 2003). This time coincides with the maximal invasion of CTB and the increased production of matrix metalloproteases and urokinase–type plasminogen activator (Cohen et al., 2006; Xu et al., 2000). The central role of PAR1 in tumor invasiveness and metastasis has been derived from different studies. PAR1 plays an important role during metastatic breast carcinoma cell invasion and prostate cancer progression by enhancing both angiogenesis and tumor growth (Salah et

al., 2005; Yin et al., 2003a). The pathobiology of several pregnancy disorders is associated with dysregulantion of CTB invasion (Even-Ram et al., 2003). Therefore, due to the fact that PAR1 is crucial for many important processes involved in placentation, and since polymorphism in its gene was found to correlate with different diseases and unfavorable pregnancy outcomes (Grisaru-Granovsky et al., 2007), we suggest that such polymorphism (-506 I/D and -1426 C/T) might correlate with RA.

1.3 Problem Statement

Despite the fact of high fertility rate among Palestinian females, which according to the Palestinian Ministry Of Health (MOH) in its executive summary 2003 was calculated to be 3.89 (4.7 in Gaza Strip and 3.4 in West Bank), yet**,** the problem with RA is still present. Although no statistical data is available in our country about the prevalence of RA, except the one done by the UNRWA, many women are still going to local clinics for treatment of RA with no pregnancy success. According to UNRWA, 4 - 8 % of women attending the UNRWA antenatal care unit in the north part of the west bank, suffer from RA (Abu-Hilal, 2009). While RA might be related to chromosomal abnormalities, thrombophilia, metabolic disorders, anatomical causes, and immune factors, the etiology in 50% of the cases is still unknown (Horne and Alexander, 2005). Though, more research must be focused toward the identification of further risk factors in order to find the appropriate treatment, and prevent over treatment of an enormous number of women who receive either low molecular weight heparin or hormonal treatment as prophylactic protocols to prevent RA (Dudding and Attia, 2004; Horne and Alexander, 2005). Here, we suggest testing the correlation between *Par1* polymorphism (-506 I/D and -1426 C/T) - a gene known to be involved in pathological and physiological invasion processes like placentation – and RA.

1.4 Goals

The main objective of this work is to investigate the association between *Par1* gene polymorphism in the 5′ regulatory region (-506 I/D and -1426 C/T) and RA among Palestinian females in the West Bank. This study also aims to develop a cost effective and

advanced technique for genotyping these polymorphisms using High Resolution Melting (HRM) one of the Real-Time Polymerases Chain Reaction (RT-PCR) for the -1426 C/T (rs 32934) SNP and size polymorphic RCR for the -506 I/D (rs 11267092).

1.5 Questions

- *Par1* gene polymorphisms (-506 I/D and -1426 C/T) and recurrent abortion…is there a relationship?
- High Resolution Melting (HRM) is a new molecular technique to detect single nucleotide polymorphisms (SNPs). Could it be applicable for detecting -1426 C/T (rs 32934) SNP?
- What is the allele frequency of *Par1*gene polymorphisms (-506 I/D and -1426 C/T) among the Palestinian female population?

1.6 Hypothesis

PAR-1 is highly expressed in the 7th - 10th weeks of gestation and shut down thereafter, this period is coincided with embryonic implantation and placental formation. Moreover, PAR1 has been shown in different invasion events and in processes critical for placentation. Thus, we hypothesize that *Par1* polymorphisms (-506 I/D and -1426 C/T) that might alter the expression of this gene could lead to unfavorable pregnancy outcomes, including RA.

1.7 Scientific Background

1.7.1 Recurrent abortion

Recurrent abortion (RA), traditionally has been defined as three or more consecutive pregnancy losses at less than 20 weeks of gestation (Allison and Schust, 2009; Pabinger, 2009; Perni et al., 2004). Recently, the Practice Committee of the American Society for Reproductive Medicine (ASRM) in January 2008 and the ASRM Board of Directors in February 2008 have redefined RA as “two or more failed pregnancies” (ASRM, 2008). RA affects 5% of women in their reproductive age (Kovalevsky et al., 2004; Lissalde-

Lavigne et al., 2005; Sierra and Stephenson, 2006). While RA was found to correlate with chromosomal abnormalities, thrombophilia, metabolic disorders, anatomical causes, infectious diseases, and immune factors , the etiology in half of cases remains vague, figure (1.1) (Ford and Schust, 2009; Horne and Alexander, 2005). RA could be named as recurrent pregnancy loss (RPL) (Kutteh and Triplett, 2006), recurrent miscarriage (Horne and Alexander, 2005; Li et al., 2002; Rai and Regan, 2006), recurrent spontaneous abortion (Perni et al., 2004) and habitual abortion (Gruber and Huber, 2005).

1.7.1.1 Causes of RA

1.7.1.1.1 Genetic factor

About 2% to 5% of RA is associated with a parental balanced structural chromosome rearrangement (Allison and Schust, 2009; Ford and Schust, 2009). Chromosomal translocation either reciprocal or robertsonian are most frequently encountered (Clifford et al., 1994; Horne and Alexander, 2005). Although these parents are phenotypically normal , 50% - 70 % of their gametes and consequently their embryos are unbalanced due to abnormal separation at meiosis leading to pregnancy termination (Rai and Regan, 2006). Aneuploid embryos may be another cause of RA; it is more common with advanced reproductive age reflecting decreased quality oocytes (Sierra and Stephenson, 2006; Vidal et al., 1998). X-chromosome inactivation has been reported to be a cause of RA (Uehara et al., 2001)

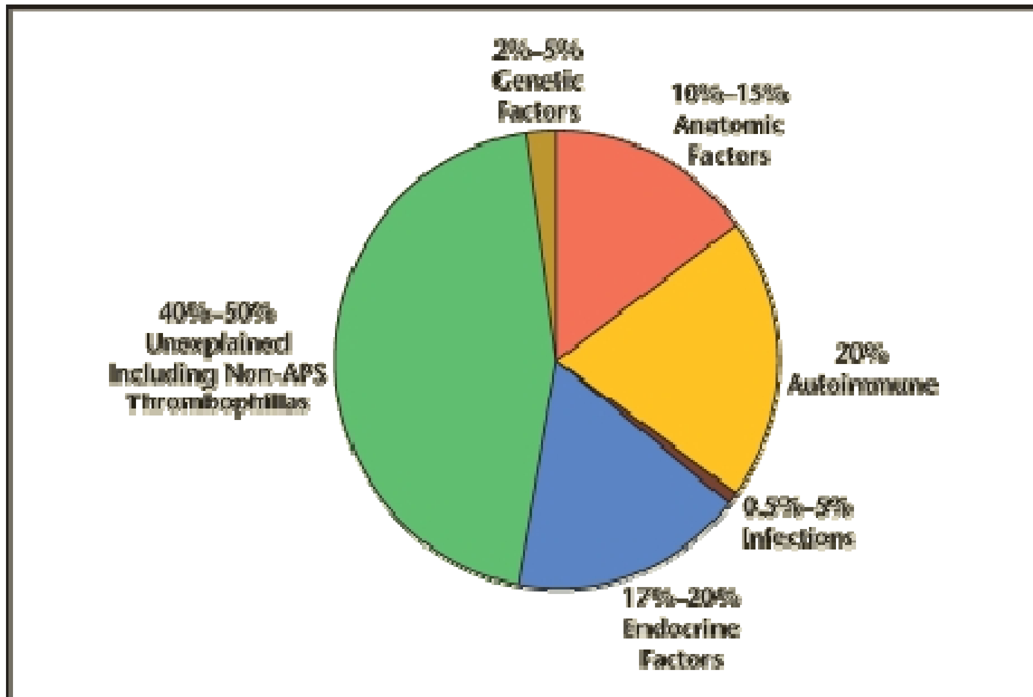


Figure 1.1: Etiology of recurrent pregnancy loss. APS, antiphospholipid syndrome. Adopted from (Ford and Schust, 209).

1.7.1.1.2 Metabolic disorders

Endocrine disorders are heterogeneous, involved in around 17% to 20% of RA (Ford and Schust, 2009). Prolactin plays a role in both ovulation and endometrial maturation (Bussen et al., 1999; Rai and Regan, 2006). Hyperprolactinaemia and lack of endometrial prolactin expression during the luteal phase of the menstrual cycle have been reported to be associated with RA (Hirahara et al., 1998; Rai and Regan, 2006). Hypo- and hyperthyroidism have also been associated with fetal loss (Kaprara and Krassas, 2008). Uncontrolled diabetes mellitus and insulin resistance is common in women with RA (Craig et al., 2002; Roberts and Murphy, 2000). Alternatively, well –controlled diabetes mellitus and treated thyroid dysfunction is not associated with RA (Clifford et al., 1994). Polycystic ovarian syndrome (PCOS) has been associated with RA (Rai et al., 2000). Recently , PCOS and its association with insulin resistance have been noted as a cause of RA (Ford and Schust, 2009; Rai and Regan, 2006). Increased androgen level in the follicular phase has also been reported to be associated with RA (Bussen et al., 1999; Okon

et al., 1998). Increased levels of luteinizing hormone (LH) is suggested to be a cause of RA (Clifford et al., 1994; Homburg et al., 1988), others however, did not recommend LH hypersecretion to be a cause of RA (Bussen et al., 1999; Li et al., 2000). These contradicting results may be related to the more specific and more sensitive methods of detection and the sampling time (Li et al., 2002).

1.7.1.1.3 Thrombophilia

Thrombophilia either acquired and/or hereditary, predispose patients to thromboembolic events (Kutteh and Triplett, 2006; Li et al., 2002). Pregnancy itself is considered as secondary hypercoagulable state (Kutteh and Triplett, 2006; Rai and Regan, 2006). Recently, several studies have been carried out to find an association between different causes of thrombophilia and RA and yielded controversial results. For that, different meta-analysis studies were carried out to reveal these contradiction (Dudding and Attia, 2004; Kist et al., 2008; Kovalevsky et al., 2004; Rey et al., 2003). These studies revealed a strong association between RA in the second /third trimester and carriers of factor V Leiden (FVL) (Dudding and Attia, 2004; Kist et al., 2008). Others revealed an association between carriers of FVL, activated protein C resistance, and prothrombin mutation in the first trimester of pregnancy with stronger association in late pregnancy trimesters (Kovalevsky et al., 2004; Rey et al., 2003). Ethnicity and disease severity may interpret these diverse results (Kist et al., 2008). Females homozygous for the methyltetrafolate reductase mutation (MTHFR) are not at risk for RA (Pabinger, 2009; Rey et al., 2003). However, patients suffering from Antiphospholipid syndrome (APS) -acquired thrombophilia- with Antiphospholipid Antibodies (APAs) are at high risk concerning RA (Kutteh and Triplett, 2006; Pabinger, 2009). As these antibodies may impair trophoblast invasion and promote trophoblast apoptosis (Rai and Regan, 2006).

1.7.1.1.4 Anatomical causes.

Congenital and acquired uterus abnormalities are another cause of RA and are related to 10% to 15% of RA causes (Ford and Schust, 2009). Congenital abnormalities as in Müllerian anomalies have been implicated with septate uterus (Propst and Hill, 2000). This will lead to RA as a result of reduced sensitivity to steroid hormones (Fedele et al.,

1996; Horne and Alexander, 2005) and inadequate blood supply to the developing embryo and placenta (Propst and Hill, 2000). Asherman's syndrome or endometrial fibrosis is an acquired problem in which uterine cavity is partially or completely obliterated , resulting in improper implantation, decreased sensitivity to steroid hormones, interruption of blood supply to the uterus and/or no space for the fetus to develop and grow (Horne and Alexander, 2005; Li et al., 2002).

1.7.1.1.5 Immune factors

Since the fetus is genetically not identical to his mother, immunological factors may be an important etiology concerning RA (Ford and Schust, 2009; Horne and Alexander, 2005). For that, the presence of cytotoxic antibodies, inappropriate sharing of human leukocyte antigens, absence of maternal blocking antibodies and disturbances in natural killer cell function and allocation may be different causes of RA (Allison and Schust, 2009). Autoantibodies are more commonly encountered antibodies in patients with RA (Horne and Alexander, 2005; Rai and Regan, 2006). APAs are being the mostly encountered (Greaves et al., 2000; Rai and Regan, 2006). The presence of thyroid antibodies may be related to increased risk of RA as these antibodies reveal a generalized activation of the immune system against the feto-placental unit (Kaprara and Krassas, 2008; Pratt et al., 1993).

1.7.1.1.6 Infections

Infection is an infrequent cause of RA (Summers, 1994). For an infective agent to be involved as a cause of RA, it must persist in the genital tract and cause some maternal symptoms (Rai and Regan, 2006). While TORCH (Toxoplasmosis, Rubella, Cytomegalo-virus and Herpes) infections have been reported to be implicated in adverse pregnancy outcome and miscarriage (Gilbert, 2002), routine screening for them is not recommended in RA (Horne and Alexander, 2005; Li et al., 2002; Rai and Regan, 2006). The presence of high titer immunoglobulin G antibody to *Chlamydia trachomatis* has been reported to be associated with RA (Baud et al., 2008; Witkin and Ledger, 1992).

1.7.1.1.7 Undefined causes.

A definite reason for the repeated miscarriage is still unknown in up to 50% of cases (Allison and Schust, 2009; Horne and Alexander, 2005; Li et al., 2002).

1.7.1.2 Placentation

Embryonic development requires a placenta that forms by implantation of the blastocyst in the maternal uterus (Tarrade et al., 2001). The placenta is an independent and temporary organ that allows nutrition and gas exchanges between the fetus and the maternal body (Tarrade et al., 2001). Placentation requires a sequence of complex, coordinated interactions between CTB and the maternal uterus (Xu et al., 2000). An important aspect of this process is the invasion of the CTB into the maternal decidua and the spiral arteries (figure 1.2) (Le Bouteiller and Tabiasco, 2006; Yin et al., 2003a). A successful pregnancy outcome depends strictly on proper placentation and maintenance of sufficient placental circulation (Dudding and Attia, 2004). Therefore, it is possible that any abnormalities in placental formation or vasculature may lead to improper placentation or inadequate feto-maternal circulation resulting in adverse pregnancy outcome (Dudding and Attia, 2004). It has been demonstrated that any dysregulation in CTB invasion will lead to several disorders of pregnancy (Yin et al., 2003a), for instance, preeclampsia and restriction of fetal growth are associated with shallow uterine invasion (Zhou et al., 1993), while partial or complete hydatidiform moles and choriocarcinoma are associated with excessive proliferation and invasion (Even-Ram et al., 2003; Yin et al., 2003a) . A critical feature in CTB invasion is their interaction with the surrounding ECM, which is characterized by specific cell adhesion molecule profile switching (Damsky and Fisher, 1998; Zhou et al., 1993). This includes the down regulation of $\alpha6\beta4$ and E-cadherin that are characteristics of stable epithelial monolayer (Zhou et al., 1993), and the up regulation of $\alpha v\beta3$, $\alpha1\beta1$ and VE cadherin that mimic the endothelial cells phenotype (Zhou et al., 1993). It has been shown that the abnormality in adhesion – related to antigen expression– may be due to the abnormal expression of regulatory molecules produced by the trophoblast or the maternal cells with which they come in contact (Zhou et al., 1993). The CTB in their invasion process share many features with tumor cells in term of metastasis, growth, search for

blood supply and ECM degradation; these properties critical for appropriate feto-maternal interaction and should be tightly controlled (Damsky and Fisher, 1998; Even-Ram et al., 2003; Flamigni et al., 1991; Yin et al., 2003a). CTB invasion, unlike tumor cells, is precisely regulated to be spatially constricted to the inner third of the myometrium and temporally restricted to early pregnancy (Xu et al., 2000). In CTB invasion, placental microenvironment remodeling and trophoblast out growth are needed where these effects are carried out by serine and matrix metalloproteases (MMPs) (Cohen et al., 2006; Xu et al., 2000). It has been reported that MMP-9 secretion increases gradually from the 7th - 11th weeks of gestation with the maximal invasive potential of CTB (Xu et al., 2001; Xu et al., 2000). This gradual increase coincides with the over expression of the Protease Activated Receptor1 (PAR1) which plays an important role in pathologic and physiologic invasion processes (Even-Ram et al., 1998; Grisaru-Granovsky et al., 2007).

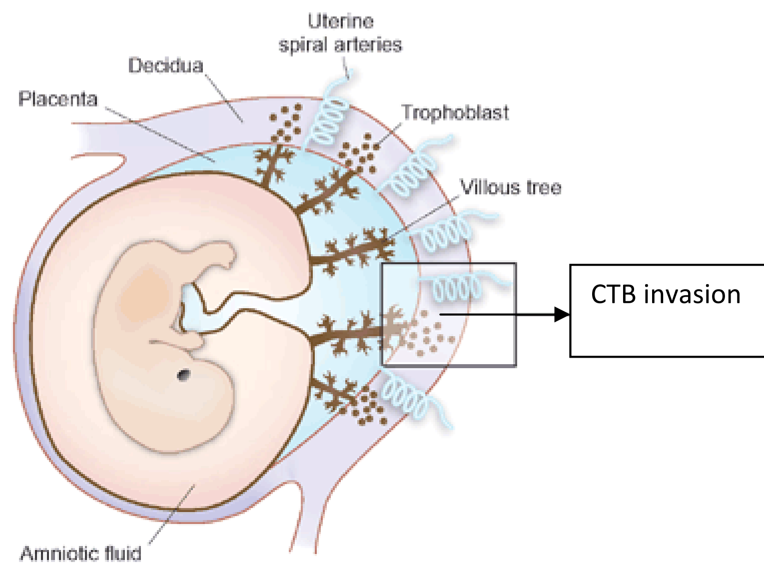


Figure 1.2 Placentation. Cytotrophoblasts (CTB) differentiate, proliferate, and invade into the maternal decidua and the spiral arteries. Adopted from (le Bouteiller, 2006), with modification.

1.7.1.3 Protease Activated Receptors

PAR1, a seven transmembrane G-protein coupled receptor (GPCR), belongs to the PAR family (Coughlin, 2000; Hirano and Kanaide, 2003; Vu et al., 1991). PARs are found to be expressed on a variety of cell types and tissues such as platelets, endothelial cells, sensory neurons, fibroblast, vascular and nonvascular smooth muscles (O'Brien et al., 2008). Multiple extracellular proteases can activate PAR*s*; thrombin is the physiological activator of PAR1, PAR3 and PAR4 (Coughlin et al., 1992; Grand et al., 1996). Other proteases can activate the receptors and may contribute to their function *in vivo* such as activated protein C (APC) (Mosnier et al., 2007; Riewald et al., 2002), anticoagulant proteases plasmin, cathepsin G (Traynelis and Trejo, 2007) and MMPs family (Boire et al., 2005; Trivedi et al., 2009). PAR-2, on the other hand, cannot be activated by thrombin and require trypsin-like protease for activation as trypsin (Nystedt et al., 1994), tryptase (Molino et al., 1997), factor VIIa and factor Xa (Camerer et al., 1996). PARs are activated by an N-terminus cleavage that exposes a tethered ligand which autoregulates the receptor by coupling it to the G protein (Chen et al., 1994; Traynelis and Trejo, 2007). This activation is important for the hydrolysis of the structural proteins at the ECM, engagement of integrins, cell adhesion and mitogenesis (Boire et al., 2005; Grand et al., 1996; Martorell et al., 2008).

1.7.1.3.1 PAR1

Human thrombin receptor was isolated from cDNA cloned in *Xenopus* oocytes (Vu et al., 1991). PAR1 consists of seven helical hydrophobic transmembrane regions (Vu et al., 1991). This assembly gave rise to three intracellular loops with C- terminal tail and three extracellular loops with long N-terminal domain, figure (1.3) (Grand et al., 1996). This long N-terminal domain is important for receptor recognition and cleavage (Vu et al., 1991).

PAR1, also known as Factor 2 receptor (F2R) (O'Brien et al., 2008) or human thrombin receptor (Li et al., 1996). It spans about 27-kilobase (kb) on chromosome number 5q11.2 to q13.3 (Arnaud et al., 2000; Schmidt et al., 1996) , with Genbank accession number AF054633.1 (http://www.ncbi.nlm.nih.gov/nuccore/3309036). It consists of two exons

and a single intron (around 22 kb in size) (Dupont et al., 2003; Schmidt et al., 1996). PAR1 encodes 425 amino acids (Vu et al., 1991). Of which, 29 amino acids are encoded in the first exon, while the majority of the coding sequence and the thrombin cleavage site are located in the second exon (Schmidt et al., 1996). Within the proximal promoter, multiple transcription initiation sites are located with the absence of a TATA box sequence (Li et al., 1996).

1.7.1.3.1.1 PAR1 activation

PAR 1 is considered as a peptide receptor that carries its own ligand which become exposed upon receptor cleavage (Coughlin, 1999a). PAR1 cleavage site resides in the amino-terminal extracellular domain between amino-acids arginine 41 and serine 42 (Vu et al., 1991). Once cleavage takes place, a new receptor amino terminal started with the sequence SFLLRN (Serine- phenylalanine- leucine- leucine – arginine - aspargine) is generated and functions as a tethered ligand (Vu et al., 1991). This newly exposed N-terminus interacts with a binding site located on amino acid 244- 268 in the second extracellular loop and activates the receptor (Bahou et al., 1994; Gerszten et al., 1994; O'Brien et al., 2001) (figure 1.3). Synthetic hexapeptide SFLLRN or pentapeptide SFLLR, that resembles the newly formed amino-terminus, or modified sequence TRAPs (thrombin receptor-activating peptides), can fully activate the receptor independent of receptor cleavage (Anderluh and Dolenc, 2002; Chen et al., 1994; Sabo et al., 1992). Although thrombin and TRAPs can activate PAR1, different concentration from them are required to elicit the same response (Grand et al., 1996). Although thrombin is considered the main physiological activator for PAR1 (Vu et al., 1991), other proteases can activate the receptor and elicit intracellular signaling. Among these proteases, APC (Riewald et al., 2002), anticoagulant proteases plasmin, cathepsin G (Traynelis and Trejo, 2007) and MMPs family (Boire et al., 2005; Trivedi et al., 2009).

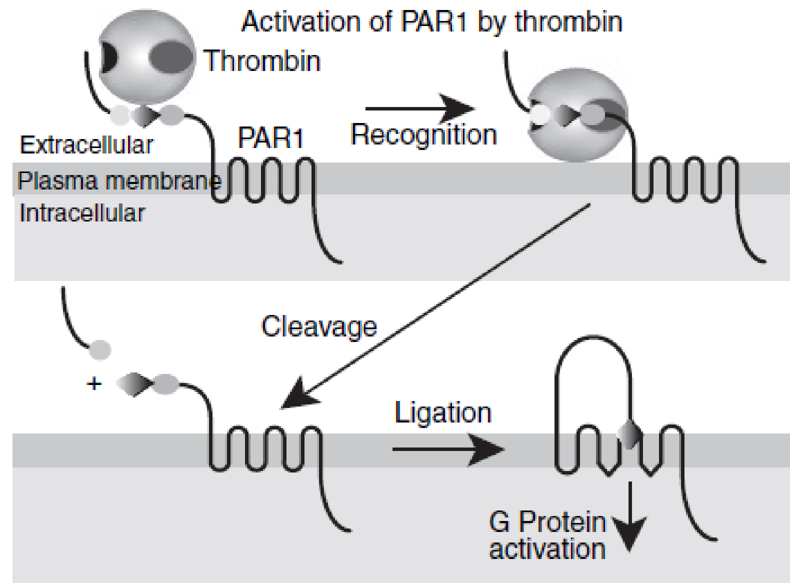


Figure1.3: PAR1 structure and activation mechanism. PAR1, a seven transmembrane bound receptor. Thrombin recognition site resides in the amino-terminal extracellular domain; the newly formed amino-terminal will bind to the second extracellular loop and activates the receptor. Adopted from (Coughlin, 2005).

1.7.1.3.1.2 PAR1 signaling

Once activated, PAR1 undergo conformational changes to facilitate coupling to heterotrimeric G-protein (Coughlin, 1999a). PAR1 couples to Gαq, Gα i, Gα12/13 and Gβγ families (Coughlin, 2000). This will induce activation of diverse signaling cascades and elicit different cellular responses figure (1.4) (Coughlin, 2005; Hirano and Kanaide, 2003). Coupling to Gα12/13 subunits will lead to guanine nucleotide exchanging factors (GEFs) binding and activation of Rho kinase, serine/threonine-specific protein, resulting in platelets' shape change (Angiolillo et al., 2010). On the other hand, coupling to Gαq activates phospholipase Cβ that leads to phosphoinositide hydrolysis and the production of inositol (1, 4, 5)-triphosphate (IP3) and diacylglycerol (DAC) (Arora et al., 2007; Coughlin, 2005). This will cause mitogen-activated protein (MAP) kinases activation, mobilization of intracellular $Ca2^{+,}$ and protein kinase C activation (Arora et al., 2007;

Coughlin, 2000). Adenylyl cyclase inhibition is a result of Gα i coupling (Coughlin, 2005). Coupling to Gβγ will activate phosphoinositide-3 kinase (PI3 kinases), ions channels, and G-protein coupled receptor kinases (GRKs) (Coughlin, 2005).

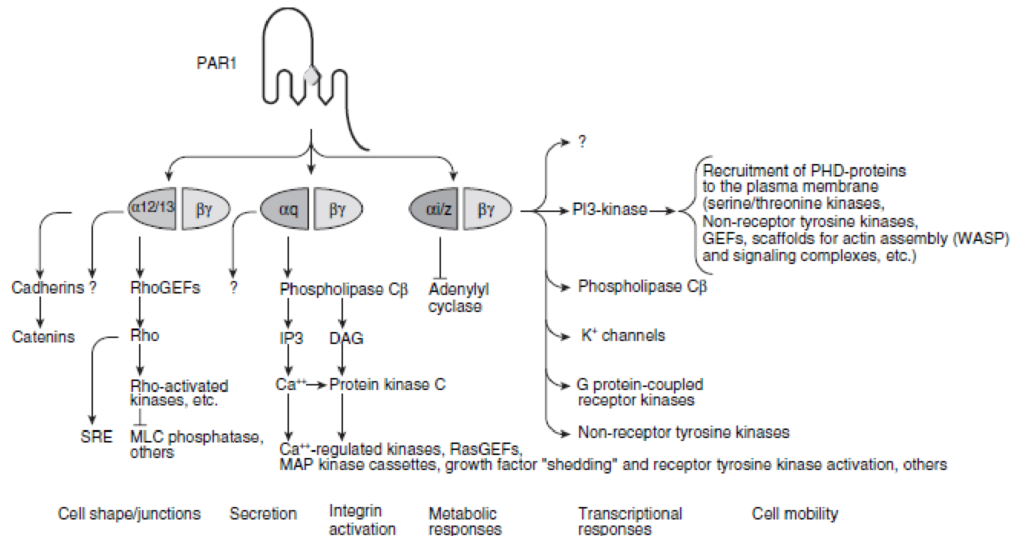


Figure1.4: PAR1 signaling. Once the newly formed amino terminal binds to its receptor, coupling to PAR1 Gαq, Gα i, Gα12/13 and Gβγ families takes place. This will lead to activation of diverse signaling cascades and eliciting different cellular responses. Adopted from (Coughlin, 2005).

1.7.1.3.1.3 PAR signal regulation

Activation of PAR1 by proteases is irreversible and thus termination of signaling is important to prevent uncontrolled signaling (Coughlin, 1999b; O'Brien et al., 2001). PAR1, like other classical GPCR, once activated is rapidly phosphorylated and internalized, but instead of recycling, activated PAR1 is sorted to lysosomes (Chen et al., 2004; Coughlin, 1999a; Trejo et al., 1998). The desensitization of PAR1 signaling is regulated by multiple independent mechanisms including C-tail phosphorylation and binding of β-arrestins independent of phosphorylation (Chen et al., 2004). Uncleaved PAR1 on the other hand, cycles constitutively between the cell surface and an intracellular compartment, by this, a protected receptor pool is generated (Arora et al., 2007). This pool can repopulate the cell surface by receptors after proteases exposure independent of *de novo*

receptor synthesis (Arora et al., 2007; Coughlin, 1999a), thereby, rapid resensitization to proteases will be achieved (Arora et al., 2007; Coughlin, 1999a).

1.7.1.3.1.4 PAR1 tissue distribution and function

Since the discovery of RAR1, extensive research studies have identified it as an important player in different pathologic and physiologic situations. PAR1 is the main thrombin receptor on vascular cells (Coughlin, 2005; Grand et al., 1996). Its activation on the platelets' surface results in the change of platelets' shape, the release of Adenosine diphosphate (ADP) and thromboxane A2 (TxA2) (Coughlin, 2000). PAR1 activation on endothelial cells results in the recruitment of immune cells, disruption of the endothelial cell barrier and migration and release of growth factors (Hirano and Kanaide, 2003). These functions suggest that PAR1 plays an important role in haemostasis, inflammation and vascular development (Coughlin, 2000; Traynelis and Trejo, 2007). In vascular smooth muscle cells, PAR1 activation results in direct contractile effect mainly in atherosclerosis by mediating hypertrophy, proliferation, migration and production of extracellular matrix (Hirano and Kanaide, 2003; Martorell et al., 2008). Hyperalgesia and edema as a result of neurogenic inflammation is PAR1 mediated (Coughlin, 2005). It has been shown that PAR1 prevent apoptosis in neurons (Guo et al., 2004). PAR1 has also been reported to be expressed on Hepatic stellate cells (Martinelli et al., 2008).

Recent studies have advanced our knowledge about the important role that PAR1 plays in tumor development, metastasis and angiogenesis (Yin et al., 2003a; Yin et al., 2003b). It has been reported that PAR1 is highly expressed in malignant tissue biopsy specimens and metastatic tumor cell lines (Even-Ram et al., 1998). In normal epithelial tissue or cell lines of low metastatic properties, PAR1 showed little or no expression (Even-Ram et al., 1998; Grisaru-Granovsky et al., 2009). The central role of PAR1 in tumors invasiveness and metastasis has been derived from different studies. PAR1 plays an important role during metastatic breast carcinoma cell invasion and prostate cancer progression by enhancing both angiogenesis and tumor growth (Salah et al., 2005; Yin et al., 2003a). Moreover, it has been noticed that PAR1 is exclusively expressed between the 7th- 10th weeks of gestation and completely shut off thereafter (undetectable by the 12th week) (Even-Ram et

al., 2003) suggesting a temporal and critical relationship between the expression of PAR-1 and early placentation (Even-Ram et al., 2003; Grisaru-Granovsky et al., 2009).

1.7.1.3.1.5 PAR1 in embryonic development

It has been shown that 50% of *Par1* Knockout mouse embryos die by E9-10 (Griffin et al., 2001). Death occurs as a result of multiple bleeding and cardiovascular failures suggesting that PAR1 and coagulation factors may contribute to normal blood vessel development (Griffin et al., 2001). In the time period restricted for placentation, the expression of PAR1 on CTB peaks *in vivo* between the 7th -10th weeks of gestation and totally shuts off later (Even-Ram et al., 2003). This period coincides with the maximal invasion of CTB and the increased production of matrix metalloproteases and urokinase –type plasminogen activator (Cohen et al., 2006; Xu et al., 2000). The pathobiology of some pregnancy disorders is related to dysregulation in CTB invasion (Even-Ram et al., 2003), for example, preeclampsia and restriction of fetal growth are related to shallow uterine invasion (Zhou et al., 1993), while partial and complete hydatidiform moles and choriocarcinoma are associated with extreme proliferation and invasion (Even-Ram et al., 2003; Yin et al., 2003a). Consequently, PAR1 is crucial to many important processes involved in placentation and the over all of normal pregnancy.

1.7.1.3.1.6 Polymorphisms in *Par1*

Three different polymorphisms in human *Par*1 have been reported to have physiological significance (Arnaud et al., 2000; Dupont et al., 2003; Grisaru-Granovsky et al., 2007). The heavily studied, and the ones that found to have functional relevance include; an A/T single nucleotide polymorphism (SNP) in the 3′ end of the large intron near a putative splicing acceptor site (IVS-14 A/T) (rs168753) (Arnaud et al., 2000), a C/T transition at 1426 upstream the translation start site (-1426 C/T) (rs32934) and a 13-base pair (bp) insertion repeat preceding -506 5′-CGGCCGCGGGAAG -3′ sequence (-506 I/D) (rs11267092) where I means insertion and D means deletion (Arnaud et al., 2000). It has been shown that the polymorphism in IVS-14 A/T in the intron affects the density of PAR1 receptors at the platelets surface; the presence of the T allele is associated with decreased expression (Dupont et al., 2003). This suggest that IVS-14A/T SNP may be clinically

relevant in individuals who are at increasing risk of bleeding as mild hemophilia, surgical patients or von Willebrand disease (Dupont et al., 2003).

It has been demonstrated that a 13bp I in the -506I/D polymorphism was under represented in male cases with venous thromboembolism (VTE) (Arnaud et al., 2000). This result suggests a gender –dependent VTE protective effect of this insertion (Arnaud et al., 2000). It has been reported that the II genotype in -506 I/D polymorphism may be an essential predictive factor for esophageal adenocarcinoma (EA) and was associated with developing tumor recurrence (Lurje et al., 2010). The -1426 C/T polymorphism, on the other hand, has been demonstrated to be implicated in chronic hepatitis C liver fibrosis, preterm delivery (PTD) and Gaucher disease. Rate of Liver fibrosis (RF) has been reported to be faster in patients having the TT genotype compared with those having the CT or CC genotype (Martinelli et al., 2008). Additionally, mothers heterozygous for this SNP were at risk concerning PTD (Grisaru-Granovsky et al., 2007). Moreover, this SNP and its T allele have also been implicated with thrombocytopenia in Gaucher disease patients (Altarescu et al., 2010).

1.7.1.3.1.7 *Par1* Polymorphism detection methods

Different PCR based mutation detection methods were used to detect PAR1 polymorphism. For instance, Restriction Fragment Length Polymorphism (RFLP) was used to detect -506 I/D (Altarescu et al., 2010; Arnaud et al., 2000; Grisaru-Granovsky et al., 2007) and the -1426 C/T (Arnaud et al., 2000; Grisaru-Granovsky et al., 2007) polymorphism with the suitable restriction enzymes. Others used size polymorphic PCR to detect -506 I/D (Dupont et al., 2003). -1426 C/T polymorphism was detected by DNA sequencing (Altarescu et al., 2010; Dupont et al., 2003) or Amplification –Refractory Mutation System (ARMS) PCR (Martinelli et al., 2008). In view of that**,** -1426 C/T polymorphism as other similar SNPs, can be detected by different PCR based SNP detection methods. Even though, DNA sequencing is considered the gold standard technique, its high relative cost is the main disadvantage (Krypuy et al., 2006). RFLP and ARMS on the other hand, is time consuming and expensive. For that, we aim to assess the applicability of High resolution

melting (HRM) which is an application of Real-Time PCR (RT-PCR) in detecting this SNP.

Melting curve analysis in conjunction with real-time PCR was introduced in 1997 (Wittwer et al., 2003). In one- tube, changes in florescence behavior can be monitored due to the release of an interchelating DNA dye from a DNA duplex as it is denaturated by increasing temperature (Krypuy et al., 2006). Because different genetic sequences melt at slightly different rates, they can be viewed, compared, and detected using these curves. Our SNP of interest (-1426 C/T) is classified as class 1 SNP according to the duplexes that are produced after PCR amplification (Liew et al., 2004). It can be simply recognized by melting analysis (Liew et al., 2004). It has been reported that HRM analysis can detect mutations in clinical samples (Krypuy et al., 2006), and genotypes SNP as those for FVL, prothrombin and MTHFR (Liew et al., 2004).

1.8 Literature review

PAR1 and its association with human pregnancy is a new issue in which some studies were carried out, the results have highlighted the importance of temporal expression of PAR1 and spatial regulation of PAR1 signaling for proper embryonic growth, migration, and development.

A series of studies by Evan-Ram and colleagues have clearly shown that PAR1 is involved in restricted and unrestricted pathological trophoblast invasion (Even-Ram et al., 2003). Their studies showed that PAR1 is highly and spatially expressed between the 7th and 10th weeks of gestation in CTB and shut off after that (Even-Ram et al., 2003), moreover, they have shown that PAR1 is highly expressed in the CTB of completely hydatidiform mole (CHM) compared to very low expression in normal age-matched placenta (Even-Ram et al., 2003).

A study by O'Loughlin *et. al* showed that vasodilation effect of thrombin in umbilical artery vasculature is PAR1 mediated, this finding is important for feto-placental circulation in normal pregnancy (O'Loughlin et al., 2005). Adverse pregnancy outcome such as

preeclampsia (PE) and intrauterine growth retardation may be related to non-thrombotic effect of thrombin through PAR1 (O'Loughlin et al., 2005).

A pilot case-control study performed by Grisaru-Granovsky *et.al* proved an association between *Par1* polymorphism (-506 I/D, -1426C/T and -14 IVS A/T) and unfavorable pregnancy outcome. Polymorphisms in the 5′ regulatory region and intervening sequence were investigated in PE, preterm delivery (PTD) and small for gestational age babies (SGA). This study showed that there is an association between maternal heterozygous allele of the -1426 C/T polymorphism of *Par*1 and PTD (Grisaru-Granovsky et al., 2007) and did not show an association between neonatal *Par1* polymorphism and adverse pregnancy outcome (Grisaru-Granovsky et al., 2007).

In a cross-sectional study, Erez *et. al.* demonstrated a strong PAR1 expression in placentas from preterm PE complicated pregnancies compared with placentas from spontaneous preterm labor (Erez et al., 2008).

Another recent study carried by Grisaru-Granovsky and colleagues has demonstrated for the first time the causal association between PAR1 and β– catenin stabilization in placental CTB. They showed that silencing of *Par1* significantly inhibits proliferation and invasion of CTB (Grisaru-Granovsky et al., 2009).

A case control study by Arnaud and coworkers was carried out to find an association between PAR1 polymorphism and VTE. While genotypes and allele frequencies of the -506 I/D, -1426 C/T and the IVs -14 A/T were similar between cases and controls, the allele I was under represented in male cases with VTE. This suggests a possible gender-dependent protective effect of the insertion in males (Arnaud et al., 2000).

A study by Dupont and colleagues was carried out to examine the effect of *Par1* polymorphism on the expression and function of PAR1 on platelet surface. While the - 506 I/D and the -1426 C/T were not associated with the platelets PAR1 phenotype, the IVs -14 A/T was associated with PAR1 expression level. Carriers for the homozygous A allele had

a significantly higher PAR1 level than heterozygous carriers, and the presence of the T allele showed a decreased platelets response to SFLLRN (Dupont et al., 2003).

Martinelli *et. al.* in their study showed a relationship between the TT polymorphism in the -1426 C/T polymorphism and the rate of liver fibrosis. Patients homozygous for the T allele showed higher RF compared with heterozygous (CT) or homozygous for the C allele. However, the IVS -14 A/T and the - 506 I/D showed no difference on RF among the different genotypes (Martinelli et al., 2008).

Recently, a study carried out by Altarescu *et. al.* aimed to describe *Par1* polymorphism in Gaucher disease. The prevalence of -1426 C/T SNP with the TT genotype were found to be statistically significant among Gaucher disease patients compared to controls. Although this SNP was not found to be associated with disease severity, need for enzyme replacement therapy or splenectomy, it was associated with low platelets' count. On the other hand, homozygous -506 I/D polymorphism with the II genotype was found to be correlated with lower bone mineral density at the femoral neck area compared with patients having other genotypes (Altarescu et al., 2010).

Lurje *et. al*. in their study concerning EA and genetic variations in angiogenesis related pathways showed that -506 I/D polymorphism with the II genotype may be an important prognostic factor for EA. The II genotype was significantly related to tumor recurrence. This polymorphism may increase the angiogenic potential that is vital for the development of EA tumor progression (Lurje et al., 2010).

To the best of our knowledge, no data on the association between *Par1* gene polymorphism and RA is available so far.

Chapter Two

Methodology

2.1 Sampling

2.1.1 Inclusion criteria

Samples from Palestinian females in child bearing age suffering from RA (two or more consecutive spontaneous abortion) in the first trimester matched with females with two or more normal deliveries were analyzed for polymorphisms in the 5′regulatory region of *Par1*. All women were provided with a written informed consent prior to the collection of blood samples. This research was performed in accordance with the declaration of Helsinki used by the Palestinian Ministry of health.

2.1.2 DNA Controls

Previously genotyped samples by DNA sequencing for each polymorphism were used as DNA control in this study.

2.1.3 Research place

The research was carried out at Al-Quds Nutrition and Health Research Institute (ANAHRI), Al –Quds University, Abu Dies.

2.2 Sample processing:

2.2.1 Blood sample collection

Blood samples were collected from participants for DNA extraction as whole blood in EDTA tubes (5ml). The samples were stored at 4 ^{0}C up to three days.

The samples were collected from:

- Primary Health Care Center/ Ramallah.
- NGOs (Red Crescent Hospital/ Jerusalem).
- Private Centers.

2.2.2 Genomic DNA extraction

DNA extraction was done using MasterPure™ (according to the manufacturer's instruction) DNA Purification Kit for Blood (Epicentre Biotechnology, Madison, USA). DNA quantity and quality were measured using Nanodrop 1000 spectrophotometer (Thermo Scientific, Bancroft, USA), soft copy version (3.7.1). The following parameters were obtained; DNA concentration, the 260/230 ratio (for any DNA contamination by phenolate ion, thiocyanates since both solvents used in DNA extraction procedure) and the 260/ 280 ratio to evaluate nucleic acid contamination by protein. DNA was then stored at -20 ^{0}C until used.

2.3 Polymorphisms identification

2.3.1 Size polymorphic PCR

This technique was used to test for -506 I/D (rs11267092) polymorphism. PCR primers were designed according to (http://frodo.wi.mit.edu/primer3/) to give PCR products of 100 and 113bp for the deletion (D) and insertion (I) alleles, respectively. The primer sequences are shown in table (2.1). Their location on *Par1* gene is shown in figure (2.1). PCR amplification was performed using PCR-Ready ™ High Yield Kit (Syntezza Bioscience, Jerusalem). To each tube a mixture of 22.5 µl distilled water (DEPC treated, Beit Haemek, Israel), from each primer (Rmut30 and Del13Ins), 0.5µl (0.5 uM) (Syntezza Bioscience, Jerusalem), 0.5 µl formamide (Promega, Madison, USA) and 1µl (100 ng/µl) DNA were added. Formamide was added to enhance PCR specificity. PCR amplification was done on preheated Gene Amp PCR system 9700 (PE Applied Biosystem) according to the following conditions: initial denaturation at 95 ^{0}C for 3 minutes followed by 35 cycles of denaturation at 95 ^{0}C for 30 seconds, annealing at 55 ^{0}C for 30 seconds, extension at 72 ^{0}C for 40 seconds and final extension at 72 ^{0}C for 7 minutes. The products were analyzed using 3% agarose gel (Sea-Kem^R LE agarose, lonza, USA), and visualized by Image

Master VDS (Pharmacia Biotech) and documented using Fujifilm, thermal imaging system FTI-500. Previously sequenced DNA samples were used as controls. Moreover, twenty percent of the samples were repeated randomly. The products were evaluated according to the following criteria: The homozygous insertion allele (II) was one band with 113bp in length. The heterozygous allele (ID) was two bands, 113bp and 100bp in length. On the other hand, the homozygous deletion allele (DD) was one band with 100 bp.

2.3.2 Restriction Fragment Length Polymorphism (RFLP)

This technique was used for the -1426 C/T (rs32934) polymorphism detection. This method was proposed to be done according to Arnaud et al.(Arnaud et al., 2000), however, the restriction criteria were totally different when compared with the Nebcutter. While the C allele was sited to remain uncut and the T allele was sited to lose 39 bp, the Nebcutter revealed a totally different digestion criterion. According to the Nedcutter, both alleles will lose 15 bp and the C allele will also lose another 25 bp, consequently the difference between the two alleles was only 25 bp (418 / 393 bp) but not 39 bp (433/ 393 bp) as sited in the article. This difference between the two alleles was difficult to detect, for that, modifications were performed.

2.3.2.1 RFLP modification

Newly designed Primers were performed to allow proper detection of this SNP. This modification was performed in order to obtain shorter PCR product to get better resolution and to see if there is another suitable restriction enzyme. Two sets of PCR primers were designed according (http://frodo.wi.mit.edu/primer3/) to give PCR products of 223 bp or 293 bp before digestion. The reverse primer was used according to Arnaud et. al. (Arnaud et al., 2000), and newly designed one was used that allow digestion by another restriction enzyme. The restriction enzyme was chosen according to NEBcutter V2.0 (http://tools.-neb.com/NEBcutter2/). The primer sequences with the suitable restriction enzyme used for the modification are shown in table (2.2) and their locations on our gene are shown in figure (2.1). PCR was done as mentioned above without formamide addition. PCR amplification was done on Mastercycler gradient PCR machine (Eppendrof, North America, USA) according to the following conditions: initial denaturation at 96 ^{0}C for5

minutes followed by 38 cycles of denaturation at 95 ^{0}C for 30 seconds, extension at 72 ^{0}C for 50 seconds and final extension at 72 ^{0}C for 6 minutes. We tested annealing at 58 ^{0}C, 60 ^{0}C, and 62 ^{0}C for 30 seconds. The PCR products were analyzed on 1.5% agarose (Sea-Kem^R LE agarose, lonza, USA). According to the analyzed product we decided to continue our optimization and work with the shorter PCR products.

After amplification using our newly designed primers and optimized annealing temperature, digestion of PCR products was performed according to the following protocol: 20µl PCR product, 1µl of the corresponding restriction enzyme, *HpaII* or *BstN1* (Fermentas, Burlington, Canada), 2.5 µl of 10X of the corresponding buffer with BSA (Fermentas, Burlington, Canada). PCR products were digested for 2 hours at 37^{0}C and then analyzed on 3% agarose gel (Sea-Kem^R LE agarose, lonza, USA). PCR products were visualized and documented as stated above. The products were evaluated according to the criteria clarified in tables (2.3-2.4) using the NEB cutter web site V2.0 (http://tools.neb.-com/NEBcutter2/).

After optimization, we decided to work with the primers that gave a PCR product of 223bp and digested with *HpaII* restriction enzyme. Furthermore, 10% of the samples were repeated randomly with the primers that gave PCR product of 223 bp and digested with *BstN1* enzyme as a confirmatory step.

2.3.3 DNA sequencing

For quality assurance purposes, direct DNA-sequencing was performed to confirm validity of the methods used. These samples were used as a positive control in HRM genotyping technique.

2.3.3.1 PCR product cleaning

Before sequencing, PCR products were purified using High pure PCR product purification Kit (Roche, Germany) according to the manufacturer's instruction. Samples were then sequenced from both directions (5′ and 3′) with FCTPara-sh forward primer (10 uM) and HpaIICTR reverse primer (10 uM).

2.3.4 Real Time-PCR (RT-PCR) and HRM

2.3.4.1 RT-PCR preparation

It has been noted that shorter amplicon in HRM is more sensitive in detecting mutation than larger one (Krypuy et al., 2006). Therefore, primers with 76 bp product to detect the C/T mutation were designed using (http://frodo.wi.mit.edu/primer3/) web site. The primer sequences are shown in table (2.1) and their locations on our gene are shown in figure (2.1). PCR amplification was performed in a 20 μl reaction volume. To each tube a mixture of 3.4 μl distilled water, 4μl primer mix (10 pM), 10 μl of hot start PCR master mix (X2) (Thermo Scientific, Bancroft, USA), 0.6 μL SYTO9 (1.5mM) (Invitrogen, Carlsbad, CA, USA), 2μl DNA (100- 200 ng/reaction). PCR cycling and HRM analysis were carried out using Rotor-Gene 6000 (Corbett Research, New South Whale, Australia) and its software version 1.2 according to the following conditions: after an initial denaturation at 95 ^{0}C for10 minutes, 40 cycles were carried out; 95 ^{0}C for 5 seconds, 60 ^{0}C for 10 seconds, 72 ^{0}C for 20 seconds and melting from 75 - 95 ^{0}C rising at 0.2 ^{0}C per second.

To make sure that the amplified product matched with the estimated size, PCR products were analyzed by gel electrophoresis. PCR products were visualized and documented as stated above. To every run negative and positive controls were included. All PCR reactions were performed in duplicates.

2.3.4.2 Melting curve analysis

Melting curve analysis was performed directly on the same instrument after amplification with its provided software. Different curves were obtained and all of them aimed to discriminate between different genotypes. Derivative melting curves were obtained for direct comparison after melting. In theses curves the fluorescence signal is plotted against the temperature, as the double stranded DNA becomes single stranded, the fluorescence intensity decreases and the dye is released. The melting temperature (Tm) at which 50% of the DNA is double stranded can be obtained from them (Erali et al., 2008). Normalized curves were another design for analysis. In these curves, linear baselines before and after

melting transition were defined for each sample. Then fluorescence intensity was normalized between 0% and 100%. Fluorescence acquisition at each temperature was calculated as the percentage of fluorescence between the top and the bottom baselines for each sample (Wittwer et al., 2003). The melting profile was also demonstrated as difference plots, in which the melting curve for one C/T sample (that was further confirmed by sequencing) was chosen as a reference and became a horizontal line at zero as it is subtracted from itself. The difference between each curve and the reference was plotted against temperature. These plots allow different genotypes to cluster in groups for easier visual inspection (Wittwer et al., 2003).

Table 2.1: PCR primers used in *Par1* polymorphism analysis.

SNP site and method of detection	Sequence 5′- 3′	Primer direction
-506 I/D Size polymorphic	CACTGTGGACGTCTCCACAT	Forward (Del 13 Ins)
	GCGAAGCTGTCAGTGA	Reverse (R mut 30)
-1426 C/T RFLP	GCTGCAGCTTATCAACCACA	Forward FCT Para-sh
	TGCTAAGGGCCCCCAGGGGCGTCG CGGCTGGGGGTGGGCG	Reverse (HpaIICTR)
-1426 C/T HRM	ACTTAAGCTGCAGGCTCCTC	forward HRMCT PAR1
	ACACTCGCTTGGCAGTCTG	Reverse (PCR2 CT)

Table 2.2: PCR primers used in RFLP modification.

Primer 5′-3′	Sequence 5′-3′	Band size	Restriction enzyme
Forward HpaIICTR	TGGGGGAATGATACTCTTCG	293	*BstN1*
Reverse TR2Lmod	TGCTAAGGGCCCCCAGGGGCGTC GCGGCTGGGGGTGGCC		
Forward FCT Para-sh	GCTGCAGCTTATCAACCACA	223	*BstN1*
Reverse TR2Lmod	TGCTAAGGGCCCCCAGGGGCGTCG CGGCTGGGGGTGGCC		
Forward HpaIICTR	TGGGGGAATGATACTCTTCG	293	*HpaII*
Reverse HpaIICTR	TGCTAAGGGCCCCCAGGGGCGTC GCGGCTGGGGGTGGGCG		
Forward FCT Para-sh	GCTGCAGCTTATCAACCACA	223	*HpaII*
Reverse HpaIICTR	TGCTAAGGGCCCCCAGGGGCGTC GCGGCTGGGGGTGGGCG		

2.3.5 Agarose Gel Preparation and Electrophoresis

The 4, 3, 1.5 % agarose gel was prepared by dissolving 4, 3, 1.5 grams agarose (Sea Kem ^{R} LE agarose, Lonza, USA) respectively in 100 ml 1X TAE electrophoresis buffer (0.04 M Tris –Acetate, pH 8.0 and 0.001 M EDTA), boiled in microwave, and when it cooled to about 50°C, 5µl of ethidium bromide (0.5 ug/ml) (Sigma) was added.

The PCR products were analyzed on horizontal gel electrophoresis SUB- Cell ^{R} GT (Medi Pharm, Beit-Sahour). The applied voltage was 120 volt in the first 10 min then at 100 volt for 1 hour.

Table 2.3: The virtual product size after digestion according to Neb cutter for the C allele.

PCR length (bp)	Enzyme	Ends	Coordinates	Bands size (bp)
293	BstN1	(LeftEnd)-BstNI	1-253	253
		BstNI-BstNI	254 -278	25
		BstNI-(RightEnd)	279 -293	15
293	HpaII	HpaII-HpaII	62 -252	191
		(LeftEnd)-HpaII	1 -61	61
		HpaII-(RightEnd)	253 -293	41
223	BstN1	(LeftEnd)-BstNI	1 -183	183
		BstNI-BstNI	184 -208	25
		BstN1(RightEnd)	209 -223	15
223	HpaII	(LeftEnd)-HpaII	1-182	182
		HpaII-(RightEnd)	183 -223	41

Table 2.4: The virtual product size after digestion according to Neb cutter for the T allele.

PCR length(bp)	Enzyme	Ends	Coordinates	Bands size (bp)
293	BstN1	(LeftEnd)-BstN1	1-278	278
		BstN1-(RightEnd)	279-293	15
293	HpaII	(LeftEnd)-HpaII	1-61	61
		HpaII-(RightEnd)	62-293	232
223	BstN1	(LeftEnd)-BstNI	1-208	208
		BstNI-(RightEnd)	209-223	15
223	HpaII	LeftEnd-RightEnd	Uncut	223

```
1081 AAATCGCCTC TGATCGTACT TTCTCCACGG ATGTAAGTGT CCGGGCTCTA GTGGGGGAAT
1141 GATACTCTTC GTGCGAAATT CACTTTTAAA AAAGGCTTAG AAAACTGACC ACCGGCTCTC
1201 AGCTGCAGCT TATCAACCAC AGAACTCCGA ATATGCCCGC ACATCAGTGC GCTGGGTTAA
1261 GAGGAGAAGG GGCTGCGGCT GAGCTTTCCT AGAAACAGCT ATTTGGGGAC CCATTTCCTG
1321 TTGGAGTCTG AAAGGCGCAC GCACCAGAGC CAGTGGCAAA GCAACTTAAG CTGCAGGCTC
1381 CTCCTGCCCA CCCCCAGCCG CGACGCCCCT GGGGGCCCTT AGCAGACTGC CAAGCGAGTG
1441 TCCCCGCCCT GTGCCTTGAA TTTAGCCAAA CTCCTTTAAT ATCAGGCCTG GCGCGGTGAT
1501 TCACGCCTGT AATCCCCGCA CTTTAGGAGG CTGAGGCAGG AGGATCGCTT GAGGCCAGCA
1561 GTGAAATTAG CCGGACATGG TGGCGCGCGC CTTTCTGTGG TCCCAGGTAA TCCGGAGGCT

2221 GCTGCAGGGC GTGGACGCAT CCTGGCCGGG GCGTCCACTG TCGACGTCTC CACATCCCAG
2281 GAGGGTCGAG ACGGCCGCGG GAAGCAGCCT GCGAGCCGTG CGGCCCCATT CCAAGGACCC
2341 CGCCAGTGTG AGTCACTGAC AGCTTCGCGA ATCAACGGTG CCCAGAGGAA AAAACTTCTC
```

Figure 2.1: Human *Par1* gene, exon 1 and partial cds (accession no. AF054633.1**.).** Red primers are for the -1426 C/T SNP with the HRM. The green primers are for the -506 I/D primers. The blue primers are for the -1426 C/T SNP with RFLP, the grey nucleotide A was shared between the RFLP and HRM primers. The C nucleotide is the site of the SNP, The T near the site of SNP was changed in the reverse primer to G in order to be cut with *HpaII* enzyme. The C near the site of SNP, it was changed to G in the reverse primer in order to be cut with *BstN1* enzyme. CGGCCGCGGGAAG is the site where insertion takes place in the -506 site.

2.4 Statistical analysis

The association between different genotype in females with recurrent abortion and those with normal pregnancies was done using Pearson Chi-square or fisher exact whenever appropriate. A p-value of < 0.05 was considered statistically significant. The analysis was performed using SPSS V17.0. Allele frequencies were calculated manually. Distribution of the alleles was tested for departure from Hardy-Weinberg equilibrium (HWE) using the (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl) website. The allele frequencies were observed within the probability limits ($P < 0.05$, for HWE) using Pearson Chi-square or fisher exact whenever appropriate.

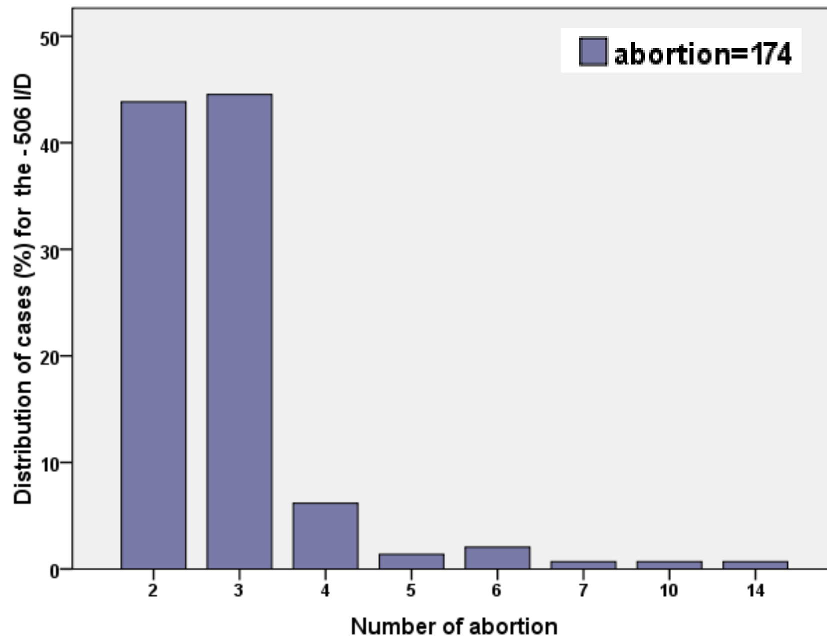
Chapter Three

Results

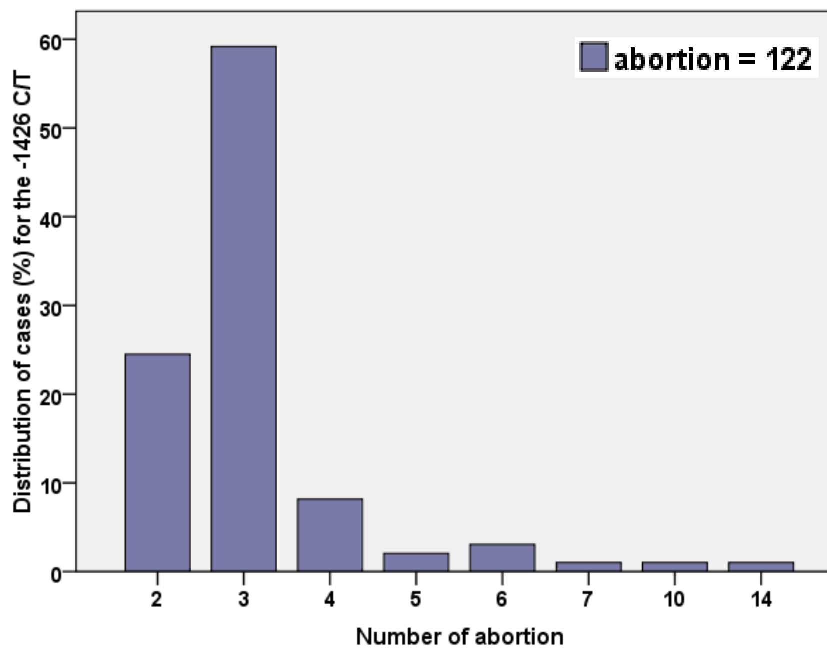
3.1 Population Study

This study was performed to find out the association between polymorphism in *Par 1* gene and recurrent abortion. To achieve our goal, we have genotyped Palestinian females for *Par1* polymorphism in the 5′ regulatory region, namely -1426C/T (rs32934) SNP and -506 I/D (rs 11267092) polymorphism. A total of 415 participants were evaluated for the -506 I/D polymorphism consisting of 174 (41.9%) cases and 241 (58.1%) controls. 230 participants were evaluated for the –1426 C/T polymorphism, consisting of 122 (53%) cases and 108 (47%) controls. For the -506 I/D polymorphism detection , the case group was women who had two (43.8%) or more (56.2%) consecutive first trimester miscarriage (figure 3.1a,) with a mean age (32.82 years) as shown in Table 3.1. For the -1426 C/T polymorphism, the case group was women who had two (24.5%) or more (75.5 %) consecutive first trimester miscarriage (figure 3.1 b) with mean age (32.89 years) as shown in Table (3.1). The control group for both polymorphisms was women who had two or more successful pregnancy outcome with mean age 28.53 years for -506 I/D and 27.96 years for -1426 C/T (Table 3.1). Our study subjects belonged to the same ethnic group. They were distributed among the north and center area of Palestine. For the -506 I/D, 77.8% of participants were from the north and 22.2% were from the center (figures 3.2 a). For the -1426 C/T, 64.3% of the participants were from the north and 35.7% of the participants were from the center (figure 3.2 b). Any case suffered from second or third trimester pregnancy loss was excluded from the study. The cases did not exclude for other abnormalities or mutations

except in cases from the center area of Palestine because the cost of the study will be highly elevated.

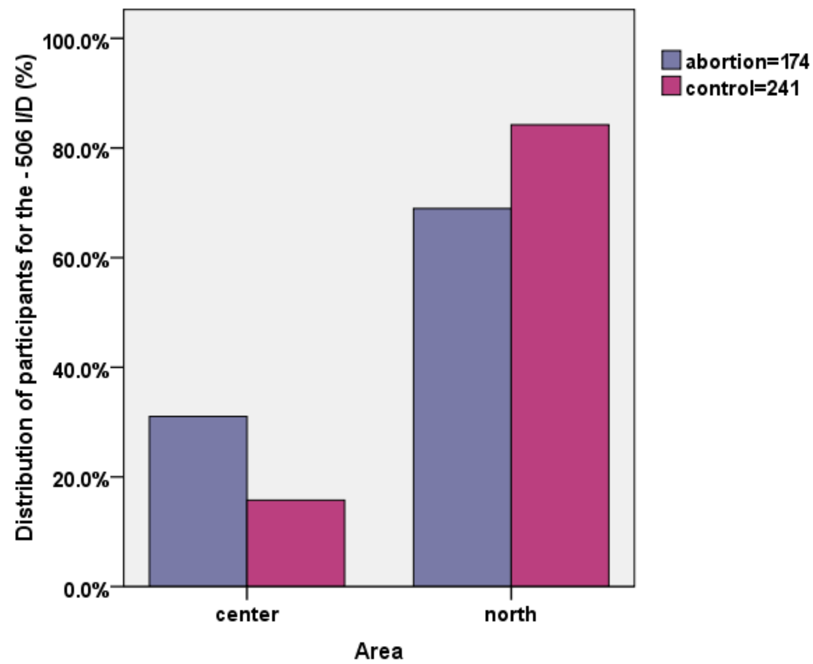


(a)

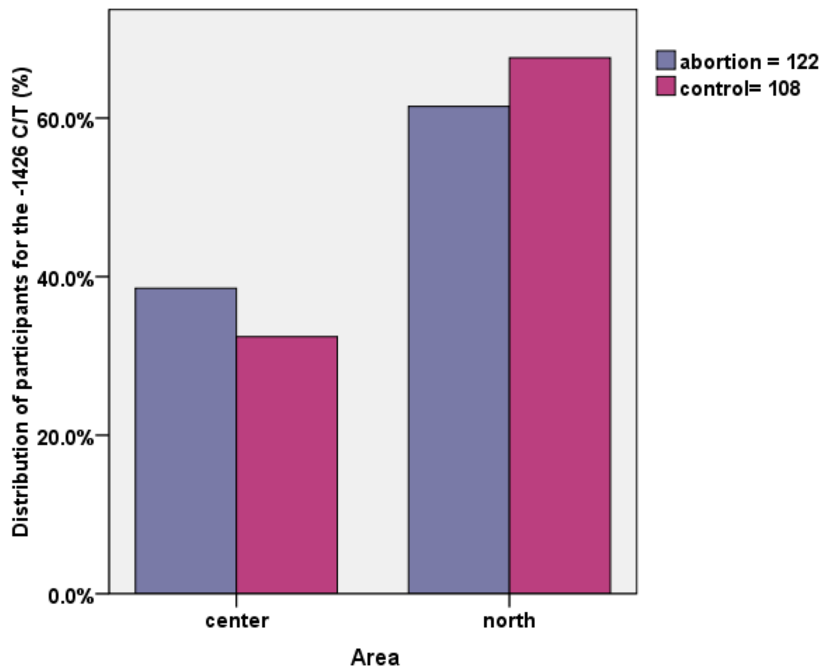


(b)

Figure 3.1: Distribution of cases (%) by number of abortions. a) -506 I/D polymorphism participants (cases, n = 174). B) -1426 C/T SNP participants (cases, n= 122).



(a)



(b)

Figure 3.2: Distribution of participants (%) among the north and center area of Palestine. a) Participants in -506 I/D polymorphism genotyping. b) Participants in -1426C/T polymorphism genotyping.

Table 3.1: Distribution of participants by age.

Polymorphism	Mean age of cases	Mean age of controls
-506 I/D	32.82 ± 6.6 8	28.53 ± 5.84
-1426 C/T	32.89 ± 6.78	27.96 ± 6.03

Mean (years) ± SD.

3.2 Genotyping results.

3.2.1 Screening for the -506 I/D (11267092) by size polymorphic PCR

In order to genotype the -506I/D polymorphism we used size polymorphic PCR. This technique unlike the previously used RFLP technique (Altarescu et al., 2010; Arnaud et al., 2000; Grisaru-Granovsky et al., 2007) or Dynamic Allele Specific Hybridization (DASH) was easy and rapid. In this technique the DD, ID, and II genotypes give PCR products of 100, 100 and 113, respectively as clearly seen in figure (3.3). We tested our technique validity by genotyping previously sequenced samples and found it valid in all the cases.

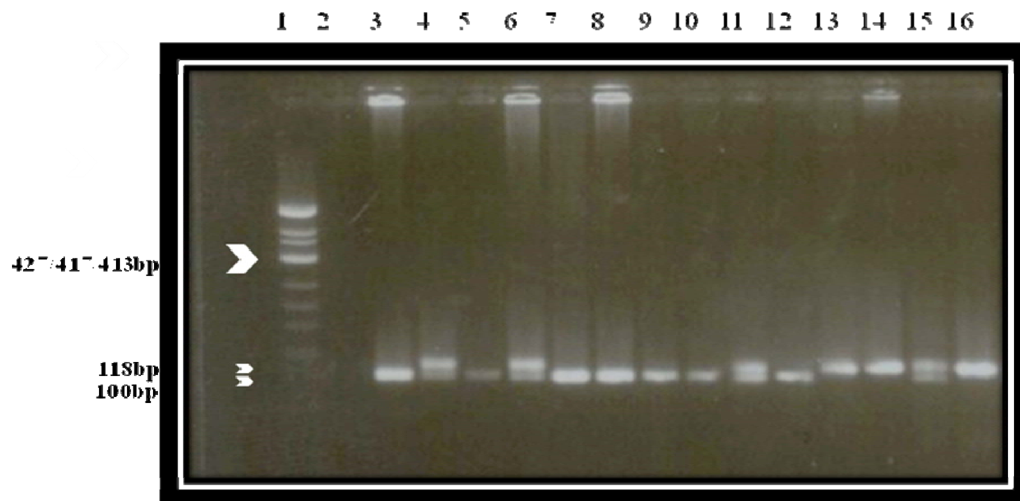


Figure 3.3: -506 I/D polymorphism genotyping by size polymorphic PCR. Lane 1 represents Hinf l digest DNA ladder. Lane 2, negative control. Lanes (3, 5, 7-10, 12) show DD genotype with 100 bp. Lanes (4, 6, 11,15) show ID genotype with 113/100 and lanes (13, 14, 16) show II genotype with 113bp.

3.2.2 Screening for the -1426 C/T (rs32934) SNP by RFLP

Different techniques were used to detect this SNP; RFLP (Arnaud et al., 2000; Grisaru-Granovsky et al., 2007), DNA sequencing (Altarescu et al., 2010; Dupont et al., 2003) and Amplification –Refractory Mutation System (ARMS) PCR (Martinelli et al., 2008). We have chosen to detect this SNP by RFLP according to Arnaud et. al. (Arnaud et al., 2000), because the difference between the two alleles were only 25 bp (418 / 393 bp) and not 39 bp as sited in the article (433/ 393 bp) we modified this method. For this modification, different pairs of primers that can be cut by different restriction enzymes (*HpaII* and *BstN1*) were used. Optimization for the PCR was done using different annealing temperatures with the selected pairs of primers (figure 3.4). The 60^0C annealing temperature – second group - has the minimal nonspecific bandings with the pairs of primers that gave PCR products of 223 bp and can be cut by *BstN1* and *HpaII* enzyme.

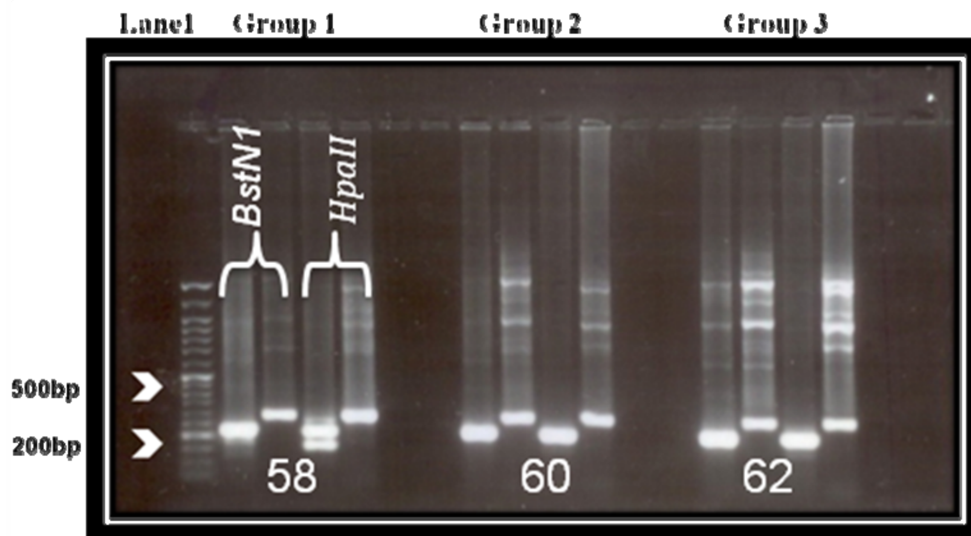


Figure 3.4: RFLP optimization using different sets of primers combinations and different annealing temperature. Lane 1 represents the 50bp DNA ladder. Each group is composed of PCR products that can be cut by *BstN1*, short (223bp), long (293) and by *HpaII* short (223bp), long (293bp), respectively. Annealing temperature was 58, 60,and 62 ^{0}C, for each group respectively.

After optimization, we decided to work with the primer pairs that gave products size of 223bp before restriction as shown in figure 3.5. Although our modified RFLP method was able to differentiate CC genotype from the CT genotype with both enzymes, the *HpaII* enzyme gave better resolution, as shown in figure 3.6. Ten percent (10%) of the samples were repeated randomly with *BstN1* enzyme to confirm the results (figure 3.7). The TT genotype was absent among our participants. As seen in figure (3.5, 3.6, 3.7), the theoretically calculated band sizes agreed with the ones appeared in the gel. The results obtained with our modified RFLP method were confirmed by DNA sequencing.

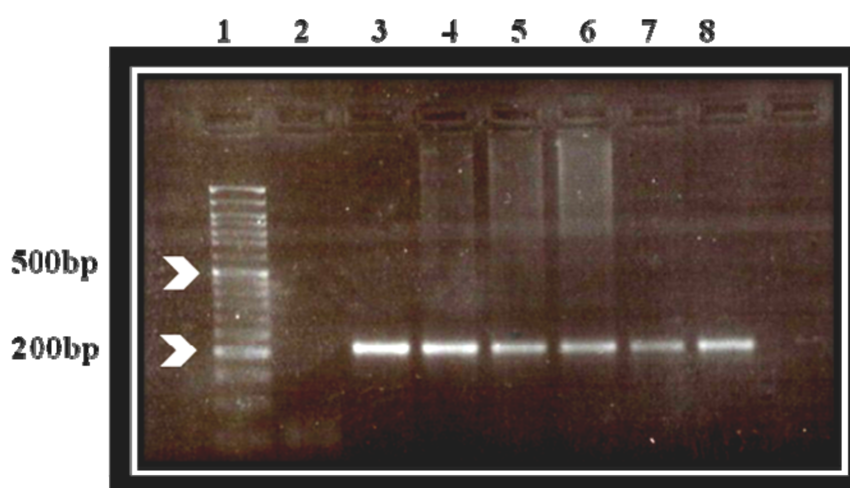


Figure 3.5: PCR products for the -1426 C/T before digestion. Lane 1 represents the 50 bp DNA ladder. Lane 2 is the negative control. The rest of lanes represent the PCR products before restriction with 223bp band size.

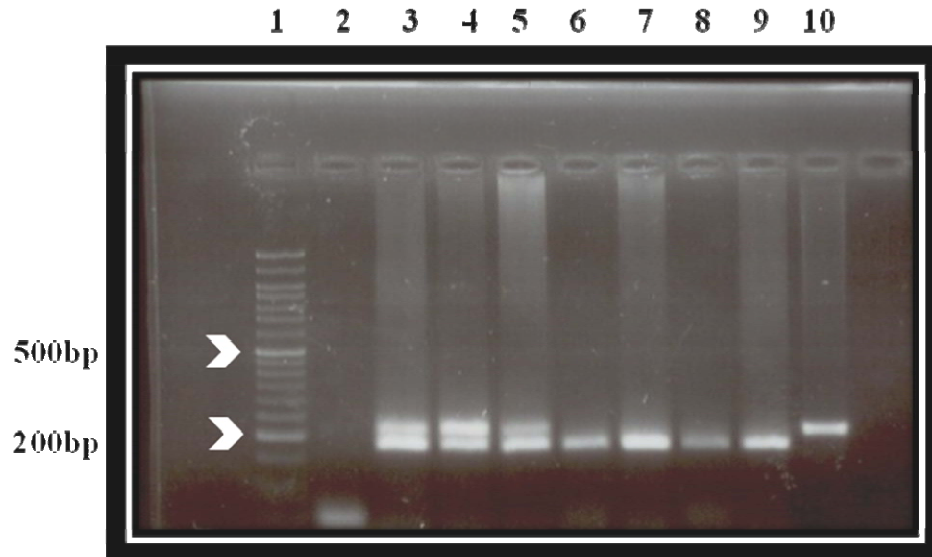


Figure 3.6: -1426 C/T SNP genotyping by RFLP with *HpaII* enzyme. Lane 1 represents the 50 bp DNA ladder; Lane 2 is the negative control, lanes (3-5) show CT genotype with 223/182 bp bands , lanes (6-9) show CC genotype with 182 bp band and lane 10 shows the uncut products with 223 bp band size.

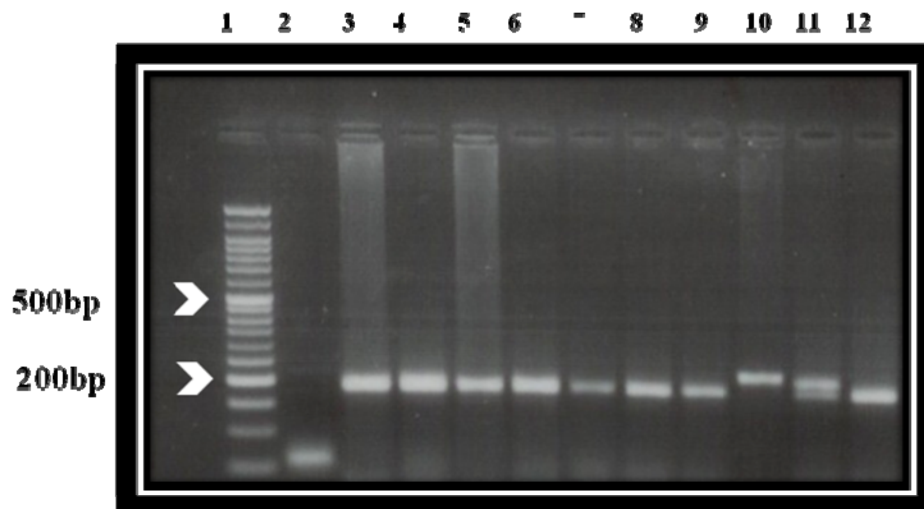


Figure 3.7: -1426 C/T SNP genotyping by RFLP with *BstN1* enzyme. Lane 1 represents the 50 bp DNA ladder; Lane 2 is the negative control, lanes (3-9,12) show CC genotype with 183 bp band, and lane (10) shows the uncut products with 223bp band , lane (11) shows CT genotype with 208/183 bp bands size.

3.2.3 DNA sequencing

DNA sequencing was used for genotyping -1426 C/T SNP for quality assurance purposes. The results of the RFLP matched with the results of sequencing. Figure (3.8), represents the chromatogram of the CC genotype with one peak for the C nucleotide at the -1426 site. In contrast, T and C peaks are seen at the -1426 site with CT genotype (figure 3.9).

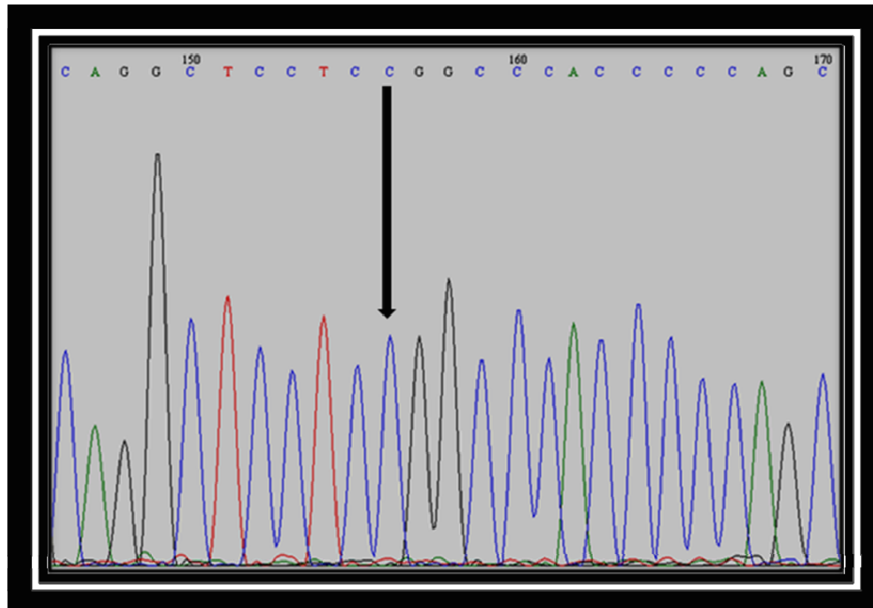


Figure 3.8: -1426 C/T SNP genotyping by DNA sequencing. The chromatogram shows the C nucleotide peak in CC genotype.

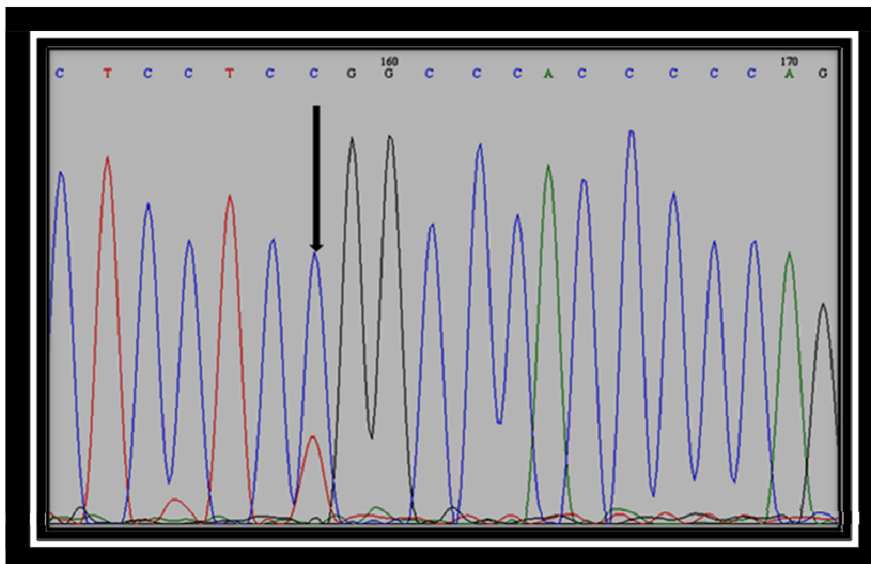


Figure 3.9: -1426 C/T SNP genotyping by DNA sequencing. The chromatogram shows both C and T nucleotide peaks in CT genotype.

3.2.4 Real Time-PCR and HRM

In this study, we aimed to assess the applicability of HRM methodology in detecting -1426 C/T. For this purpose, samples tested by RFLP and DNA sequencing were used as controls for the HRM. By HRM the CC can be clearly distinguished from CT genotypes in a simple, rapid manner. Different graphs were used to evaluate genotype as shown in figure (3.10, 3.11, 3.12). Different genotypes can be easily discriminated by normalized (figure 3.11) and fluorescence difference (figure 3.12) curves in these curves each genotype tends to be in one cluster. Each genotype has different melting behavior as clearly seen in derivative melting curve (figure 3.10), this figure also reflect the stability of the CC genotype. For quality assurance, HRM PCR products were analyzed by gel electrophoresis and the obtained bands (78bp) matched with expected band size as shown in figure (3.13).

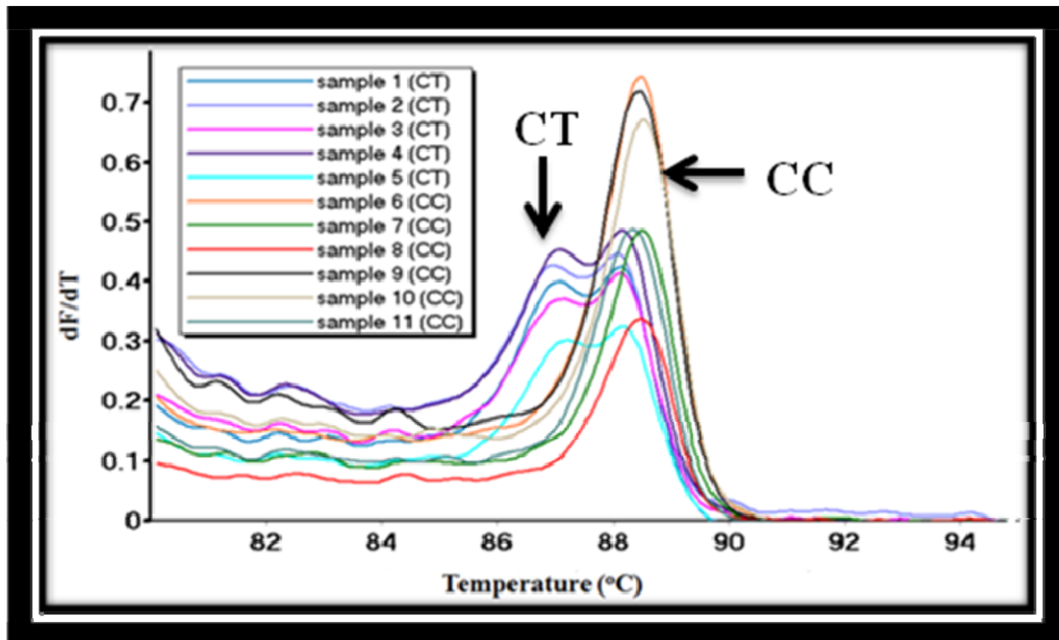


Figure 3.10: Derivative melting curve for *Par 1* (-1426 C/T) SNP. PCR products with CT genotype show two melting peaks, while the CC genotype shows only one peak.

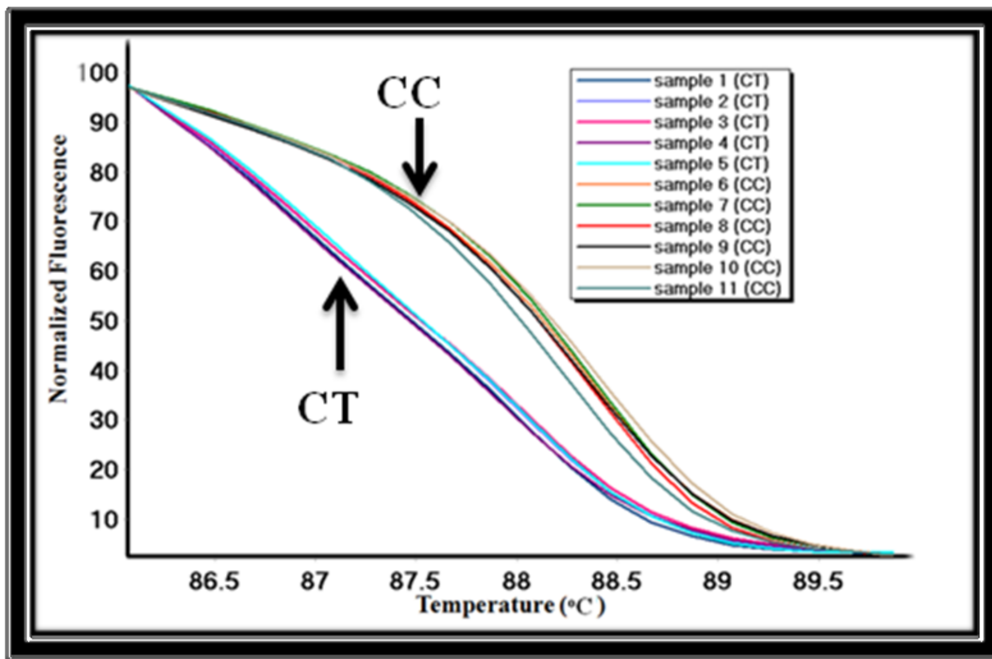


Figure 3.11: Normalized HRM curves for -1426 C/T SNP genotyping. Samples with the same genotype tend to be in one cluster.

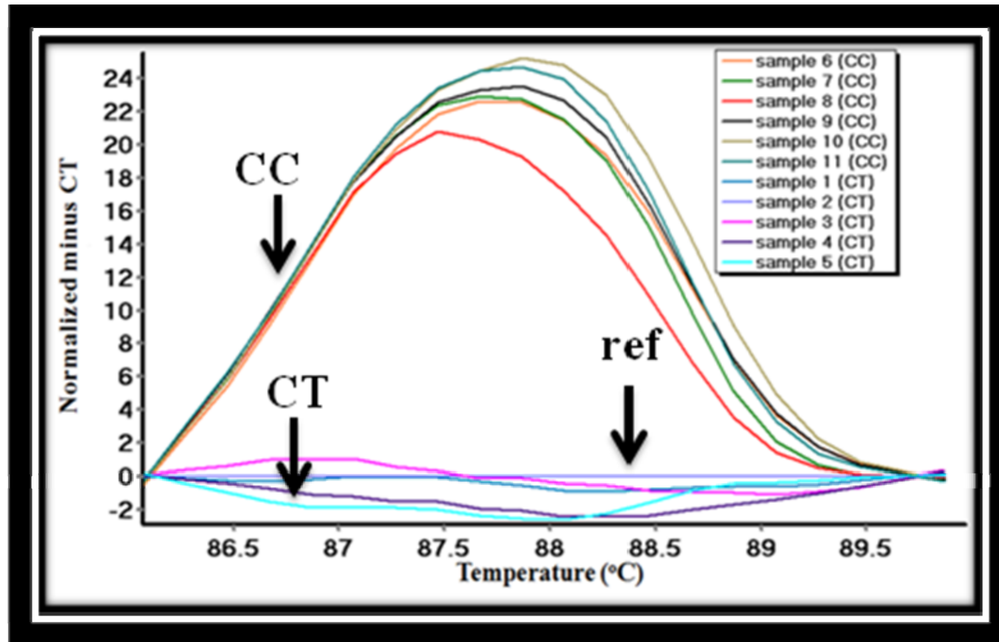


Figure3.12: Fluorescence difference curves. Difference in fluorescence compared to one of the heterozygous samples (C/T), curves were plotted against the temperature, leading to clear separation of sample groups with the same genotype (ref: reference sample).

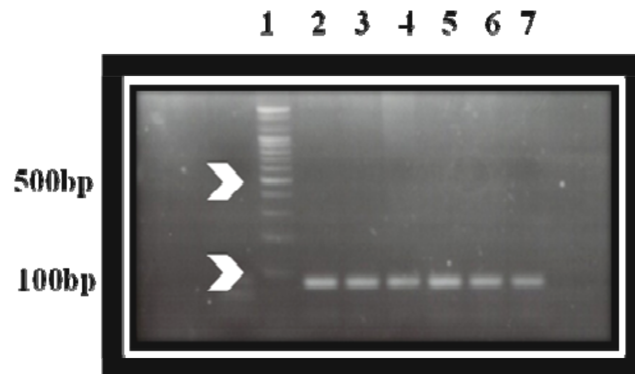


Figure 3.13: HRM PCR products for the -1426 C/T SNP. Lane 1 represents is the 100 bp DNA ladder, lane (2- 7) are HRM products with 78bp size.

3.3 Distribution of *Par1* genotyping among Palestinian females

We have genotyped females for PAR1 polymorphism in the 5′ regulatory region, namely -506 I/D (rs11267092) and -1426 C/T (rs32934). As shown in figure (3. 14), the DD was present in 48.3% among cases and 41.9% among controls. The ID genotype was present in 37.9% among cases and 41.1% among controls while the II genotype was present in 13.8% in cases and 17.0% in controls. For the -1426 C/T SNP only two genotypes were present since the TT genotype is not detected in our participants. As shown in figure (3.15), the CC genotype was present in 97.5% in cases and 98.1% in controls. The CT genotype was present in 2.5 % among cases and 1.9% among controls. The distribution of - 506 I/D and -1426 C/T genotypes of *Par1* polymorphisms and minor allele frequencies is described in table (3.2, 3.3) respectively. The polymorphisms inspected in this study showed no deviation from Hardy – Weinberg equilibrium (HWE). The prevalence of -506 I allele was 51.7% and 58.1 % of cases and controls, respectively and -1426 T allele was 2.5% and 1.9% of cases and controls, respectively. The prevalence of these two alleles was not significantly different between the two groups. Table (3.4) represents the allelic odds ratio with the 95% confidence interval.

Patients with known abnormalities were only excluded from the center area of Palestine, for that, the genotyping results were further analyzed according to their distribution (the north and the center area of Palestine). Table 3.5 and 3.6 represent the distribution of - 506 I/D and -1426 C/T genotypes of *Par1* polymorphisms and minor allele frequencies. According to the center area of Palestine, the prevalence of -506 I allele at the center was 48.1 % and 42.1 % of cases and controls, respectively and -1426 T allele was 4.3 % and 2.9% of cases and controls, respectively. The prevalence of these two alleles was not significantly different between the two groups. Table (3.4) represents the allelic odds ratio with the 95% confidence interval.

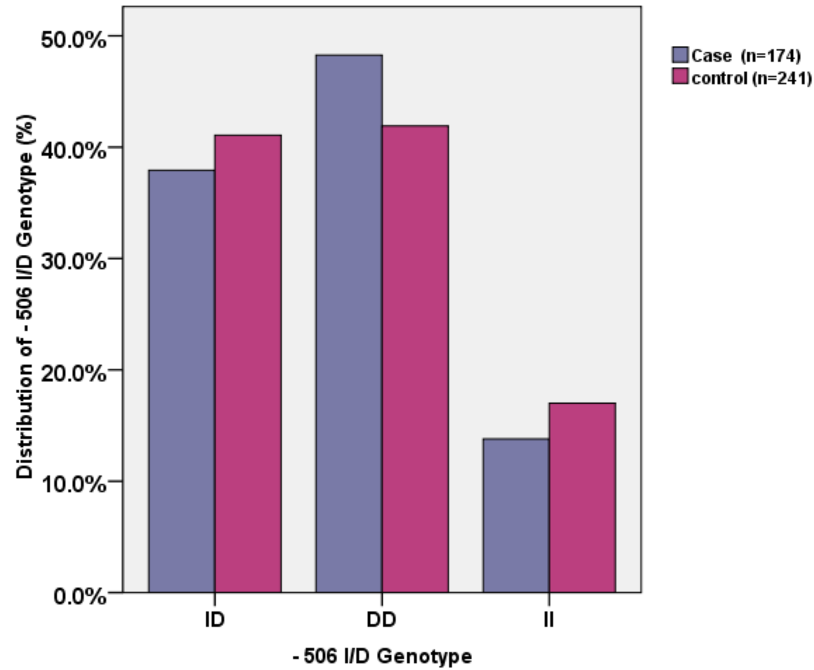


Figure 3.14: Distribution of -506 I/D Polymorphism among Cases and Controls. No significant association between different genotypes and the two groups (p= 0.399).

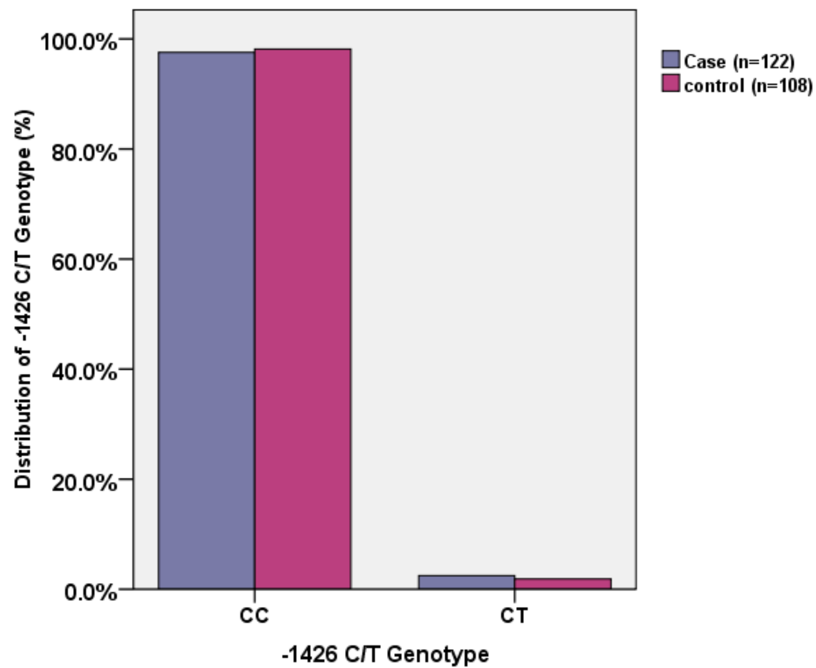


Figure 3.15: Distribution of -1426 C/T SNP among Cases and Controls. No significant association between different genotypes and the two groups (p=1.0).

Table: 3.2 Genotype and Minor Allele Frequencies for the -506 I/D Polymorphisms in Cases and Controls.

Genotype	Cases, n (%)	Controls, n (%)
DD	84 (48.3)	101 (41.9)
ID	66 (37.9)	99 (41.1)
II	24 (13.8 %)	41 (17.0)
Total	174	241
Allele I frequency	0.328	0.376
P = 0.399[a]		

[a]: Chi square test. n: number

Table 3.3: Genotype and Minor Allele Frequencies for the -1426 C/T SNP in Cases and Controls.

Genotype	Cases, n (%)	Controls, n (%)
CC	119 (97.5)	106 (98.1)
CT	3 (2.5)	2 (1.9)
TT	0 (0)	0 (0)
Total	122	108
Allele T frequency	0.012	0.009
P =1.0 (F)[a]		

[a]: Fisher Exact Test. n: number

Table 3.4: Allelic odds ratio for *Par1* polymorphism.

polymorphism	OR	95% CI	P Value
-506 I/D	0.8	0.6 - 1.1	0.155 (P)[a]
-1426 C/T	1.3	0.2 - 8.0	1.0 (F)[b]
-506 I/D (center)	1.2	0.6 – 2.2	0.668 P)[a]
-1426 C/T (center)	1.5	0.1 – 16.9	1.0 (F)[b]

[a]:Chi square test . [b] :Fisher Exact Test. CI : Confidence Interval. OR: odds ratio

Table 3.5: Genotype and Minor Allele Frequencies for the -506 I/D Polymorphism in Cases and Controls according to their distribution.

	Center of Palestine, n (%)		The North of Palestine, n (%)	
Genotype	Cases	Controls)	Cases	Controls
DD	28 (51.9)	22 (57.9)	56 (46.6)	79 (38.9)
ID	19 (35.2)	11 (28.9)	47 (39.2)	88 (43.4)
II	7 (12.9)	5 (13.2)	17 (14.2)	36 (17.7)
Total	54	38	120	203
Allele I frequency	0.306	0.276	0.338	0.394
	P = 0.812[a]		P = 0.371[a]	

[a]: Chi square test. n: number.

Table 3.6: Genotype and Minor Allele Frequencies for the -1426 C/ T SNP in Cases and Controls according to their distribution.

	Center of Palestine, n (%)		The North of Palestine, n (%)	
Genotype	Cases	Controls)	Cases	Controls
CC	45 (95.7)	34 (97.1)	74 (98.7)	72 (98.6)
CT	2 (4.3)	1 (2.9)	1 (1.3)	1 (1.4)
TT	0 (0)	0 (0)	0 (0)	0 (0)
Total	47	35	75	73
Allele T frequency	0.021	0.014	0.007	0.007
	P = 1 (F)[a]		P = 1 (F)[a]	

[a]: Fisher Exact Test. n: number

Chapter Four

4.1 Discussion

Even though different causes of RA are known to date, the cause in half of the cases are still unknown (Allison and Schust, 2009). Hence, RA is an annoying condition for physicians and upsetting for patients. For that, more efforts should be done on the way to identify further risk factors to find an appropriate treatment and prevent over treatment of an enormous number of women who receive either heparin or hormonal treatment as prophylactic protocols to prevent RA (Dudding and Attia, 2004; Horne and Alexander, 2005).

In order to solve such a problem, it is mandatory to know the pathophysiology of pregnancy in the different trimesters. One of the important aspects of pregnancy in the first trimester is the implantation of the blastocyst and placental formation (Cross et al., 1994; Damsky and Fisher, 1998). During this process, CTBs proliferate, become motile and invade the maternal uterus (Cross et al., 1994). Although little is known at the molecular level concerning implantation (Flamigni et al., 1991; Inagaki et al., 2003), the over expression of PAR1at the time restricted to the invasion period (between the 7th to 10th weeks of gestation) is well documented (Even-Ram et al., 2003). For that, we hypothesized that -506 I/D and -1426 C/T polymorphisms in *Par*1 might alters the expression of this gene and increases the risk of RA.

To reach our goal, a case –controlled study was performed among participants from the same ethnic group. While the case was females suffered from two or more consecutive pregnancy loss in the first trimester of pregnancy, the control was females who gave birth

normally for two or more babies. Those participants were genotyped for *Par1*polymorphisms in the 5′regulatory region that affect gene expression, namely -1426 C/T (rs32934) and - 506I/D (rs11267092) polymorphisms. There was no statistically significant difference between the two groups in the two examined polymorphisms. PCR based detection methods were used successfully for genotyping purposes. Moreover, the applicability of HRM was examined for the -1426 C/T SNP genotyping. This method successfully differentiates different genotypes and can be considered as a cost effective, rapid technique for detecting and screening this SNP.

The -506 I/D polymorphism was genotyped using size polymorphic PCR technique. Our designed primers were able to detect the different genotypes (DD, ID and II) (figure 3.3) effectively and the results obtained by this method matched well with previously DNA sequenced samples. Unlike RFLP -the most used method detect this polymorphism (Altarescu et al., 2010; Arnaud et al., 2000; Grisaru-Granovsky et al., 2007) or Dynamic Allele Specific Hybridization (DASH) (Gigante et al., 2009), our method can detect the different genotypes in one step PCR without restriction enzymes or labeled probes, which lowers the cost and reduces time required to test this polymorphism. This method was also used by Dupont *et. al*. with different sets of primers (Dupont et al., 2003).

The -1426 C/T SNP was genotyped using RFLP technique. We planned to work according to Arnaud *et. al*. (Arnaud et al., 2000), however, we faced difficulties in detecting the different genotypes (CT and CC) despite the long time given for the PCR products to be separate on agarose gel electrophoresis. Moreover, Altarescu *et. al*. and coworkers did not work according to Arnaud *et. al.* for technical reasons too, and DNA sequencing was used instead (Altarescu et al., 2010). We successfully solved this problem with our newly designed primers and their corresponding restriction enzymes. Our modified method gave better resolution for the different genotypes (CC and CT) (figure 3.6, 3.7) and the results obtained with it matched well when further confirmed by sequencing (figure 3.8, 3.9).

Different PCR based SNP detection methods were used to detect -1426 SNP and similar SNPs. RFLP is a simple method, can be performed at every standard laboratory that has a

thermal cycler and electrophoresis system and can be considered as a reliable method. Nevertheless, it is labor, expensive and time consuming because of post-PCR manipuation. ARMS although do not require post PCR manipulation, the need of two separate PCR reactions is also expensive and time consuming (Ye et al., 2001). DNA sequencing is considered as the gold standard technique (Krypuy et al., 2006). However, its high cost is the main disadvantage (Krypuy et al., 2006). Accordingly, rapid, simple, low cost and high throughput methods are mandatory for scanning purposes and SNP genotyping.

In the current study, we assessed the ability of HRM to detect -1426 C/T SNP, its results were in parallel with the RFLP and sequencing methods with no observed discrepancies. HRM analysis of PCR products depends on DNA melting in the presence of saturating SYTO9, a DNA binding dye (Erali et al., 2008). Among the three PCR based mutation detection methods we used for -1426 C/T SNP genotyping, HRM has many advantages as also indicated by Liew *et. al.* (Liew et al., 2004). In a close- tube method, the analysis is performed immediately once amplification is completed in the same machine. Thus, contamination of the amplified product is eliminated as no further processing nor separation steps are required. Moreover, small amplicons used in HRM allow better differentiation for the different genotypes as it maximize the difference in Tm between them and also reduces the overall time require for cycling when compared with the conventional PCR (Liew et al., 2004; Wittwer et al., 2003). For example, genotyping results can be obtained at maximum 90 minutes of performing the reaction even with high sample number (up to 72 sample/ run). Also, when it was compared with other methods, it can be considered as inexpensive molecular technique. Add to that, with the progress and improvement of HRM, no probe –based system (labeled primers) is required. Finally, the used interchelating dye- SYTO9- had low toxicity when compared with other DNA binding dyes such as ethidium bromide (Monis et al., 2005).

In HRM, the genotype is determined by melting behavior of each allele. In our study we used different melting curve analyses to discriminate between the different genotypes. For example, while the CC genotype shows a one peak stable melting pattern, the CT genotype shows less stable double peak phenotype as clearly seen in the melting curve analysis,

figure 3.10. Other types of melting behavior analysis, used to differentiate between the different genotypes, such types include, the normalized fluorescence curve which allow visual clustering of different genotypes, as seen in figure 3.11, and amplicon melting difference curves figure 3.12, in which samples with genotypes similar to the reference genotype cluster around it, while those with different genotypes fall in the opposite side.

In light of the increased role that *Par1* polymorphism plays in different diseases, we recommend the use of HRM in research areas that require genotyping of-1426 C/T SNP. For those laboratories that do not have RT- PCR machine, our modified RFLP method will help in screening and genotyping the -1426 C/T SNP in a more simple and comfortable way.

Hardy–Weinberg (HW) law or as known HW equilibrium (HWE), indicates that the frequency of two alleles in a single locus are predicted to remain in equilibrium and balance from generation to generation in the absence of mutation or selection (Stern, 1943). In the current study, no deviation from HWE was seen among cases and controls in the two tested polymorphisms. The allele frequencies among the two groups suggest no association between *Par* 1 polymorphism and recurrent abortion ($p = 0.154$, $p = 1.0$ for the -506 I/D and -1426 C/T, respectively) (Table 3.4). The same results (no association) were obtained when samples were analyzed according to their geographical distribution (north and center district of Palestine).

The frequency of the I allele in the -506 I/D polymorphism in our population was close to its frequency in other populations (Altarescu et al., 2010; Arnaud et al., 2000; Dupont et al., 2003; Grisaru-Granovsky et al., 2007). While the I allele has been shown to have a protective effect regarding VTE among French males (Arnaud et al., 2000), no evidence for a protective effect of this allele from VTE among Californians of European ancestry even when analyzed by sex (Pecheniuk et al., 2008). These contradicting results may be related to the small sample size in the later study (114 subjects) when compared with 1462 subjects in the former one. The absence of a protective effect of the I allele among French females might be related to sex hormones that might cause a decrease in PAR1 expression

in vascular cells (Arnaud et al., 2000). It has been shown that II genotype in -506 I/D polymorphism may be an essential predictive factor for EA and was associated with developing tumor recurrence (Lurje et al., 2010). As this polymorphism has no effect on the outcome of pregnancy in our study (RA) the effect of such polymorphism may be tissue specific and not only sex related.

In -1426 C/T SNP genotyping, the TT homozygous genotype was absent among all subjects (Table3.3). The scarcity of this genotype is not only evident in our study. The study carried out by Grisaru-Granovsky *et. al*. among the Jewish people did not show this genotype (Grisaru-Granovsky et al., 2007), while in Arnaud's *e.t al.* study among the European people, only one case out of 1464 had this genotype (Arnaud et al., 2000). Other studies, on the other hand, had this genotype but in low frequencies (Martinelli et al., 2008). The only study that had an aberrant TT genotype was done by Altarescu and colleagues among the Jewish people (Altarescu et al., 2010). This can be interpreted in different ways; studies concerning *Par1* polymorphism was carried out among different populations, for that, ethnicity may affect the frequency of this genotype. Concerning ethnicity and -1426 C/T, two different studies were carried out on the same ethnic group (Jews) (Altarescu et al., 2010; Grisaru-Granovsky et al., 2007). In these two studies the results obtained concerning the TT genotype were totally different, except in the heterozygous state. This difference may be related to the method used in SNP detection. While Altarescu *et al*, used sequencing to detect this SNP (Altarescu et al., 2010), Grisaru-Granovsky et. al. used RFLP to do so (Grisaru-Granovsky et al., 2007). Incidentally, sequencing is considered the gold standard for genotyping (Krypuy et al., 2006), and in fact, Altarescu *et. al.* in 2010 reported that for technical reasons, they used sequencing and not RFLP in their study. As we demonstrated earlier in our discussion, we modified the RFLP method. Upon modification, we could easily differentiate between the genotypes compared to Arnaud *et. al*. and the difference in the digestion critiria between Arnaud's article and the Nebcutter web site cause difference in interpreting the different genotype. This difference in ease in interpreting the digestion criteria may be the cause of totally different distribution of genotypes among the Jews people. Although it came out to have no correlation with RA in our study, the T allele in this polymorphism has been reported to

be associated with high rates of liver fibrosis in men suffering from hepatitis C (gender related) (Martinelli et al., 2008). Females carrying the T allele were at increased risk concerning PTD (Grisaru-Granovsky et al., 2007). The TT genotype also correlate with low platelets count in patients with Gaucher disease (Altarescu et al., 2010). This discrepancy in its correlation with different disease situation could be gender related, tissue specific or different biological process.

While our study did not reveal an association between polymorphisms in *Par*1 at the 5′ regulatory region, the role of *Par*1 polymorphism in the intervening sequence still need to be evaluated, since only the T allele of -14 IVS A/T (rs168753) SNP was reported to be associated with decreased platelets PAR1 expression level (Dupont et al., 2003).

In the current study the role of *Par*1 polymorphisms (rs11267092 and rs32934) at the maternal side was evaluated, and this was one of the limitations in our study, the fetal factor concerning *Par*1 polymorphism must be taken in consideration as placentation is a complex and shared process between microenvironment (maternal factor) and CTB (fetal factor).

4.2 Recommendations

In conclusion, little is known and much remains to be learned about the molecular events and vital signaling pathways that take place during placentation. How placental- maternal cell type cooperate in an integrated way to control decidual invasion and spiral artery remodeling in spatial and temporal manner.

As RA is a frustrating condition for physicians and upsetting for patients, we recommend testing *Par1* polymorphisms (rs11267092, rs32934 and rs168753) as a fetal factor that may lead to RA and evaluation of the -14 IVS A/T SNP (rs168753) before ruling out the *Par*1 gene ppolymorphism as a risk factor for RA in our population. We also recommend the use of our methods when screening for *Par1* polymorphisms in the 5′ regulatory (rs11267092 and rs32934) region in other diseases since they provide simple and easy ways for analysis.

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Appendices

Recurrent abortion study, relation to Homeostasis

Questionnaire

Patient Name:________________________ Patient ID:____________________

Parity_____ G_____ Phone No:_____________________

No. Abortions:____________ Age: ____________________

Successive abortions:________________ Place:_____________________

Neonatal death: _________________

Pre- maturity: ________________

Doctor's Name:_______________________

Patient selection: Recurrent pregnancy loss will be defined as two or more consecutive spontaneous abortions.

Normal control: females with two or more normal deliveries.

Test which the patient has already done before:

Test Name	Normal	Abnormal
Factor V	☐	☐
Factor II	☐	☐
Factor XIII	☐	☐
Beta fibrinogen	☐	☐
Anticardiolipn antibody	☐	☐
Protein S	☐	☐
Protein C	☐	☐
Antithrombin III deficiency	☐	☐
Uterine Shape	☐	☐
Infectious screen	☐	☐
Couples karyotyping	☐	☐

إقرار خطي

أنا الموقعة أدناه

....

أوافق على اخذ عينة دم مني لغرض إجراء بحث يتناول أسباب حدوث الإجهاض المتكرر، وقد تم شرح الأهداف من إجراء البحث من قبل طبيبي المعالج
الدكتور/ة..
....

Sample 3-5ml EDTA whole blood sample, was withdrawn under sterile conditions. Sample was taken and kept at $4^{o}C$ for one week.

Lab's signature ……………………….

علاقة الإجهاض المتكرر مع تعدد أشكال الجين (*Par1*) عند النساء الفلسطينيات

إعداد: مرفت محمود أبو غزاله

إشراف: الدكتور زيدون صلاح

أ. د .زياد عابدين

ملخص:

الإجهاض المتكرر من احد مشاكل الحمل ويصيب 5% من النساء في سن الإنجاب خلال الأشهر الثلاثة الأولى من الحمل. وبالرغم من وجود أسباب كثيرة للإجهاض المتكرر إلا أن السبب الحقيقي لا يزال مبهما عند نصف الحالات.

لقد لوحظ ارتفاع نسبة المستقبل (PAR1) في نسيج المشيمة عند تكوينها في الفترة الممتدة بين الأسبوع السابع إلى الأسبوع العاشر من الحمل ثم اختفاء هذا المستقبل بعد ذلك، ولقد أثبتت الدراسات أن (PAR1) مهم في عملية تكون المشيمة و أن تعدد أشكال هذا الجين (polymorphism) له علاقة مع بعض مشاكل الحمل، فكان هدف الدراسة الرئيسي إيجاد العلاقة بين تعدد أشكال هذا الجين و الإجهاض المتكرر.

اشتملت الدراسة على نساء من وسط و شمال فلسطين، حيث اعتبرت كل امرأة (174 حالة) تعرضت لاجهاضين متكررين أو أكثر في الأشهر الثلاث الأولى من حملها حالة مرضية . أما المجموعة الضابطة (241) فكانت تضم نساء أنجبن مرتين أو أكثر بدون مشاكل تذكر.

تمّ إجراء هذا البحث في معهد البحوث الصحية و التغذية التابع لجامعة القدس في أبو ديس في الفترة الزمنية 2009 إلى 2010، حيث تم عزل الحمض النووي من عينات دم أخذت من المشاركات في الدراسة. تمت دراسة منطقتي تعدد الشكل (I/D) Insertion / Deletion 506− و C/T 1426- في الجين (*Par1*) و استخدمت طريقة اختلاف طول القطعة الناتجة عن التفاعل السلسلي (PCR) المسماة (size polymorphic PCR) للكشف عن I/D 506-. أما C/T 1426− فقد تم الكشف عنها بطريقة قص القطعة الناتجة عن التفاعل السلسلي (PCR) والمسماة (RFLP) و الطريقة المسماة ذوبان عالي الدقة (HRM) وتسلسل الحمض النووي (DNA sequencing).

لقد تم في هذه الدراسة إجراء تعديل على طرق منشورة لفحص الأشكال المتعددة لهذا الجين وكانت هذه التعديلات تهدف إلى تسهيل عملية فحص تعدد أشكال (*Par1*) ، ولقد نجحت هذه التعديلات حيث كانت الطرق المستخدمة أسهل و أفضل من الطرق السابقة. وكان لطريقة (HRM) بالذات ميزة كبيرة من حيث أنها توفر الوقت و الجهد والتكلفة .

لم تثبت هذه الدراسة وجود علاقة بين أشكال (*Par1*) والإجهاض المتكرر حيث لم يشاهد أي اختلاف لتعدد أشكال (*Par1*) عن التوازن الطبيعي ل Hardy – Weinberg وكان تردد الاليل I في 506I/D- هو 0.328 و 0.376 للحالة المرضية و المجموعة الضابطة ، على التوالي. وكانت نسبة الخلاف (OR) = 0.8 و فترة الثقة (CI) =(0.6 – 1.1) (p 0.155) بينما كان تردد الاليل T في C/T 1426− هو 0.012 و 0.009 للحالة المرضية والمجموعة الضابطة على التوالي. وكانت نسبة الخلاف (OR)= 1.3 و فترة الثقة (CI) = (0.8-1.8) (p1.0).

وبالرغم من عدم وجود علاقة لتعدد أشكال (*Par1*) مع الإجهاض المتكرر إلا أننا نثمن دراستنا، حيث قمنا بتطوير وسائل أسهل و أوفر لفحص أشكال (*Par1*) خصوصا أن هذا الجين له علاقة مع كثير من الأمراض حسب ما ورد في عدة دراسات سابقة. وبما أن هذه الدراسة لم تثبت العلاقة مع الإجهاض المتكرر في المجتمع الفلسطيني بالرغم من وجود علاقة لتعدد أشكال (*Par1*) مع مشاكل أخرى في الحمل وكذلك مع أمراض أخرى في مجتمعات مختلفة، فان السؤال لا يزال مفتوحا لإيجاد علاقة بين تعدد أشكال (*Par1*) مع مشاكل أخرى للحمل والإجهاض المتكرر في مجتمعنا إذا تم فحص الجنين أيضا وليس فقط الأم.