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Interleukin-6, Interleukin-10 and Interleukin-17 Gene Polymorphisms in Palestinian Women With Recurrent Miscarriage

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Dedication

I dedicate my dissertation work to my family especially to my beloved husband (Rami), and my kids Qusay, Basel, and Ayham, for their tolerance and for inspring me to do the best. I also dedicate this work to my parents and all my family for their support and encouragement.

Niveen Nizam Sublban

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:

Niveen Nizam Sublban

Date: 8/5/2018

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Abstract

Background: Recurrent Miscarriage (RM) is defined as two or more consecutive pregnancy losses before 20th week of gestation, it is one of the most common pregnancy complications, affecting up to 2–4% of women of reproductive age. Several contributing factors have been identified in the risk of recurrent miscarriages, such as genetic defects, the immune system, endocrine and anatomical systems. The etiology in approximately 50% of cases of RM is unknown, but it has been assumed that a proportion of these recurrent pregnancy losses may be due to immunological reasons.

During normal pregnancy, there is a cooperative interaction between the maternal immune system and fetal antigen, the failure of this immune system adaptation causes alloimmune rejection of the fetus, leading to loss of pregnancy.

After encountering the antigen, T helper (Th) precursors differentiate into functionally Th1 and Th2 cells, with unique patterns of cytokine production. Cytokine production levels are partly under genetic control, and their gene expression can be changed by nucleotide variation. Several studies have shown that cytokines play a major role in reproductive phenomena, where the predominant response of Th2 has been associated with normal pregnancy, and the Th1 response has been linked to pregnancy failure. The expression of pro-inflammatory cytokines in the uterus such as IL-17, TNF and IL-6 has been associated with fetal loss. Anti-inflammatory cytokines such as IL-10 appear to protect against inflammation-induced miscarriage and protect against abortion caused by inflammation.

Objective: To investigate the association of RM with polymorphisms of an anti-inflammatory IL-10 (-592 A/C, -819 C/T), and two pro-inflammatory IL-17A, IL-17F and IL-6 (174 C/G) cytokine genes in RM compared to normal Palestinian women in the West Bank.

Methods: This study included 107 women from different areas of the West Bank, (Ramallah, Al-Khalil, Jerusalem, Bethlehem, Nablus, Tulkarem, and Jercho. 55 women with recurrent miscarriages without a specific cause, and 52 women who do not have any pregnancy-related risks. All samples were analyzed by polymerase chain reaction

(PCR) targeting IL-10,IL-6, and IL-17 gene polymorphisms followed by RFLP, using specific restriction enzymes for each gene site

Results: This study has proven a significant association of polymorphism in the IL10-819 C/T, and increased frequency of recurrent miscarriage among Palestinian women (p=0,0009) and the lack of association between polymorphisms in IL10-592C / A, IL6-174G / C, IL-17, and recurrent miscarriage in the studied group.

Conclusions: There is not only one genetic factor, but possibly several that are involved in the abortion disease etiology. There are about 50% are idiopathic factors. If the relationship between genetic factors and immune system disorders is explained, genetic polymorphisms as the one that is studied may represent markers for choice of therapeutic options and for counseling patients with repeated spontaneous abortions.

There are significant differences in the results of previous studies conducted in different regions of the world on this subject, these discrepancies may be explained by ethnic differences of the study groups as well as the variation in number of the subjects that are included in the research

To the best of our knowledge, this was the first report in the West Bank at Palestine that examines the relationship between IL-10, IL-6, and IL-17 gene polymorphisms and spontaneous abortion, which may be considered as supportive of previous studies in the same field. However, the true mechanism of this relationship remains unknown and requires more investigation.

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List of Abbreviations

μL: Microliter

μM: Micromolar

ACE: angiotensin-converting enzyme

AD: Alzheimer's disease

BioEdit: Biological sequence alignment editor

BLAST: Basic Local Alignment Search Tool

BMI: body mass index

Bp: Base pair

DNA: Deoxyribonucleic acid

EDTA: Ethylenediaminetetraacetic acid

F: Forward

FOXP3: Forkhead box protein P3

G: Gram

hCG: Human chorionic gonadotropin

HLA: Human leukocyte antigen

IL-10: Interleukin 10

IL-6: Interleukin 6

IL-17: Interleukin 17

IFN-γ: interferon-gamma

iNOS: Nitric oxide synthase

MHC: Major histocompatibility complex

mL: Millilitre

NCBI: National center for Biotechnology Information

NOS3: endothelium-derived nitric oxide synthase 3

PAI-1: Plasminogen activator inhibitor 1

PCR: Polymerase chain reaction

PIBF: Progesterone-induced blocking factor

pPROM: preterm premature rupture of the membranes

R: Reverse

RFLP: Restriction fragment length polymorphism

RM: Recurrent miscarriage

SNP: Single Nucleotide Polymorphism

TAE: Tris acetate EDTA

TGF-β1:Transforming growth factor β1

Th: T Helper cells

TNF: Tumor Necrosis Factor

TREG cell: Regulatory T cell.

V: Volt

Chapter One

Introduction

1.1 Background

Recurrent miscarriage (RM) is defined as two or more consecutive pregnancy losses before 20th week of gestation. It is one of the most common pregnancy complications affecting up to 2–4% of women of reproductive age (Zidan, Rezk, Alnemr, & Moniem, 2015). Approximately 1% of all women trying to conceive have RMs. When RM is defined as two previous miscarriages, the proportion rises to 5% (Alalaf, 2012). This is a devastating problem, particularly to Palestinian families who are fond of having large families. Several identified risk factors, such as genetic, immune, endocrine, anatomical and infective defects, have been postulated to contribute to the risk of RM (Branch, Gibson, & Silver, 2010). The aetiology of RM in approximately 50% of cases of is unknown. However it has been postulated that a proportion of these repeated pregnancy losses may be due to various immune causes (Laird et al., 2003).

During normal pregnancy, there is cooperative interaction between maternal immune system and fetal antigen, in which failure of the immune system adaptation leads to alloimmune rejection of the fetus resulting in loss of pregnancy (Kumar, 2014)
After encountering the antigen, T helper (Th) precursors differentiate into functionally T-cell lineages, including Th1 and Th2 cells, with unique patterns of cytokine production (Mosmann & Sad, 1996). Cytokine production levels are partly under genetic control, and their gene expression can be changed by nucleotide variation. Several studies have shown that cytokines play a major role in reproductive phenomena, where Th2- dominant response has been associated with normal pregnancy, and Th1 response has been related to pregnancy failure (Alkhuriji, Alhimaidi, Babay, & Wary, 2013).

Enhanced uterine expression of pro-inflammatory cytokines such as tumor necrosis factor (TNF), interferon-gamma (IFN-γ), interleukin (IL)-1β, and IL-6 has been associated with embryo loss (Haddad, Duclos, Antecka, Lapp, & Baines, 1997). Anti-inflammatory cytokines such as IL-10 appear to protect against inflammation-induced miscarriage (Robertson, Care, & Skinner, 2007). It is generally accepted that a successful pregnancy is dependent on a shift away from Th1-type and a bias towards Th2-type immune responses (El-Shazly, Makhseed, Azizieh, & Raghupathy, 2004)

IL6 is a multifunctional cytokine with pivotal roles in the inflammatory response and in directing T cell differentiation in adaptive immunity. IL6 is widely expressed in the female reproductive tract and gestational tissues, and exerts regulatory functions in embryo implantation and placental development, as well as the immune adaptations required to tolerate pregnancy (Prins, Gomez-Lopez, & Robertson, 2012). A common C/G polymorphism located within the IL-6 gene promoter at nucleotide position 174 bp, upstream from the start site of transcription, has been reported to affect IL-6 expression and may contribute to miscarriage (Jasper, Tremellen, & Robertson, 2007). However the predictive value of IL-6 for pregnancy outcome remains unclear.

IL 17 a pro-inflammatory cytokine, it is predominantly produced by distinct subset of T helper cells (Th17) (Harrington et al., 2005). Th17 cells are known to be involved in the pathogenesis of autoimmune and inflammatory diseases, immunological rejection of non-self tissue and may play role in the pathogenesis of RM (Fu, Tian, & Wei, 2014).

Interleukin-10 (IL-10), defined as a pleiotropic anti-inflammatory cytokine, is produced by activated Th2 cells, B cells, monocytes and macrophages. (IL-10) promotes embryonic development by maintaining the maternal–fetal tolerance (Wang, Hao, & Lin, 2011). The IL-10 gene is located on chromosome 1q31–q32 region, of which the promoter is highly polymorphic, Many single-nucleotide polymorphisms (SNPs) were reported in the proximal at position−1082A/G (dbSNP ID rs1800896), −819C/T (dbSNP ID rs1800871), and −592C/A (dbSNP ID rs1800872) regions of IL-10 gene (Chaouat et al., 2002).

Study Justification

Spontaneous recurrent miscarriages represent an important medical problem. Numerous researches have investigated the causes of abortion disease. However, in a significant percentage of patients, the exact cause of spontaneous abortions could not be identified. Immunological factors play an important role in execution and the physiological evolution of pregnancy. Few studies have been conducted to explain the relationships between the codifying genes for cytokines and the abortion disease.

Research hypothesis

There is an association between polymorphisms of two pro-inflammatory (IL-6 and IL-17) and and anti-inflammatory (IL-10) cytokine genes with RM women in the West Bank/ Palestine.

Study objective

Considering the potential role of cytokines and cytokine polymorphisms in RM patients, the aim of this study was to investigate the association of RM with polymorphisms of an anti-inflammatory IL-10 (-592 A/C, -819 C/T), and two pro-inflammatory IL-17A, IL-17F and IL-6 (174 C/G) cytokine genes in RM compared with normal pregnancy women in the West Bank.

1.2 Literature review

1.2.1 Recurrent miscarriage:

Recurrent miscarriage (RM), which is defined as 2-3 or more consecutive pregnancy losses before 20 weeks, affects 0.5–2% of pregnant women (Ma, Xu, Wang, Xian, & Liu, 2012). RM is a genetically heterogeneous condition resulting from both maternal and embryonic regulating factors (Rasti, Z., Nasiri, M., & Kohan, L. 2016)

The exact pathophysiology of RM is still unclear. However, several etiological factors such as chromosomal abnormality, anatomic defects, hormonal problems, thrombophilic disorders, infections and immune system factors have been proposed to contribute to RM (Bahadori et al., 2014).

There are environmental factors contributing to recurrent miscarriage. Studies interesting about the effect of maternal cigarette smoking and exposure to environmental tobacco smoke on spontaneous abortion have been carried out in the past decades. Many studies indicated that a body mass index (BMI) of 24 or greater, passive smoking, and family history of miscarriage among women have an increased risk effect for RM (B. Y. Zhang et al., 2010).

1.2.2 Recurrent miscarriage among Palestinian women:

In the Palestinian community, the incidence of abortion is relatively high, estimated at 4 -8% among women at the age of reproduction (Hussein, Darwish, & Shelbayeh, 2010).
Hussein et al. have reported the association between factor V Leiden mutation and poor pregnancy outcomes among Palestinian women from the West Bank region of Palestine suffering from recurrent miscarriages with unknown cause, compared to control women with uncomplicated pregnancies and deliveries. Many studies have been conducted on the consideration of Thrombophilia as a significant factor that play a major role in 40-60% of unexplained multiple miscarriages (Brenner et al., 1997). Inherited Thrombophilia is a multi-factorial disorder caused by inherited and acquired factors including mutations in genes that code for natural anticoagulants such as anti-thrombin, protein C, and protein S, or clotting factors like prothrombin and factor V (Walker, 2000).
The study of Hussein et al. provides evidence for the significant association between factor V leiden mutation and poor pregnancy outcome among the Palestinian population. The association of the mutation has an effect on miscarriages that happen after week 10 and becomes greater effect after 12 week of gestation. However, some early foetal losses reported in the study seem to have no association with factor V variant (Hussein et al., 2010).
Another study has also been published in 2010 conducted to investigate the correlation between spontaneous (RM) and common polymorphisms in angiotensin-converting enzyme (ACE), plasminogen activator inhibitor 1 (PAI-1) and endothelium-derived nitric oxide synthase 3 (NOS3) genes among women experiencing RM in the Gaza Strip (Al Sallout & Sharif, 2010). There is a belief that Urokinase plasminogen activator, its receptor, and PAI-1 are control proteolysis and remodelling of maternal tissue during trophoblast invasion (Lockshin, 1999), (Floridon et al., 2000).

1.2.3 Immune cells and molecules in women with normal pregnancy and recurrent miscarriage:

There is associated increase in tolerance promoting T regulatory cell (Treg) activity and a proportional decrease in the pro-inflammatory Th17-cell activity. Trophoblast acts as an allogenic tissue due to the parental genetic contribution during normal pregnancy (Fig 1.1). It induces an immunomodulatory effect, suppresses abortigenic maternal B and T cell responses leading to adaptation of the fetus (Y. K. Choi & Kwak-Kim, 2008). The maternal immune tolerance is likely to be modulated by both adaptive and innate immunity during pregnancy. The natural killer cells − macrophage, dendritic cell and Treg cell − migrate and increase in the endometrium during the implantation window. Deletion of these cells has deleterious effects on implantation and placental development (Liang et al., 2015). There is an association between pregnancy loss and autoimmune phenomena such as the presence of antiphospholipid antibodies—Lupus anticoagulant and anticardiolipin antibody (Allahbadia & Allahbadia, 2003)
Human chorionic gonadotropin (hCG) is also of very importance in the establishment of the early embryo in the endometrium. Its role has been defined in promoting angiogenesis and placentation and in recruiting and promoting maternal Treg cell function. B- hCG is important in maintaining pregnancy by promoting a down regulation of harmful maternal immunity (Kumar, 2014).

Natural killer cells floading the uterus at the time of implantation carries receptors that interact with HLA-C, HLA-E on surface of trophoblast, triggering the secretion of cytokines that help trophoblast to invade or limit the extent to which it invades, (Fig1.1) (Bohiltea & Radoi, 2014a). The serum cytokine levels for adverse pregnancy outcomes such as preterm delivery, low–birth weight babies and small-for-date babies showed a significant difference only at the time of recruitment (Kumar, Begum, Prasad, Aggarwal, & Sharma, 2014), even during early pregnancy, plasma levels of pro-inflammatory cytokines are higher, while levels of anti-inflammatory cytokines are lower in women who miscarry than in those who have normal pregnancy (Alkhuriji et al., 2013).

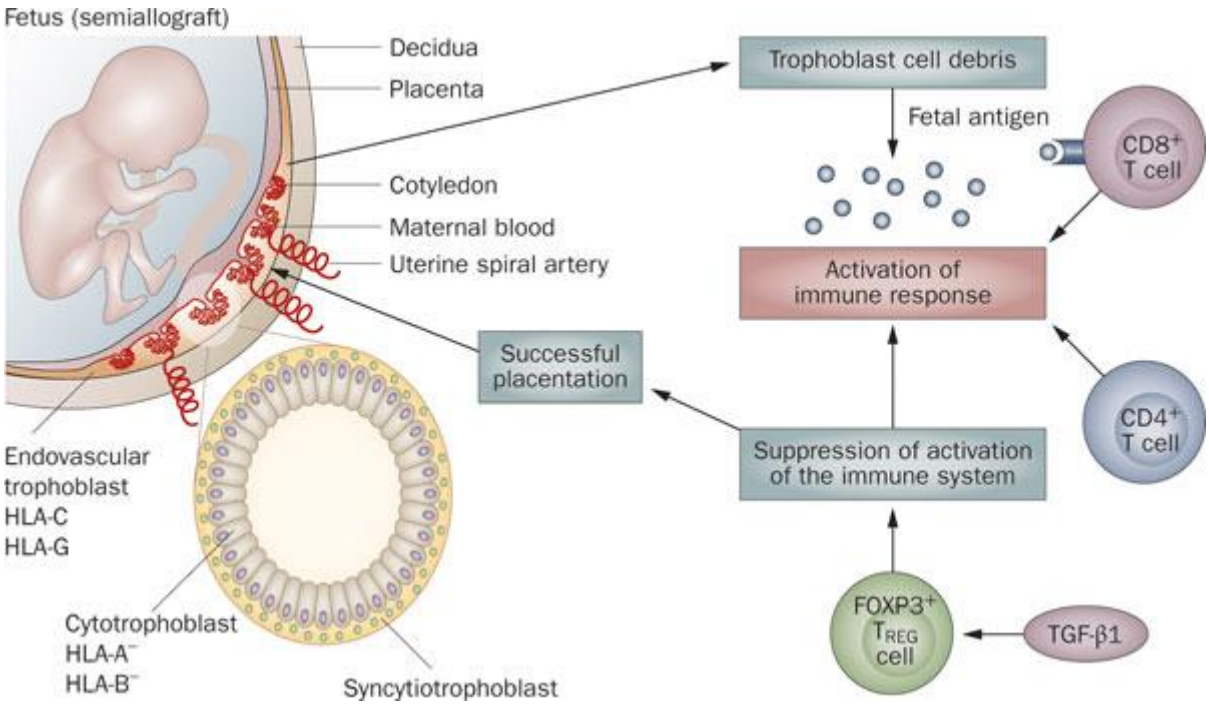

Figure 1.1 Maternal tolerance of the fetus. MHC II antigens and polymorphic MHCI antigens such as HLA-A and HLA-B are not expressed on trophoblasts of placental origin. By contrast, the invasive extravillous cytotrophoblast and endovascular trophoblast cell subsets express a unique subset of MHCI products, HLA-C and HLA-G. Debris from apoptotic or necrotic trophoblast cells is released into maternal peripheral blood and activates the maternal immune system. Subsequently, activated FOXP3+CD4+CD25+ TREG cells induce tolerance to specific fetal antigens by suppressing immune system activation (Tower, Crocker, Chirico, Baker, & Bruce, 2011)

1.3.1 Cytokines:

Cytokines are signalling, soluble proteins or glycoproteins, complex, pleiotropic group that are secreted by different immune cells, leukocytes and other cell types. They act as chemical communicators between cells, but are not effector molecules in their own right. Some can be expressed on the cell membrane, others are held in reservoirs in the extracellular matrix. Cytokines bind to specific receptors on the surface of target cells. These receptors are coupled to intracellular signal transduction and second messenger pathways. Most cytokines are growth and/or differentiation factors (Calleja-Agius & Brincat, 2008).

Cytokines are also hormones of the hematopoietic system which can affect the biologic behaviors of hematopoietic cells and processes, such as inflammation, wound healing and septic shock (Chau, Markley, Juang, & Tsen, 2016). They are also involved in

immunological, inflammatory and infectious diseases (Rasti, Z., Nasiri, M., & Kohan, L. 2016).

Cytokines include chemokines (direct immune cells via chemotaxis to sites of inflammation), interferons (mediate cellular responses predominately to viral infections), interleukins (promote cell proliferation, maturation, migration, differentiation, and activation) or colony-stimulating factors (stimulate proliferation and differentiation of other target cells) (Chau et al., 2016).

1.3.2 The pro-inflammatory and anti-inflammatory cytokines in recurrent miscarriage:

Some women with idiopathic RM have increased production of pro-inflammatory cytokines such as tumour necrosis factor- (TNF-α) and interferon-γ (IFN-γ_) and decreased levels of anti-inflammatory cytokines such as interleukin-10 (IL-10) by peripheral blood mononuclear cells. Genetic polymorphisms associated with high and low production of a number of cytokines, including TNF-α, IFN-γ, and IL-10 have been found (Babbage et al., 2001).

Although the mechanism of pro-inflammatory polarization is unknown, it may affect embryo implantation through a number of mechanisms. Excess pro-inflammatory cytokine (especially IFN-γ) could activate macrophage and induce its differentiation, which may express nitric oxide synthase (iNOS) and mediate directly the damage to trophoblasts. It was reported that both extravillous trophoblast proliferation and invasion were inhibited in vitro by the combined pro-inflammatory cytokines.

For these reasons, the dominance of pro-inflammatory cytokines was probably one of the important factors affecting embryo implantation (Liang et al., 2015) (Fig 1.2).

Failure to enable the shifting from a pro-inflammatory (Th1) to an anti-inflammatory (Th2) pattern, as evidenced by a persisting or dominant Th1 immune response, is associated with complications such as multiple implantation failures, RM, pre-eclampsia, preterm delivery, and intra-uterine growth restriction (Y. K. Choi & Kwak-Kim, 2008).

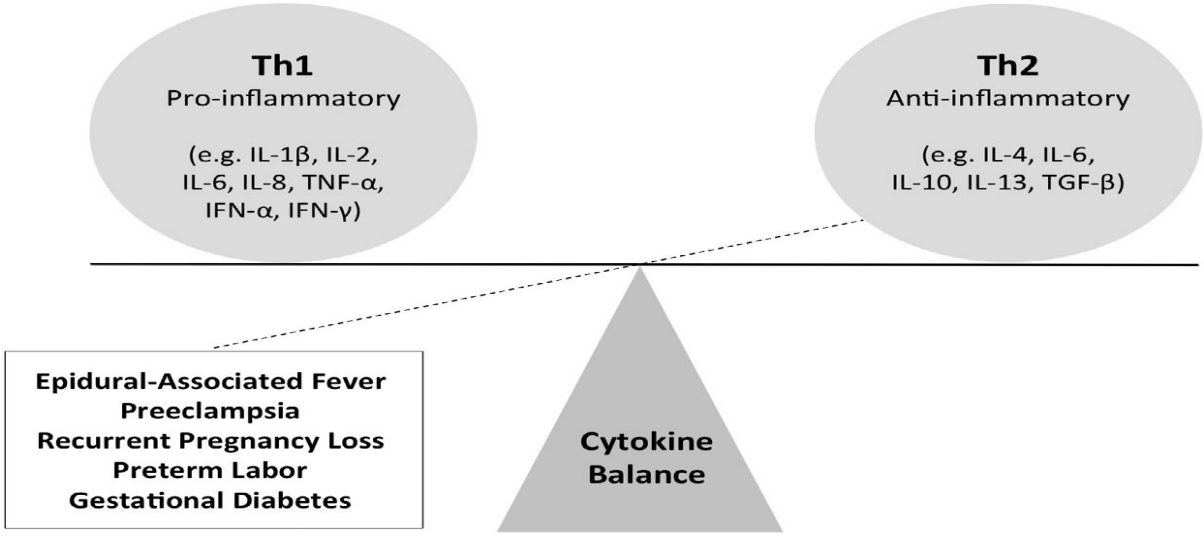

Figure 1.2: Cytokine balance during pregnancy. The pro-inflammatory Th1 response is important for maternal host defences whereas the Th2 reaction is important for inducing fetal immune tolerance. Imbalance of the immune responses may lead to adverse pregnancy outcomes such as gestational diabetes, preeclampsia, recurrent pregnancy loss, preterm labor and development of labor epidural-associated fever (Chau et al., 2016)

1.4 Medications that can be effective to prevent recurrent miscarriage

Many Progesterone containing drugs are now available for treating women with RM. These medications reduce the chances of spontaneous miscarriage in women with unexplained RM improving the pregnancy outcome. Progesterone may promote the development of a cytokine microenvironment supporting pregnancy maintenance. It increases the expression of Th2-type cell responses and leukemia inhibitory factor. Thus the elevated concentrations of progesterone characteristic of early pregnancy may promote an immune environment sustaining pregnancy maintenance (Haas & Ramsey, 2013).

Progesterone-induced blocking factor (PIBF) prevents inflammatory and thrombotic reactions toward the fetus. PIBF, a protein synthesized by activated lymphocytes in the presence of progesterone, promotes this shift toward Th2 cytokines (Kumar et al., 2014). One report from Egypt proved that progesterone have a great effect in reducing the risk of miscarriage if administered in the luteal phase of the cycle, before confirmation of pregnancy in women with history of unexplained RM (Ismail, Abbas, Ali, & Amin, 2018). In contrast other studies have proved that there was no evidence

that first-trimester progesterone therapy improves outcomes in women with a history of unexplained RM (Coomarasamy et al., 2016).

1.5 Cytokine gene polymorphisms:

Certain cytokine gene polymorphisms in the promoter regions, exons or introns influence the level of cytokine secretion, (Y. K. Choi & Kwak-Kim, 2008)

There are now many well documented instances where nucleotide polymorphisms occur within the regulatory region of cytokine genes, some of these are associated with an altered rate of gene expression (Unfried et al., 2003). Many studies have shown that polymorphism of cytokine genes is associated with susceptibility to certain inflammatory and infectious disease (Y. K. Choi & Kwak-Kim, 2008).

Genotype and allele frequencies of cytokine polymorphisms show significant differences between different populations. Allele frequency variation was such that, within each cytokine, there was a tendency for at least one population to present itself as _different_ from the other groups: for example the Caucasian Northern Ireland group with a lower IL-6 -174G (high) allele frequency, relative to the other populations, and the complete absence of genotype IL-6 -174 CC (low) in the African group. Also those of Asian lineage exhibited an increase in IFN-y genotype + 874 A/A (L) that resulted in low expression as compared with whites. These differences show that the heredity of certain cytokine gene polymorphisms is strongly associated with ethnicity. It will be necessary to evaluate global frequencies for the variant alleles that may cause cytokine dysfunction and establish a data bank of information for future disease studies (Prigoshin, Tambutti, Larriba, Gogorza, & Testa, 2004) .

Research efforts have focused on single nucleotide polymorphisms (SNP) in cytokine genes, and various SNPs have been reported to be related with infectious and inflammatory conditions, including the risk of pre-labour rupture of the amniotic membranes and preterm labor (Alkhuriji et al., 2013).

1.6 Interleukin-10 gene and idiopathic recurrent miscarriage:

IL-10 is defined as a pleiotropic cytokine, is produced by activated Th2 cells, B cells, monocytes and macrophages. IL-10 is the most potent immunosuppressive and anti-inflammatory molecule, one of anti- inflammatory cytokines that appear to protect against inflammation that leads to pregnancy loss (Alkhuriji et al., 2013).

IL-10 gene is located on human chromosome 1 (1q31-q32) and plays an important role in the Th2 dependent immune responses. The processes including antigen presentation, T-cell proliferation, and selectively Th1-mediated cellular responses can be interfered by IL-10 through down-regulating the chemokines and pro-inflammatory cytokines, especially tumor necrosis factor-α and interferon-γ.

Many single-nucleotide polymorphisms (SNPs) were reported in the proximal (at position -1082A/G, -819C/T and -592C/A) and distal regions of this gene and were reportedly associated in IL-10 transcription rate, thereby directly affecting its production level (Alkhuriji et al., 2013).

Mutations in IL-10 have been detected at several loci, and the association between IL-10 SNPs and disease risk has been heavily studied, Numerous molecular epidemiological studies have investigated the association betweenIL-10 gene polymorphisms and several types of cancer risk. One study published in 2006, explained the negative association between three IL-10 promoter polymorphisms and the risk of nasopharyngeal cancer in Italian populations (Niu et al., 2015).

IL-10 (increase during labour), IL-10 and IL-4, named as protective agents during pregnancy, show a constant presence at the first two trimesters with IL-10 showing a peak of production during labour. This cytokine shows a minimal presence in the serum of pregnant or non-pregnant women at levels ranging from 0.14 to 0.88 ng/ml, implying a possible maintenance role during the gestation period (Vassiliadis, Ranella, Papadimitriou, Makrygiannakis, & Athanassakis, 1998).

Many evidences suggests that IL-10 is elevated in some reproductive pathologies. IL-10 levels have been shown to be significantly increased in amniotic fluid of small-forgestational- age pregnancies as well as in severe pre-eclampsia (Y. K. Choi & Kwak-Kim, 2008). Q. Liu et al revealed that individuals carrying the CC genotype of IL-10 -819T/C were associated with an increased risk of preeclampsia compared to the TT genotype (Q. Liu et al., 2015)

During pregnancy, IL-10 is produced locally in the fetoplacental unit by cytotrophoblasts, and it up regulates the human leukocyte antigen (HLA)-G expression of cytotrophoblasts at the feto-maternal barrier.
IL-10 gene polymorphism screening might have some relevance in RM patients. It was suggested that increased IL-10 expression was associated with successful pregnancy, whereas low levels were linked to recurrent fetal loss (Karhukorpi, Laitinen, Karttunen, & Tiilikainen, 2001). Previous in vitro studies showed that the GCC haplotype was related to increase IL-10 production, whereas the ATA haplotype was correlated with low levels of IL-10 production (Su et al., 2016)

In humans, IL-10 production varies according to the presence of a specific allele at the1082 position where G⁄G variation determines a high cytokine production phenotype. Lin et al. concluded that the IL-10 592 AA genotype is correlated with a decreased risk of acute graft versus host disease; thus, the AA genotype may be associated with higher IL-10 production. Also, Temple et al. found that 819T and 592A alleles may increase stimulated transcriptional activity by influencing transcription factor binding. Furthermore, Hoffmann et al. indicated that the GCC haplotype (1082 ⁄819 ⁄592) is associated with decreased IL-10 production (Y. K. Choi & Kwak-Kim, 2008).
Results of a study conducted in Iran demonstrated an association for IL-10 gene polymorphisms including IL-10-592 A/C (rs1800872) and IL-10-819 C/T (rs1800871) But not IL-10-1082 A/G (rs1800896) promoter polymorphisms in idiopathic RM. Lack of any association between IL-10-1082 A/G polymorphism and RM was consistent with previous studies (Bahadori et al., 2014). In the same context, a study in Finland suggests that the IL-10 -1082 (G/A) polymorphism is not a major genetic regulator in RM (Karhukorpi et al., 2001).

Kamali-Sarvestani et al. reported a significant association between the presence of IL-10-1082 A/G polymorphism and the occurrence of RM in Iranian women (63% in women with RM and 46% in controls) (Kamali-Sarvestani, Zolghadri, Gharesi-Fard, & Sarvari, 2005). Zammiti et al. demonstrated an association between IL-10-592C/A and -819C/T promoter polymorphisms among Tunisian RM patients (Zammiti et al., 2006).

A study in Romanian population demonstrated a role for -819 C/T but not -592 C/A IL10, -1082 A/G IL10polymorphisms in idiopathic recurrent miscarriage (Bohiltea & Radoi, 2014a).

Other studies have shown that genotype and allele frequencies do not show any significant association between the unexplained RM patients and controls, and hence cannot be considered as a clinically important polymorphism linked to unexplained RM (Babbage et al., 2001), (Prigoshin et al., 2004) among Argentinian women, (Alkhuriji et al., 2013) among Saudi women, (ALHindi & Sharif) among Palestinian women in Gaza Strip, and (Kaur & Kaur, 2011) among Indian women. All of these studies demonstrated that there is not any association between RM patients and controls concerning the IL-10 gene polymorphisms.

Many case–control studies analyzed the relationship between the IL-10 promoter haplotypes (GCC, ACC, and ATA) and RM. The pooling results demonstrated no significant associations between the three haplotypes and RM risk based on the random-effects model. The result indicated that the ATA haplotype was associated with increased RM risk (Su et al., 2016). This meta-analysis suggested that both rs1800896 and rs1800871 polymorphisms have a positive relationship with RM, and women with A and C alleles for the rs1800896 and rs1800871 polymorphisms, respectively, could have effects on preventing RM. On the other hand, no sufficient evidence was found to detect the association between rs1800872 polymorphism and RM either in the overall or in the subgroup (Su et al., 2016).

Discrepancy between results of genetic association studies like those encountered here could be due to many reasons including population genetic variation (background) unrelated to the investigated alleles, presence of nucleotide polymorphism somewhere else in the examined gene e.g., in the coding or intronic regions, epigenetic alterations and linkage disequilibrium to other sequence variants in the vicinity of the studied loci (Kaur & Kaur, 2011)

Collectively, these reports highlight the need to analyze the association between IL-10 gene polymorphisms and RM in different geographic regions and ethnic groups.

1.7 Interleukin-6 gene and idiopathic recurrent miscarriage:

Interleukin-6 is a pleiotropic cytokine produced by a different cell types, including lymphocytes, monocytes and endothelial cells (Ma et al., 2012).
IL-6, contributed in the inflammatory response and in the modulation of immune responses including B cell and T cell differentiation, functions as both a pro- and anti-inflammatory cytokine (Lee, Choi, & Ji, 2015)

Secretion of IL-6 leads to a stimulation of the hypothalamic, pituitary, adrenal axis during inflammatory processes, promotes osteoclastogenesis and participates in the development of osteoporosis associated with estrogen withdrawal. IL-6 is not constitutively expressed, but is highly inducible and produced in response to a number of inflammatory stimuli. Animal studies found that an increase in lL-6 concentrations precedes uterine contractions, suggesting that lL-6 plays a role in the physiological mechanisms involved in the propagation of labour. In human studies, IL-6 production has been described in the decidua during early pregnancy. Also, IL-6 has been shown to induce the release of hCG from trophoblasts, leading to a subsequent cascade of progesterone production, release of Th2 cytokines, and suppression of TH1 cytokines (Unfried et al., 2003)

The IL-6 gene is located on chromosome 7p21, and of the several known polymorphisms in its promoter region, −174 G/C (rs1800795) and −634 G/C (rs1800796) have been the most frequently studied in RM. These two polymorphisms known to influence IL-6 expression, they are functionally significant and are known to display weak linkage disequilibrium (Lee et al., 2015).

IL-6 levels in maternal serum, amniotic fluid, vaginal fluid, and placenta1have been found to increase during the process of normal labor compared with the non-labor state (Y. K. Choi & Kwak-Kim, 2008). In other words, It was suggested that increased IL-6 expression was associated with successful pregnancy, and increase significantly at the time of delivery, whereas low levels were linked with recurrent foetal loss. Additionally, decreased expression of IL-6 mRNA was demonstrated in the mid-secretory phase of the menstrual cycle associated with habitual abortion (Ma et al., 2012).

This is compatible with an anti-inflammatory role for IL-6 in pregnancy. On the other hand, elevated levels of IL-6 and pro-inflammatory cytokines, e.g. IL-1, TNF-a, and IL-8 in the placenta, amniotic cells, and deciduas have been demonstrated in pregnancies complicated by preterm premature rupture of the membranes (pPROM), intrauterine infection and prematurity (Y. K. Choi & Kwak-Kim, 2008)
This cytokine is regulated principally at the transcriptional level by regulatory elements in its 5 flanking region, within which, the −174 G/C polymorphism acts as an important regulator of transcription (Lee et al., 2015)

The first report on an IL-6 polymorphism in RM was in 2003 by Unfried, suggested that the IL-6 polymorphism was not associated with RM and alterations in IL-6 serum levels in a Middle-European Caucasian population (Unfried et al., 2003).

The other study has investigated the relationships between RM and single nucleotide polymorphisms at -634C/G in the promoter region of the IL-6 gene in the Japanese population. The results have shown a significant difference in the -634C/G genotype frequency (CC versus CG⁄GG) between women with RM and controls. The risk of RM was lower in the carriers of the G allele than in women with the wild type (CC) (Y. K. Choi & Kwak-Kim, 2008).

A study of IL-6 polymorphism 634C／G with RM in Chinese Han population showed an association between -634C⁄G polymorphism and RM. This study demonstrated that RM risk in carriers of the G allele and the GG genotype was lower than that in women with the wild-type (Ma et al., 2012), this findings are entirely consistent with the previously published data by (Saijo et al., 2004) who suggested Japanese women carrying the-634G allele of IL-6 gene had a decreased risk of RM.
Kitamura et al. (2002) has indicated the presence of the -634G allele is associated with an elevated production and secretion of IL-6 protein by PBMC in vitro (Ma et al., 2012). Demirturk et al. reported that the -174G/C genotypic and -174C allelic frequency of IL-6 was higher in RM Turkish patients than healthy controls, emphasizing the significant association of this SNP with RM in Turkish women. (Demirturk et al., 2013). On the other hand, a study in Suadi females population, demonstrated that the IL-6 (-634C/G) polymorphism was not associated with recurrent miscarriage (Alkhuriji et al., 2013).

Furthermore, two studies in 2014 on IL-6 -174G/C, from Romanian (Bohiltea & Radoi, 2014a), and Iranian women with RM (Bahadori et al., 2014), showed that there is no statistically significant differences in the frequencies of IL-6 (-174 C/G) polymorphisms between RM women and controls.
Results of a meta analysis study in 2015 showed that IL-6 −174 G/C and −634 G/C variants are associated with susceptibility to RM in non Caucasian and Asian populations, and a negative association was detected between the GG+GC genotypes of these polymorphisms and this disease (Lee et al., 2015)

Based on the anti-inflammatory role of the IL- 6 in reproduction and due to lower IL-6 expression, the polymorphic GG genotype might be observed in the women with RM. This assumption was confirmed by the results of a study carried out in Iran showing the higher frequency of GG+GC vs. CC genotypes among patients in comparison to control group. These results are inconsistent with data published from the Japanese experiment, as they introduce the G allele as a protective factor against recurrent pregnancy loss (Rasti, Nasiri, & Kohan, 2016).

Several studies have shown the correlation of IL-6 with some diseases. A study by Brosseron et al. showed that there was increase of IL-6 plasma levels in patients with severe Alzheimer's disease (AD) compared with patients having less severe disease or healthy controls (Brosseron, Krauthausen, Kummer, & Heneka, 2014). This could be interpreted in the way that peripheral levels of IL-6 slightly increase over the time course of AD (Kalman et al., 1997).

An individual's IL-6 genotype may be relevant to other conditions, such as juvenile systemic onset arthritis, development of Kaposi sarcoma and atherosclerosis. These clinical data suggest that carriage of the mutated IL-6 allele C confers protection against the development and course of inflammatory diseases (Unfried et al., 2003)

1.8 Interleukin-17 gene and idiopathic recurrent miscarriage:

The gene encoding IL-17 was discovered in 1993 in a rodent T cell library by subtractive hybridization (Gaffen, 2008)
IL-17 (also known as IL-17A) is most homologous to IL-17F (~60%), and the genes encoding them are proximally located on chromosome 6. IL-17 and IL-17F exist as

homodimers (Gaffen, 2008). These two cytokines show high protein sequence similarity, bind to the same receptor and display similar biological activities. Binding IL-17 to its receptor initiates signalling pathways which induce the production of pro-inflammatory cytokines and chemokines and induce the recruitment of neutrophils (Zidan et al., 2015). In human recurrent pregnancy loss, excessive TH17 cells numbers and high levels of IL-17, IL-6 and IL-1beta have been identified, indicating that uncontrolled TH17 cells may emerge as an important mediator (Fu et al., 2014). These cells are actively involved in the pro-inflammatory immune responses at the maternal-fetal junction at the time of implantation which could subsequently lead to the development of RM (Zidan et al., 2015)

The shift in the balance between the regulatory subpopulations of IL-17 producing lymphocytes (Th17)/T-regulatory lymphocytes (Tregs) towards Tregs exhibiting immunosuppressive activity could be important to maintain the immunological tolerance during pregnancy and explained the high IL-17 level in RPL (Heikkinen, Möttönen, Alanen, & Lassila, 2004)

Few studies have examined the association of IL-17 A, IL-17 F, gene polymorphisms and their levels with RM. Bahadori et al did not find statistically significant association between IL-17 gene polymorphisms and the incidence of recurrent pregnancy loss.

Chen et al. reported that IL-17 levels were not significantly different between RM women and normal fertile women (Zidan et al., 2015)

Th17 are a novel subset of CD4+ T cells, which can be effective on tolerance during pregnancy. The proportion of IL-17 lymphocytes in the decidua is significantly higher than that in the peripheral blood in 1st trimester pregnant women (Fu et al., 2014).

Some reports showed that allele A of IL-17A (rs2275913) presented with high serum levels of IL-17, and the expression and/or activity of IL-17 might be suppressed in IL-17 F (rs763780) C allele carriers.

A study by Saifi et al. reported significantly higher expression of IL- 17 in the unexplained RPL group compared to the normal non-pregnant group. Another study done by Wang et al. examined IL-17 cells and detected their prevalence increase in the peripheral blood and the decidua in unexplained recurrent pregnancy loss women (Zidan et al., 2015)

A study carried out in India showed that the proportions of Th17 cells and IL-17A concentration in peripheral blood in patients with RM were significantly higher than those in non-pregnant women and normal pregnant women, and Treg frequencies were significantly lower in patients with RM than in normal pregnant patients which conforms to the results demonstrated by Yang et al (Y. S. Liu et al., 2011).

Najafi et al. found that IL-17 F (rs763780) TT genotype might be associated with a high risk of RM in Iranian women. On the other hand, no association found between of IL-17A(rs2275913) polymorphism and the risk of RM (Najafi, Hadinedoushan, Eslami, & Aflatoonian, 2014)

Zidan et al. suggested that there was association between IL-17 F (rs763780) polymorphism with decreased risk of RM in Egyptian females, but IL-17 A (rs2275913) SNP was associated with an increased risk of RM. Their results also demonstrated that the IL-17 levels was elevated in the women with RM in comparison to control group (Zidan et al., 2015).

Wang et al. Studied IL-7A and IL-7F polymorphisms in Chinese Han women with breast cancer. they found an association between the SNPs in IL-17A but not IL-17F and breast cancer risk.

Quan et al. demonstrated that there was a significant correlation between IL-17A rs2275913 and the risk for developing cervical cancer but no relation between IL-17F rs763780 gene polymorphism and cervical cancer was observed. Hayashi et al. investigated the association between gastro-duodenal disease and polymorphisms of rs763780 and rs2275913 in Japanese population. Their results indicated that rs2275913 influences the susceptibility to gastro-duodenal ulcer. A study on Polish patients with rheumatoid arthritis showed that this SNP might be associated with increased disease activity in rheumatoid arthritis patients. Moreover, Epinoza et al. have reported an association between rs2275913 genotype in the recipient side with the development of acute graft versus-host disease following bone marrow transplants (Najafi et al., 2014)

Since IL-17 has potent pathogenic properties, on the other hand, there are some mechanisms contribute to decrease its production or function. Both Th1 and Th2 cytokines as IL-27 and IL-2 suppress the activity and Th17 development. Th17 cells produce IL-10, which reduces their pro-inflammatory function (Gaffen, 2008).

Chapter Two

Materials and Methods

2.1 Study population:

A total of 107 convenient blood samples were collected from Palestinian women. Fifty five women aged 20-47 (mean 31.5 years) with a history of at least two miscarriages referred to the case group. The control group included fifty two healthy women, aged 20-44 (mean 32.7 years) with at least two normal pregnancies and no pregnancy complications such as miscarriages, still births, pre-term babies, and preeclampsia.

All the samples were collected from patient and healthy women during period between January 2017 and February 2018 from Palestine Medical Complex, Palestine Red Crescent Sociaty, Al Hiba IVF Center, Dr Omar Faisal Amr Medical Specialist Center, and Zughayer Medical Centers. Study patients were from different districts in the West Bank.

2.2 Study Questionnaire:

Interviews-based questionnaires were constructed for this study which aimed to collect general demographic and health status, patients files were reviewed also to collect medical data. Questionnaires were distributed and completed from all patients and healthy women. A copy of the study questionnaire is shown in Appendix C.

2.3 Assessment criteria:

Exclusion criteria included anatomical abnormalities, previously known systemic disease, endocrine disorders, previous venous or arterial thrombosis or a family history of thromboembolism. Chromosomal abnormalities were ruled out (karyotype) before inclusion in the study. As infection was linked with recurrent spontaneous abortion (RSA), all subjects included were confirmed to be negative for the TORCH agents Toxoplasma gondii, rubella, cytomegalovirus (CMV), herpes simplex viruses (HSV-1 and HSV-2), Varicella zoster virus (VZV) and human immunodeficiency virus (HIV-1 and HIV-2).

Inclusion criteria included women aged 20-47, divided into two groups: the control group, of healthy women with at least two normal pregnancies and no pregnancy complications such as miscarriages, still births, pre-term babies, and preeclampsia, and the case group included women with a history of at least two miscarriages without known medical causes.

2.4 Ethical considerations:

All subjects who agreed to participate in the study were briefed about the study aims and asked to sign informed consent. The principles of Helsinki declaration for scientific research were applied.

2.5 Sample Collection and Preparation:

Five ml of peripheral blood samples were collected under sterile conditions into tubes containing EDTA. Blood samples (5 ml from each sample) were centrifuged at 2000 rpm for 15 minutes. From each sample 200 µl buffy coat (the white interface between the plasma and the red blood cells) was transferred to 1.5 ml micro-tube to be used for preparation of genomic DNA.

2.5.1 DNA Extraction:

Genomic DNA was prepared from buffy coat using the DNA extraction kit (QIAamp DNA Blood, Germany) according to the manufacture instructions. Briefly, a 200 µl of

red cell Lysis solution buffer and 20 µl of Proteinase K, were added to the tube with 200µl buffy coat. The tube was vortexed vigorously, incubated at 56°C for 10 minutes. To remove the insoluble particles, 200 µl absolute ethanol was added then all the amount was transferred into spin column (filter tube), centrifuged for 1 min at 8000×g. For washing, 500 µl of first washing buffer was added and centrifuged for 1 min at 8000×g, then 500 µl of second washing buffer was added and centrifuged for 4 minutes at 14000×g. Finally, spin column was transferred into a new 1.5 ml micro-tube,150 µl of elution buffer was added and centrifuged for 1 min at 8000×g. The eluted DNA was kept at -20C until use.

2.5.2 DNA Quantification:

DNA concentration was measured spectrophotometrically using (Nanodrop 2000c; Thermoscientific). Elution buffer TE (Tris-EDTA buffer) was used as a blank, spectral measurement at 230nm, 260nm, and 280nm were also done and the ratios (260/280 nm and 260/230 nm) were calculated to assess the purity of DNA samples. DNA concentration was adjusted to (50-150 ng/µL) in each 25 µl PCR reaction. Concentrated DNA samples were diluted with ultra distilled water. In the case of low concentration of DNA in samples, the volume of DNA was increased. Samples with good purity and A260/A280 ratio about (1.6 to 2) were used in the PCR.

2.6 PCR Amplification:

2.6.1 Primers

The primers (forward and reverse) were selected on the basis on their design by previously published studies, (Table 2.1), primers were checked by Primer BLAST in Pubmed, NCBI, software(http://blast.ncbi.nlm.nih.gov/Blast.cgi)

Table 2.1: The main properties of the primers used in this study.

Gene SNP	Primer	Primer sequence	Primer size bp	Amplicon size bp	Annealing Temp °C	Ref
IL-17A	(F)	5'- TCT CCA TCT CCA TCA CCT TTG -3'	21	815 bp	57	(Najafi et al., 2014)
	(R)	5'- GTC CAA ATC AGC AAG AGC ATC -3'	21			
IL-17F	(F)	5'-CAC TGG TGC TCT GAT GAG GA- 3'	20	635 bp	54	
	(R)	5'- CATTGT GCT TTG GCT TGC T- 3'.	19			
IL-10 AC	(F)	5'-GGTGAGCACTACCTG AC TAGC -3'	21	412 bp	61	(Bahadori et al., 2014)
	(R)	5'-CCTAGGTCACAGTGAC GTGG -3'	20			
IL-10 CT	(F)	5'- TCATTCTATGTGCTGG AGATG -3'	21	209 bp	57	
	(R)	5'- TGGGGGAAGTGGGTA AGAGT -3'	20			
IL-6 174	(F)	5'-TGACTTCAGCTTTACT CTTTGT -3'	22	198 bp	57	
	(R)	5'- CTGATTGGAAACCTT ATTAAG -3'	21			

2.6.2 The PCR Protocol:

Genomic DNA was amplified by polymerase chain reaction (PCR) using IL-10 (-819 C/T and -592 A/C), IL-6(-174 C/G) and (IL-17A, IL-17F) specific primers (Table 1). Each PCR reaction with a final volume of 25 µl contained 5 µl of DNA, 7 µl distilled water,1.25 µl of each of the reverse and forward primers, and 12.5 µl PCR master mix (Ready Mix PCR Master Mix of Thermo scientific, Cat. No. AB-0575/Dc/LD/A) for IL-10, IL-6, and IL-17F genes. While Green Master Mix (green GoTaq® reaction buffer, Promega) was used for IL-17 A gene. The reaction was carried out in PCR tube (0.2 ml Axygen INC, USA) using GeneAmp PCR System 9700. Gradient PCR was run first to find the optimum annealing temperature for each primer). Thermal cycling was performed as shown in Table 2.2. Nuclease-free water was used as negative control in

each run. The reaction was then stored at 4ºC and either frozen at -20ºC or directly separated.

Table 2.2. Thermal cycling programs for amplification of IL-10, IL-17, and IL-6

PCR program	IL-6, IL-17A, IL-10C/T amplification		IL-17F amplification		IL-10A/C amplification	
	Temp	Time	Temp	Time	Temp	Time
Initial denaturation	94 ° C	5 min	94 ° C	5 min	95 ° C	5 min
Denaturation	94 ° C	1 min	94 ° C	1 min	94 ° C	1 min
Annealing	57 ° C	1 min	53 ° C	1 min	63 ° C	30 sec
Extension	72 ° C	1 min	72 ° C	1 min	72 ° C	1 min
Final extension	72 ° C	5 min	72 ° C	5 min	72 ° C	6 min
No. of cycles	32		35		35	

2.7 Gel electrophoresis:

To detect the amplified gene product of the PCR reactions, agarose gel electrophoresis was used to separate the PCR product. 2 % agarose (STANDARD AGAROSE) was prepared by dissolving 2g of agarose in 100 ml solution of 1x Tris-acetate EDTA buffer (TAE) (40 Mm of Tris acetate pH 8; and 1 mM EDTA). The agarose was boiled until it was well dissolved, and then 4µl of 10 mg/ml (0.4µg/ml) of ethidium bromide was added for DNA staining. When agarose suspension cooled down to 40°C, carefully mixed, poured into agarose gel casting system (BioRad, UK or Cleaver**,** U.S.A) and a desired comb was inserted. Five µl of the PCR product was added directly into the gel well along with the DNA size control; 5 µl from 100 bp marker (gene ruler express DNA ladder, (GeneDirex, cat number. DM001-R500) was added to detremine the correct band size. Finally, the migrated bands in the agarose gel (100 voltage for 40min) were visualized under UV light, (VILBER LOURMAT).

2.8 Restriction fragment length polymorphism (RFLP):

All PCR products were used in the PCR-RFLP analysis. Genomic DNA that was amplified was subjected to RFLP analyses using the restriction enzymes used by others, (Najafi et al., 2014) and (Bahadori et al., 2014).

A total of four restriction enzymes were used to digest the amplified products in order to screen for genetic differentiation: Rsa I, Mea III, EcoNl and NlaIII (Thermo scientific, EU) enzymes were employed to determine the IL-10 (-592 A/C, -819 C/T), IL-17A, IL-17F and IL-6 (-174 C/G) variants, respectively (Table 2.3). All restriction digestions followed the standard procedure provided by the manufacturer.

To make a final solution of 32 µl for each samples, 10 µl of PCR reaction mixture, 18 µl nuclease-free water, 2 µl 10X enzyme buffer, and 2 µl of enzyme . The mixture in the tube was then incubated in a water bath for a specific period of time as shown in Table 2.3.

Table 2.3 Restriction enzymes used for cytokine genes polymorphisms.

Polymorphisms	Amplicon size (bp)	Restriction Enzyme	RFLP Size (bp)	Temp	Time
IL-10 -592 A/C	412 bp	Rsa I	236, 176	37 °C	1-16 hours
IL-10 -819 C/T	209 bp	Mae III,	125, 84	55 °C	1 hour
IL-17 A	815 bp	EcoNl	259, 270, 286, 529	37 °C	1-16 hours
IL-17 F	635 bp	NlaIII	124, 130, 381, 511	37 °C	1-16 hours
IL-6 -174 C/G	198 bp	NlaIII	140, 58	37 °C	1-16 hours

Different concentrations of agarose gel were used in order to find an optimum gel concentration to visualise the digested fragments. The concentration of 3-3.5% was chosen to obtain a good separation of the bands. The gel was prepared and documented as mentioned above. The digested fragments were compared to a 50 bp, 100 bp DNA marker (5 µl) run on the same gel to establish the sizes of the bands cut for each sample.

2.9 Bioinformatics, Blast, and getting the FASTA sequence from NCBI

BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to get the FASTA sequences for each interleukin, by accession number used by researchers and data bases to refer to specific SNP, all sequences were recognized by entering forward and reverse primers for different interleukins genotypes severally, see appendix A. NEBcutter software (http://nc2.neb.com/NEBcutter2/) was used to make virtual cut of sequences to

be sure of restriction site position and fragments length for all genotypes. See Appendix B

2.10 Statistical analysis:

Collected data were analyzed using the Epi Info version 7.2.2.2 and statistical package for the social sciences (SPSS) software version 22 for Microsoft windows.

Genotype distributions of cytokine gene polymorphisms were compared between cases and controls by Chi-square test. Correlations between the polymorphisms were calculated by Spearman Coefficient correlation. Significance was set at P-value < 0.05.

Chapter Three

Results

A total of 107 individuals (55 patients and 52 healthy volunteers) were included in this study, questionnaires were completed from all patients and healthy volunteers, (Appendix C). Samples were recruited from different regions of Palestine: 33 samples were obtained from Ramallah, 30 from Al-Khalil, 24 from Jerusalem, 7 from Bethlehem, 6 from Nablus, 5 from Tulkarem and 2 from Jericho. Data about age, height, weight, health status, and smoking were reported from case and control groups. Tight criteria have been put to include or exclude study groups. The primary data which were included in the questionnaire were summarized in Table 3.1

Table 3.1 Clinical data and characteristics of the studied subjects

	Healthy controls (n=52)	RM patients (n=55)	Exact 95% LCL	Exact 95% UCL
Age (years)	Mean=33	Mean=31.8	21.44%	39.52%
Height (cm)	Mean=164	Mean=164.3	2.09%	11.81%
Weight (kg)	Mean=65.34	Mean=67.37	4.57%	16.52%
smokers	8	5	6.63%	19.88%
Negative smokers	29	27	43.34%	63.13%
Number of live births	Mean=4.1	Mean=2.3		
Number of miscarriages	0	Mean=3.8		
Age menses began	Mean=12.5	Mean=13.1	16.14%	33.02%
Regular menses	40 (76.9%)	38 (69%)	63.45%	81.04%

* LCL: lower control limit

* UCL: upper control limit

* n: number

As shown in table 3.1, no statically significant difference was found in the above variables (P>0.05) between the case vs. the control groups, excluding their rules as risk factor for recurrent miscarriage.

A negative statistically significant difference was found between the two groups concerning the administration of oral contraceptive pills, P= 0.0013 (Table 3.2). This negative association could be attributed to the fact that contraceptive pills are principally composed of progesterone , which has an effective role in the next pregnancy.

Table 3.2: The distribution of study population according to contraceptive pills taken

contraceptive pills	**Frequency %**		**Percent**	**Exact 95% LCL**	**Exact 95% UCL**
	case	**control**			
Yes	6 (10.9%)	20 (38.5%)	24.30%	16.53%	33.54%
No	49 (89%)	32 (61.5%)	75.70%	66.46%	83.47%
TOTAL	**107**		**100.00%**		

* LCL: lower control limit

* UCL: upper control limit

3.1 Distribution of IL-17 F, IL-17 A, genotypes and alleles in the studied groups

The undigested PCR product size was 815 bp for IL-17A SNP rs2275913. Restriction digestion for the GG genotype (homozygous wild type) generated 259, 270 and 286 bp fragments; whereas the AG genotype (heterozygous) generated 259, 270, 286 and 529 bp and the AA genotype (homozygous mutant) produced 286 and 529 bp fragments. There was no significant difference in the frequency of the G allele between the control and case group, (20.1%, and 28%, respectively). Also the A allele frequencies of the control group vs. the case group were (79.8% and 72%, respectively). So the comparison between the population of the case and the control groups in the three genotypes of AA, AG and GG revealed no significant difference (P=0.1456) (Table 3.3)

Table 3.3: The frequencies of IL-17 A, IL-17F, genotypes and alleles in RM and control group

genotypes		Healthy controls (N=52) (%)	RM case (N=55) (%)	Exact 95% LCL	Exact 95% UCL	P-value	Chi-square
IL-17 A (rs2275913)	GG	2 (3.8%)	5 (9%)	2.67%	13.02%	0.1456	3.8537
	AG	17 (32.7%)	21 (38.2%)	26.5%	45.35%		
	AA	33 (63.5%)	29 (52.8%)	48.01%	67.42%		
	G%	20.1%	28%				
	A%	79.8%	72%				
IL-17 F (rs763780)	TT	19 (36.5)	25 (45.5%)	31.7%	51.05%	0.4066	1.8
	CT	32 (61.5%)	30 (54.5%)	48.01%	67.42%		
	CC	1 (2%)	0 (0%)	0.02%	5.1%		
	T%	67.3%	72.7%				
	C%	32.7%	27.3%				

* LCL: lower control limit

* UCL: upper control limit

For IL-17F, The undigested PCR product size was 635 bp for SNP rs763780 (Fig. 3.1).

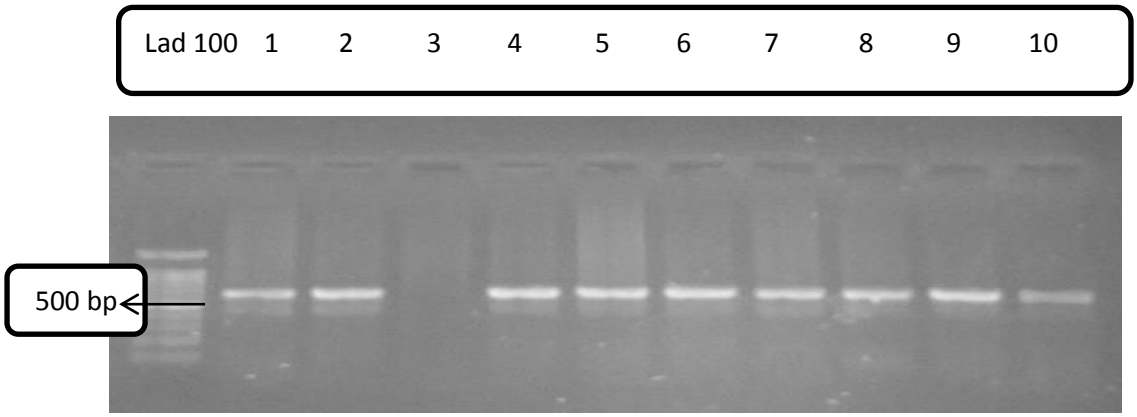

Fig 3.1: Agarose gel electrophoresis showing PCR products for IL-17F. The undigested PCR product size is 635 bp, Lane 3 is negative control

TT genotype (homozygous wild type) produced 124,130 and 381 bp fragments; the CT genotype (heterozygous) generated 124, 130, 381and 511 bp fragments and CC genotype (homozygous mutant) was characterized by 124 and 511 bp fragments (Fig. 3.2). There was no significant difference in the frequency of the C allele, it was 32,7% in the control and 27.3% in the case group. The frequency of allele T was 67.3% in the control group, and 72.7% in the case group.

The comparison between the population of the case and the control groups in the three genotypes of CC, CT and TT showed no significant difference (P=0.4066).

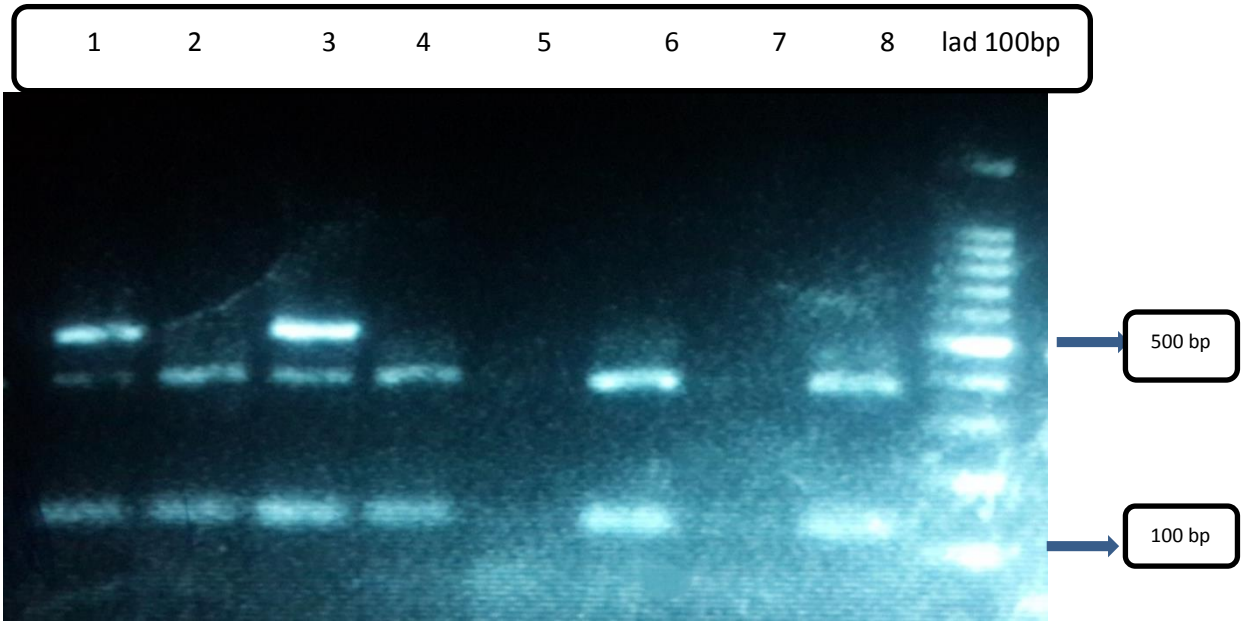

Fig 3.2: Restriction fragment polymorphism (RFLP) analysis of IL-17 F product using NlaIII enzyme. The digested product was loaded on 3% agarose gel. Lanes 5 and 7 are negative controls, lane 1and 3 are TC genotype, lanes 2, 4, 6 and 8 are TT genotype.

3.2 Distribution of IL-6-174C/G, genotypes and alleles in the studied groups

The undigested PCR product size was 198 bp for SNP rs 1800795 (Figure 3.3). Restriction digestion for the GG (homozygous mutant) genotype generated one band at 198 bp, (Figure 3.4); whereas the CG genotype generated 198, 140 and 58 bp and CC (wild type) genotype produced two bands at 140 and 58 bp fragments. NlaIII digestion enzyme cut the normal genotype, (NlaIII/ it is a type of restriction enzyme that abolishes a restriction site), this process was confirmed by retrieving the original sequences of the gene from BLAST/ FASTA website, see Appendix A, then inserting into NEBcutter which determines virtual restriction site position and fragments length. See Appendix B

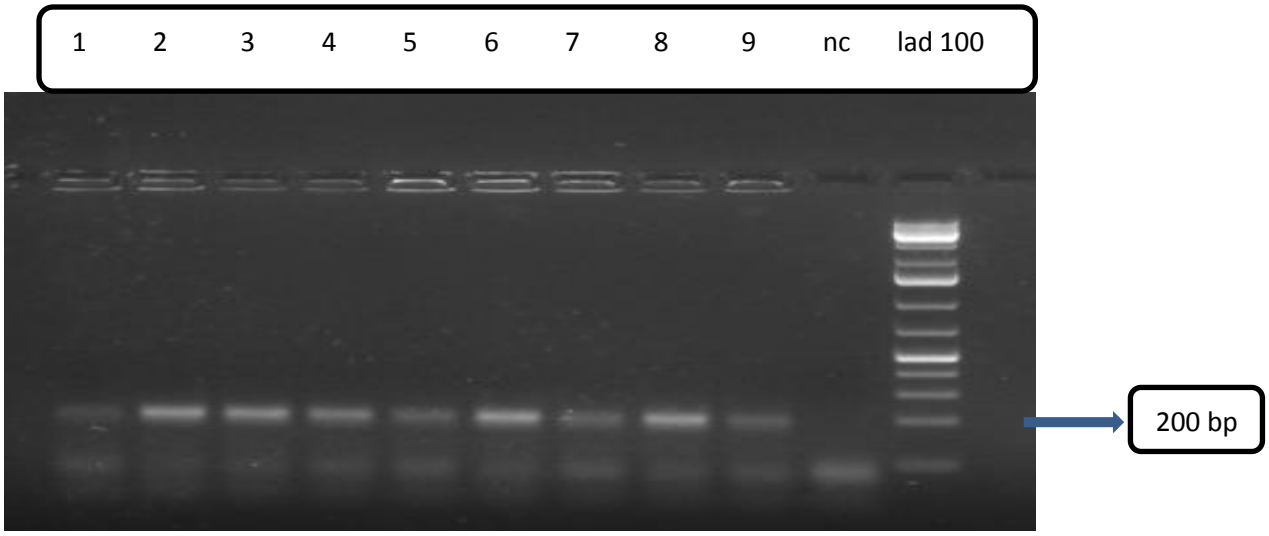

Fig 3.3: Agarose gel electrophoresis showing PCR product for SNP rs1800795. The undigested PCR product size is 198 bp, Lane 10 is negative control

The frequency of the genotypes and allelic frequencies are presented in Table 3.4. No significant differences were found between the case patients and the control groups. The CC genotype (homozygous wild type) was reported in one patient in the case group and was not reported in the control group. Our data show that there is no association between the presence of allele C in position -174 from the region of the IL6 gene promoter and recurrent pregnancy loss.

Table 3.4: The frequencies of IL- 6, genotypes and alleles in RM and control groups

genotypes		Healthy controls (N=52) (%)	RM case (N=55) (%)	Exact 95% LCL	Exact 95% UCL	P-value	Chi-square
IL-6 -174G/C (rs1800795)	GG	33 (63.5%)	37 (67.3%)	60.28%	69.8%	0.9939	0.0122
	GC	19 (36.5%)	17 (30.9%)	29.8%	36%		
	CC	0 (0%)	1 (1.8%)	0.05%	4.9%		
	G%	81.7%	82.7%				
	C%	18.3%	17.3%				

* LCL: lower control limit

* UCL: upper control limit

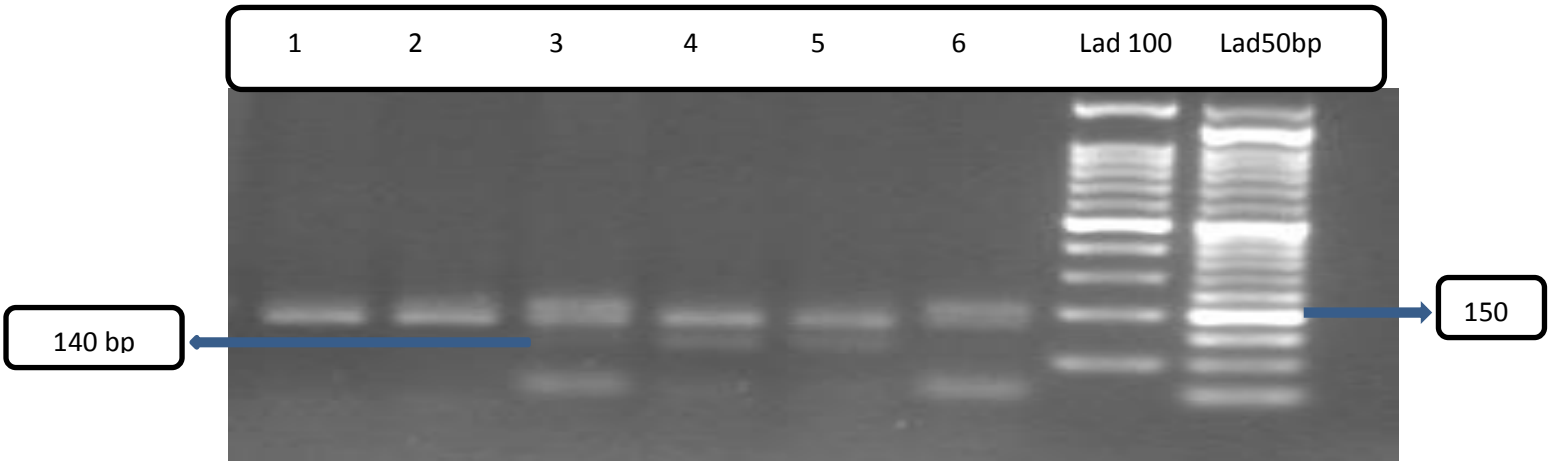

Fig. 3.4: Restriction fragment length polymorphism (RFLP) analysis of IL-6-174C/G product using NlaIII enzyme. The digested product was loaded on 3.5% agarose gel. Lanes 1, 2, and 6 are GG genotype, while lanes 3, 4, and 5 are CG genotype. M: marker, 100, 50 bp ladder

3.3 Distribution of IL-10-592 A/C and IL-10 -819 C/T, genotypes and alleles in the studied groups

The undigested PCR product size was 412 bp for IL-10 A/C, SNP rs1800872 (Fig. 3.5). Restriction digestion (RsaI enzyme), for the AA genotype (homozygous wild type) generated 236 and 176 bp fragments; whereas the AC genotype (heterozygous) generated 412, 236 and 176 bp and the indigestible fragment length 412 CC (homozygous mutant),(Fig. 3.6). The cutting here was in the normal genotype sequence, because RsaI is a type of restriction enzyme that abolishes a restriction site, this process was confirmed by getting the original sequences of the gene from BLAST/ FASTA

website, and compare it to SNP sequence, (Appendix A), then insert it into NEBcutter which determine restriction site position and fragments length. When the original sequence of IL-10 A/C was inserted to the NEBcutter website the cutting was performed with Rsa I enzyme generated two DNA fragments 236 and 176 bp, while it was uncut at the SNP site.

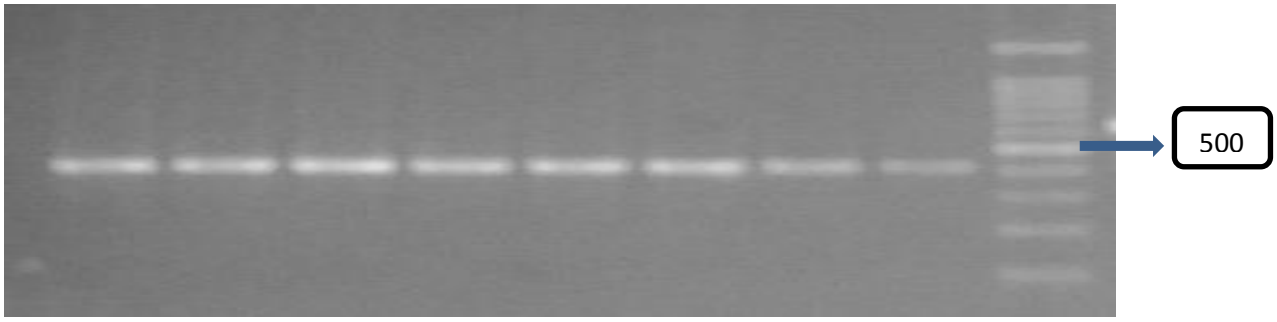

Fig 3.5: Agarose gel electrophoresis showing PCR product for SNP rs1800872. The undigested PCR product size is 412 bp.

There was no significant difference in the frequency of allele A, (with 33.66%, and 35.45% in the control and case group respectively). Also the frequency of allele C was 66.34% in the control group and 64.55% in the case group. So the comparison between the population of the case and the control groups in the three genotypes of AA, AC and CC revealed no significant difference (p=0.9626) (Table 3.5).

Table 3.5: The frequencies of IL-10 A/C, IL-10 C/T, genotypes and alleles in RM and control groups

genotypes		Healthy controls (N=52) (%)	RM case (N=55) (%)	Exact 95% LCL	Exact 95% UCL	P-value	Chi-square
IL-10 AC (rs1800872)	CC	23 (44.2%)	23 (41.8%)	33.46%	52.92%	0.9626	0.0762
	AC	23 (44.2%)	25 (45.5%)	35.23%	54.78%		
	AA	6 (11.6%)	7 (12.7%)	6.63%	19.88%		
	C%	66.34%	64.55%				
	A%	33.66%	35.45%				
IL-10 CT (rs1800871)	TT	11 (21.2%)	8 (14.5%)	11.04%	26.33%	**0.0009**	14.054
	CT	41 (78.8%)	34 (61.8%)	60.48%	78.56%		
	CC	0 (0%)	13 (23.7%)	6.63%	19.88%		
	T%	60.58%	45.45%				
	C%	39.42%	54.54%				

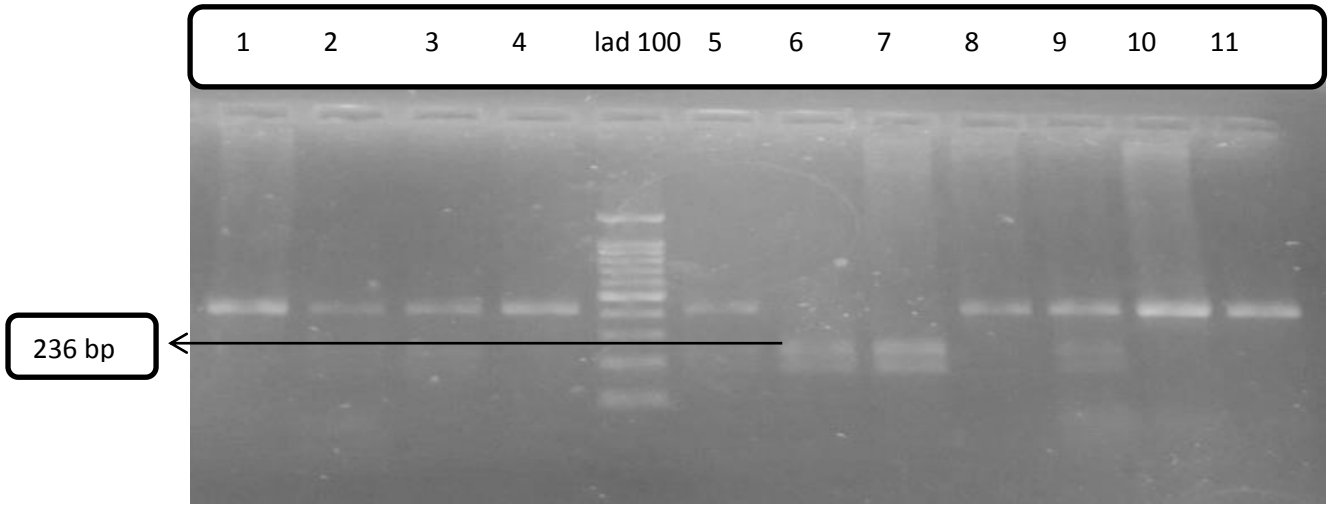

Fig. 3.6: Restriction fragment length polymorphism (RFLP) analysis of IL-10-592 A/C product using RsaI enzyme. The digested product was loaded on 3.% agarose gel. Lanes 6, 7, are AA genotype, (wild type allele) while lanes 3, 5, and 9 are AC genotype (heterozygous), the rest lanes are CC genotype.

The undigested PCR product size was 209 bp for IL-10 C/T SNP rs1800871 (Fig. 3.7). Enzymatic digestion with Mae III generated 125 and 84 bp fragments for the CC genotype (homozygous mutant); whereas the CT genotype (heterozygous) generated 209, 125 and 84 bp and the indigestible fragment length was 209bp TT (homozygous wild type),(Fig. 3.8). The digestion by Mae III enzyme was in the SNP site, because Mae III is atype of enzyme that creats a restriction site.

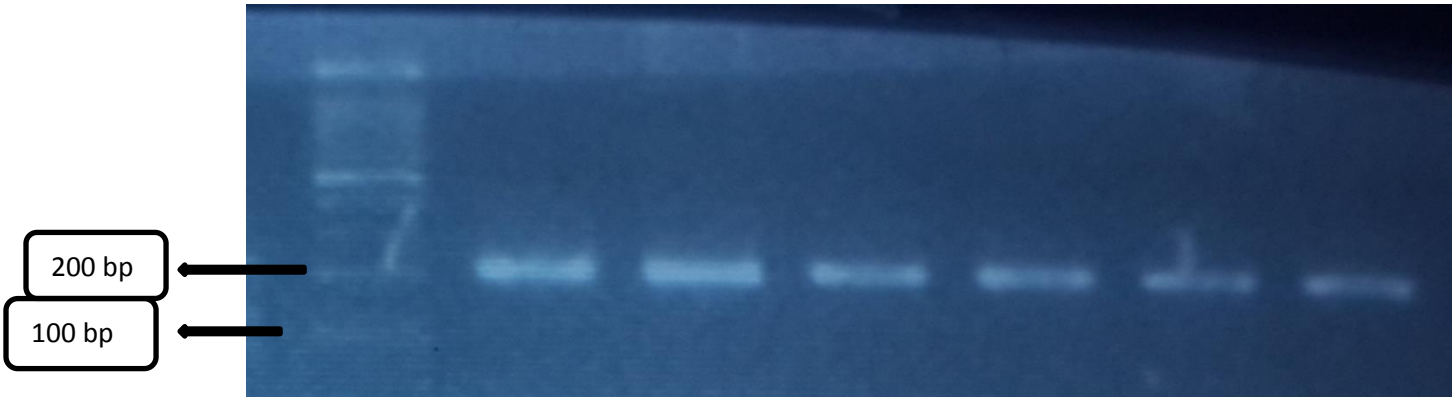

Fig 3.7: Agarose gel electrophoresis showing undigested PCR product for IL-10 C/T SNP rs1800871 at size of 209 bp.

The genotypes frequencies of polymorphism of rs1800871 in the case group were TT (14.5 %), CT (61.8 %) and CC (23.7 %). The frequencies were TT (21.2 %), CT (78.8 %) and CC (0 %) in the control group.

The frequency of allele C of SNP rs1800871 in the two groups was significantly different (P=0.0009). The frequency of mutant allele C was 54.54 % in the case group and 39.42 % in the control group. Besides, the frequency of allele T was 45.45 % in the case group and 60.58 % in the control group. The comparison between the case and the control group showed a significant difference in the three genotypes of TT, CT and CC (P=0.0009). The results are shown in Table 3.5.

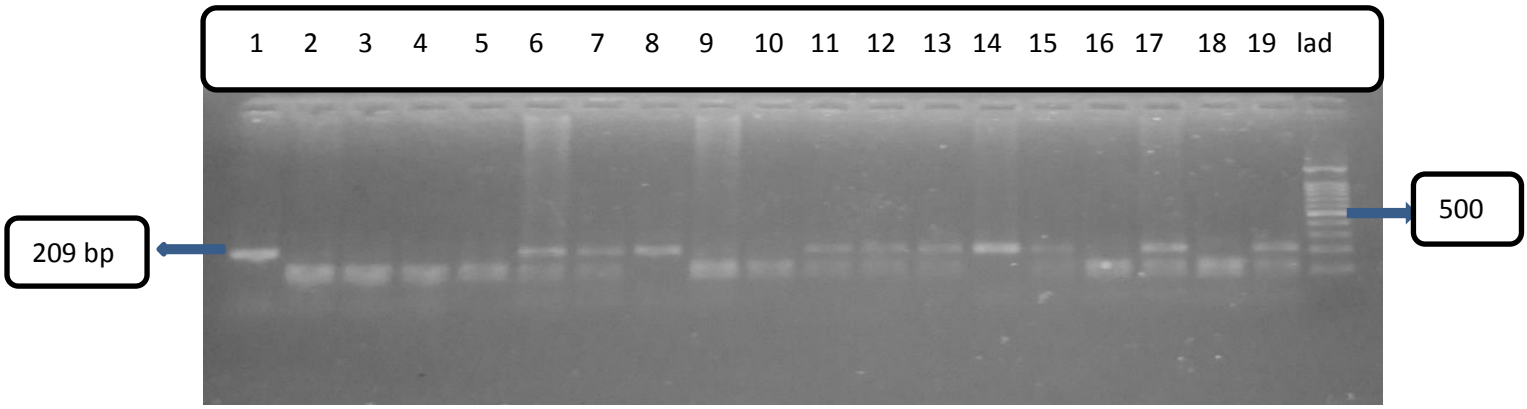

Fig 3.8. RFLP analysis of IL-10 -819 C/T polymorphism using Mae III enzyme on 2.5% agarose electrophoresis . The polymorphic region was amplified by PCR resulting in digestible fragment length 125 and 84 bp in lane 2, 3, 4, 5, 9, 10, 16 and 18 (CC mutant homozygote), indigestible fragment length 209 bp in lane 1, 8, and 14 (TT normal homozygote). Three fragments 209, 125, 84 bp (lane 6, 7, 11, 12, 13, 15,17 and 19) represent CT heterozygote. M: marker, 100 bp ladder, (all of these samples are from the case group).

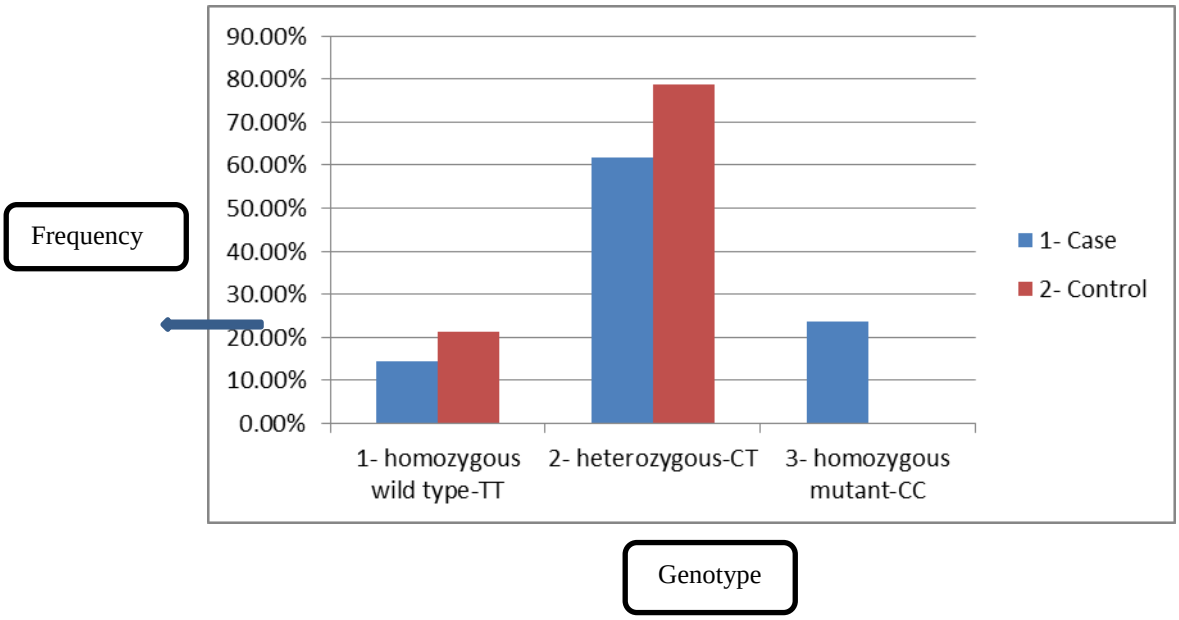

Fig. 3.9: Distribution of IL-10 -819 C/T, genotypes and alleles in the studied groups

Our results revealed a possible association of polymorphism -819 C/T and the increased frequency of recurrent abortions and the lack of association between polymorphisms IL10-592C/A, IL6-174G/C, IL-17 and the abortion disease in the studied group.

Chapter Four

Discussion

Pregnancy depends on the induction of maternal tolerance to fetal tissues; decidual cells will inhibit maternal immunity during pregnancy. Natural killer cells migrate into the uterus during implantation coordinating the secretion of cytokines that help or limit trophoblast invasion (Bohiltea & Radoi, 2014b). The etiology of recurrent abortions is heterogeneous (infectious, genetic, anatomic, hormonal factors are involved), accentuated by the acquired or birth risk factors (Bohiltea & Radoi, 2014b). Recurrent miscarriageis mediated by complex immunologic factors.

Cytokines are of vital importance in the regulation of cellular function (Su et al., 2016) Cytokines are secreted by the cells within the uterine lining and these cytokines stimulate the expression of adhesion systems that hold the embryo to the uterus. If the immune cells don't send specific signals through secretion of convenient cytokines to the embryo or if these cells don't respond to signals from the embryo, then adhesion and thus subsequent implantation will not occur (Kasap et al., 2015). Many studies are concerned in explaining the immunological and biological roles of cytokines during pregnancy, especially those in maternal peripheral blood and in uteroplacental tissues (Ozkan et al., 2015)

Interleukins are a group of immunomodulatory proteins that mediate a variety of immune reactions in the body (M. Zhang et al., 2017). Variants of genes alter the

corresponding protein expression levels. Therefore, it was necessary to assess the global frequencies of the variant alleles of interleukins that might cause RM.

To the best of our knowledges, no previous studies have been conducted in the West Bank to study the roles of cytokines polymorphisms and RM. This case control study included 107 random samples collected from Palestinian women from different cities in Palestine. In the current study we supposed a possible correlation between polymorphism IL-10-592 A/C (rs1800872), IL-10-819 C/T (rs1800871), IL-17A rs2275913, IL-17F rs763780 and IL-6 -174G/C SNP (rs1800795) and recurrent abortions. Reactive criteria have been put to include or exclude blood samples, based on specific health conditions, interviews, questionnaires for patients and healthy women volunteer to participate in this study to exclude patients with other known reason for RM. (Appendix C).

IL-6 takes part in trophoblast proliferation, differentiation and invasion and participates in follicle development and embryonic implantation. (Galazios et al., 2002) . The IL-6 protein also plays a part in the initial spiral artery remodeling that requires vascular smooth muscle cell induction and morphological change. Reduced levels of IL-6 decrease trophoblast invasion and spiral artery remodeling (Pitman, Innes, Robson, Bulmer, & Lash, 2013)

IL-6, secreted by decidual cell populations, is a potent pro-angiogenic cytokine that stimulates the proliferation of endothelial cells in vitro and regulates the behavior of the female reproductive tract and gestational tissues (Pitman et al., 2013). Based on many evidences about the role of IL-6 in the immunology of pregnancy was based on the highest level of IL-6 mRNA in the implantation and menstruation period and the presence of its receptors on the endometrium and trophoblast (Nasiri & Rasti, 2016)

During this study, we did not find any association between C/G -174 polymorphism in IL-6 gene and recurrent miscarriage. Whereas the IL-6 −174 C allele was rare in our results. Most of the patients (67.3%) and controls (63.5%) are homozygous to the mutant allele (GG). The heterozygous allele (CG) state occurs in (31%) of patients and (36.5%) of the controls, while the homo normal genotype (CC) occurs only in (1.8%) in patients and (0%) in controls. The frequencies of the genotypes and alleles do not show any significant difference between the unexplained RM patients and controls.

Our results are consistent with many previous reports (Bohiltea & Radoi, 2014b), another study showed that the frequency of allele C was 0.06% in the studied lot compared to 0.40-0.45 at the Caucasian population. Allele C has not been identified in position -174 in a study performed on 388 Japanese men and at only one person in a study performed on 259 Chinese men (Hayakawa, 2002), the IL-6 −174 C allele has not been observed in Japanese subjects (Saijo et al., 2004) and is known to be rare in Chinese and Korean populations (Lim et al., 2002), while this allele is common in Caucasians.

While other observations proved that the normal homozygous allele (CC) is common in Iran and occurs approximately (42%) in both healthy and RM women, but the study did not find any association between IL-6−174 polymorphism and RM (Bahadori et al., 2014). Lee et al. showed that IL-6 −174G/C, polymorphism is associated with susceptibility to RM, particularly in non-Caucasians (Lee et al., 2015), which was in contrast with our results. In the same context, the results of a study conducted in 2003 proved that the C allele was found to be associated with significantly lower levels of plasma,IL-6, whereas G allele was associated with higher IL-6 serum levels (Unfried et al., 2003).

Nakashima et al. showed that IL-17-positive T cells accumulated in the deciduas of women with RPL (Nakashima et al., 2010). Wang et al. found that the proportion of Th17 cells in the peripheral blood and the decidua of women with inevitable abortion were significantly higher than women with normal pregnancy (Wang et al., 2011). T helper (Th) 17 cells are a novel subset of T cells which secrete IL (Interleukin)-17. Th17 cells are known to be involved in the pathogenesis of autoimmune diseases, inflammation and immunological rejection of non-self tissue (Ivanov et al., 2006).

Few studies have examined the association of IL-17 A, IL-17 F, polymorphisms and their levels with RPL; however, their data are controversial. In addition, only few studies have been conducted so far for finding out the effect of these polymorphisms on their cytokines levels in RM patients. Therefore, further studies in different populations are essentials. Our study to find relationship between IL-17A, IL-17F polymorphisms and idiopathic recurrent miscarriage, showed that, in the RM group, the genotypes frequencies of rs2275913 polymorphism were GG (9.0 %), AG (38 %), and AA (53 %) and in the control group, were GG (1.9 %), AG (34.6 %) and AA (63.5 %).

The genotypes frequencies of rs763780 polymorphism were TT (45.4 %), TC (54.6%) and CC (0 %) in the RM group; whereas the frequencies were TT (36.5 %), TC (61.6%) and CC (1.9 %) in the control group.

Our results demonstrated that there was no significant difference between the genotypes in the two groups. Statistical analysis did not show any association between (IL-17A, nor IL-17F) polymorphisms and recurrent miscarriage, our results are consistent with a study by Bahadori et al. who did not find statistically significant association between IL-17 gene polymorphisms and the incidence of recurrent pregnancy loss (Bahadori et al., 2014).

In contrast to our findings, other reports have shown a significant association between IL-17 gene polymorphisms and risk of RM, a study conducted in Egypt found that IL-17 F (rs763780) polymorphism was associated with a decreased risk of RM in Egyptian females, but they also found that IL-17 A (rs2275913) SNP was associated with an increased risk of RM (Zidan et al., 2015). Another study indicated that IL-17F polymorphism,rs763780, might be associated with a high risk of RM in Iranian women, but there was not any association with IL-17A SNP (Najafi et al., 2014).

IL-10, produced by cytotrophoblasts and decidual T cells, protect the fetal-placental interface by reducing the cytokine secretions of Th1 cells and macrophages (Fan et al., 2011).

There are 3 famous IL-10 SNPs located in the promoter region (− 1082A > G (rs1800870), − 819T > C (rs1800871) and (− 592A > C (rs1800872)) which have been reported to regulate IL-10 transcription and expression (Kingo et al., 2005), thereby directly affecting IL-10 production levels (Alkhuriji et al., 2013). However the role of IL-10 in RM pathogenesis remains controversial (Bohiltea & Radoi, 2014b).

In this study, our results showed that the IL- 10-592 A/C polymorphism in the IL10 gene promoter is present in unexplained RM patients and controls, where most of the patients (45.5%) and controls (44.3%) are heterozygous for the genotype (AC). The homozygous mutant genotype state (CC) was reported in (41.8%) of patients and (44.2%) of the controls, while the normal genotype (AA) occurs in (12.7%) in patients and (11.5%) of controls. The genotype and allele frequencies in the above polymorphism showed no significant difference between the unexplained RM patients

and controls, thus cannot be considered as a clinically important polymorphism linked to unexplained RM.

This study was in disagreement with previous studies which reported a significant association between the presence of polymorphism and the occurrence of RM in Iranian and Tunisian women (Bahadori et al., 2014), (Kamali-Sarvestani et al., 2005), (Zammiti et al., 2006).

Statistical analysis of our results revealed that IL-10 -819 C/T polymorphism was associated with RM ($p<0.05$). In the RM group, the genotypes frequencies of (rs1800871) polymorphism were TT (14.5 %), CT (61.8 %), and CC (23.6 %) and in the control group, were TT (21.1 %), CT (78.9 %) and CC (0 %).

This finding is in agreement with previous reports which demonstrated an association for IL-10-819 C/T gene polymorphism in idiopathic RM (Su et al., 2016), (Bahadori et al., 2014), (Bohiltea & Radoi, 2014b).

Other reports revealed no association between RM patients and controls concerning the IL-10 gene (Prigoshin et al., 2004) and (Kaur & Kaur, 2011) which were in contrast with our findings.

These discrepancies may be explained by ethnic differences of the study groups as well as by number variations of the subjects that are included in the research.

Through interviews with the women who suffered from idiopathic recurrent pregnancy losses, some of them got a successful pregnant when they take progesterone medications.

Progesterone might play a significant role in establishing a convenient immune environment at early stages of pregnancy (B. C. Choi, Polgar, Xiao, & Hill, 2000). Therefore, using of progesterone in high-risk patients reduced the incidence of premature delivery and may help to establish a sufficient immune response in early pregnancy and prevent miscarriage (Kumar et al., 2014). The progesterone deficiency has been related with insufficient endometrial maturation and inappropriate regulation of inflammatory cytokines. There was a significant progressive increase in the level of IL-10 through first, second and third trimester in the progesterone treatment in women with unexplained recurrent miscarriage (Ismail et al., 2018).

No positive associations were found between risk of RM and smoking, negative smoking, and consumption of coffee. Our findings are in consistent with reports by (B. Y. Zhang et al., 2010), which did not find any relationship between smoking, alcohol

consumption, and coffee consumption and increased risk of RM. But this study found that the increased risk of RM was significant for participants with a BMI of 24.0 or greater and those with a family history of miscarriage, and that was contrary of our results.

Conclusion

Taking into account the results obtained in this study, as well as the results of previous studies we may conclude that there is not only one genetic factor, but possibly several that are involved in the abortion disease etiology, as idiopathic factors. If the relationship between genetic factors and the immune system disorders is cleared, genetic polymorphisms as the one that is studied may represent markers for selecting the therapeutic options and for counseling patients with recurrent spontaneous abortions. The recurrent spontaneous abortions etiology is a multi-factorial condition with both immune as well we non-immune causes. This study has proven a possible association of polymorphism IL-10 -819 C/T and the increased frequency of recurrent abortions and the lack of association between polymorphisms IL10-592C/A, IL6-174G/C, IL-17 and the abortion disease in the studied group.

Limitations

The size of sample may not be large enough which weakens the ability for solid statistical inference. Further studies with larger sample size from different districts are needed to identify the association between cytokines gene polymorphisms and recurrent miscarriage in the Palestinian females with RM.

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Appendix A

FASTA sequences from NCBI

Sequences of normal genotypes, and sequences of single-nucleotide polymorphism for our Interleukins. IL-10-592 A/C (rs1800872), IL-10-819 C/T (rs1800871), IL-17F (rs763780), IL-17A (rs2275913), and IL-6-174 G/C (rs1800795).

Sequence A1: sequence of normal genotype of IL-6-174G/C, digested by NlaIII.

TGACTTCAGCTTTACTCTTTGTCAAGACATGCCAAAGTGCTGAGTCACTAATAAAAGAAAAAAAGAAA
GTAAAGGAAGAGTGGTTCTGCTTCTTAGCGCTAGCCTCAATGACGACCTAAGCTGCACTTTTCCCCCTA
GTTGTGTCTTGC**C**ATGCTAAAGGACGTCACATTGCACAATCTTAATAAGGTTTCCAATCAG

Sequence A2: sequence of IL-6 -174G/C SNP rs1800795 (uncut)

TGACTTCAGCTTTACTCTTTGTCAAGACATGCCAAAGTGCTGAGTCACTAATAAAAGAAAAAAAGAAA
GTAAAGGAAGAGTGGTTCTGCTTCTTAGCGCTAGCCTCAATGACGACCTAAGCTGCACTTTTCCCCCTA
GTTGTGTCTTGC**G**ATGCTAAAGGACGTCACATTGCACAATCTTAATAAGGTTTCCAATCAG

Sequence A3: sequence of normal genotype of IL-17A.

TCTCCATCTCCATCACCTTTGTCCAGTCTCTATCCCCATTTTCAATTCCTTCCTCAAAACACCAAGTTGCT
TGGTAGCATGCAGGGTTGGAACATGCCTTTAACAGAAAATCTCGTGTCTCTTGAACCTAGTTATTTATTC
CTTGAGCAGAGTAGATATTCAACAAAAGAATTGTTAAATTCAATTAAATAGGATATATCTTATTATTAA
ATATTTTTTTCATTTTTTGTTTACTTATATGATGGGAACTTGAGTAGTTTCCGGAATTGTCTCCACAACAC
CTGGCCAAGGAATCTGTGAGGAAAAGAAAGATCAAATGGAAAATCAAGGTACATGACACCAGAAGAC
CTACATGTTACTTCAAACTTTTTCTTCCTCATGAACCATTAAAATAGAGCATAACTCTTCTGGCAGCTGT
ACATATGTTCATAAATACATGATATTGACCCATAGCATAGCAGCTCTGCTCAGCTTCTAACAAGTAAGA
ATGAAAAGAGGACATGGTCTTTAGGAACATGAATTTCTGCCCTTCCCATTTTCCTTCAGAAG**G**AGAGA
TTCTTCTATGACCTCATTGGGGGCGGAAATTTTAACCAAAATGGTGTCACCCCTGAACCCACTGCGACA
CGCCACGTAAGTGACCACAGAAGGAGAAAAGCCCTATAAAAAGAGAGACGATAGCGCTACATTTTGTC
CATCTCATAGCAGGCACAAACTCATCCATCCCCAGTTGATTGGAAGAAACAACGATGACTCCTGGGAA
GACCTCATTGGTGGTGAGTCCTGCACTAACGTGCGATGCTCTTGCTGATTTGGAC

Sequence A4: sequence of IL-17A SNP rs2275913.

TCTCCATCTCCATCACCTTTGTCCAGTCTCTATCCCCATTTTCAATTCCTTCCTCAAAACACCAAGTTGCT
TGGTAGCATGCAGGGTTGGAACATGCCTTTAACAGAAAATCTCGTGTCTCTTGAACCTAGTTATTTATTC
CTTGAGCAGAGTAGATATTCAACAAAAGAATTGTTAAATTCAATTAAATAGGATATATCTTATTATTAA
ATATTTTTTTCATTTTTTGTTTACTTATATGATGGGAACTTGAGTAGTTTCCGGAATTGTCTCCACAACAC
CTGGCCAAGGAATCTGTGAGGAAAAGAAAGATCAAATGGAAAATCAAGGTACATGACACCAGAAGAC
CTACATGTTACTTCAAACTTTTTCTTCCTCATGAACCATTAAAATAGAGCATAACTCTTCTGGCAGCTGT
ACATATGTTCATAAATACATGATATTGACCCATAGCATAGCAGCTCTGCTCAGCTTCTAACAAGTAAGA
ATGAAAAGAGGACATGGTCTTTAGGAACATGAATTTCTGCCCTTCCCATTTTCCTTCAGAAG**A**AGAGA
TTCTTCTATGACCTCATTGGGGGCGGAAATTTTAACCAAAATGGTGTCACCCCTGAACCCACTGCGACA
CGCCACGTAAGTGACCACAGAAGGAGAAAAGCCCTATAAAAAGAGAGACGATAGCGCTACATTTTGTC
CATCTCATAGCAGGCACAAACTCATCCATCCCCAGTTGATTGGAAGAAACAACGATGACTCCTGGGAA
GACCTCATTGGTGGTGAGTCCTGCACTAACGTGCGATGCTCTTGCTGATTTGGAC

Sequence A5: sequence of normal genotype of IL-17F.

CACTGGTGCTCTGATGAGGAAATATGAAAAACAAATGATAGGCATATAGAATTCCAAAATGTGTAGGG
CTACACAGTCTGTGAGTACAAGCTGGGAATGCAAACAAACACCTGAAGTATTTTTAAATTATTAATACT
TTATTATATTAGCACTGAATATATTAATTTTTCTCCTAACATTTTAGATATCAAATATAAAGTGTAGTAC
ATACACACATACATTGTGAATATTTTCTGTTTCCATCCGTGCAGGTCTTATTAAGAGTCCTGTGAAGTGG
AGGGAATTGGGGGTCAGACAGGACTTGTTGCAGAGCACTGGGTAAGGAGTGGCATTTCTACAGCTTCTT
CAGCTGAGTGGATATGCACCTCTTACTGCACA**T**GGTGGATGACAGGGGTGACGCAGGTGCAGCCAACA
GTCACCAGCACCTTCTCCAACTGGAAAGAAACAGAGCAGCCTTGGTGCTTCCTCCGGACGACCAGGGTC
TCTTGCTGGATGGGAACGGAATTCATGGAGATGTCTTCCTTTCCTTGAGCATTGATGCAGCCCAAGTTCC
TACACTGGGCCTGTACAACTTCCGAGGGGTACCGGTTGGGGTCCCAAGTGACACTGCAGGAGGAGCAA
GCCAAAGCACAATG

Sequence A6: sequence of IL-17F SNP rs763780

CACTGGTGCTCTGATGAGGAAATATGAAAAACAAATGATAGGCATATAGAATTCCAAAATGTGTAGGG
CTACACAGTCTGTGAGTACAAGCTGGGAATGCAAACAAACACCTGAAGTATTTTTAAATTATTAATACT
TTATTATATTAGCACTGAATATATTAATTTTTCTCCTAACATTTTAGATATCAAATATAAAGTGTAGTAC
ATACACACATACATTGTGAATATTTTCTGTTTCCATCCGTGCAGGTCTTATTAAGAGTCCTGTGAAGTGG
AGGGAATTGGGGGTCAGACAGGACTTGTTGCAGAGCACTGGGTAAGGAGTGGCATTTCTACAGCTTCTT
CAGCTGAGTGGATATGCACCTCTTACTGCACA**C**GGTGGATGACAGGGGTGACGCAGGTGCAGCCAAC
AGTCACCAGCACCTTCTCCAACTGGAAAGAAACAGAGCAGCCTTGGTGCTTCCTCCGGACGACCAGGG
TCTCTTGCTGGATGGGAACGGAATTCATGGAGATGTCTTCCTTTCCTTGAGCATTGATGCAGCCCAAGTT
CCTACACTGGGCCTGTACAACTTCCGAGGGGTACCGGTTGGGGTCCCAAGTGACACTGCAGGAGGAGC
AAGCCAAAGCACAATG

Sequence A7: sequence of normal genotype of IL-10-592 A/C digested by Rsa I

GGTGAGCACTACCTGACTAGCATATAAGAAGCTTTCAGCAAGTGCAGACTACTCTTACCCACTTCCCCC
AAGCACAGTTGGGGTGGGGGACAGCTGAAGAGGTGGAAACATGTGCCTGAGAATCCTAATGAAATCGG
GGTAAAGGAGCCTGGAACACATCCTGTGACCCCGCCTGT**A**CTGTAGGAAGCCAGTCTCTGGAAAGTAA
AATGGAAGGGCTGCTTGGGAACTTTGAGGATATTTAGCCCACCCCCTCATTTTTACTTGGGGAAACTAA
GGCCCAGAGACCTAAGGTGACTGCCTAAGTTAGCAAGGAGAAGTCTTGGGTATTCATCCCAGGTTGGG
GGGACCCAATTATTTCTCAATCCCATTGTATTCTGGAATGGGCAATTTGTCCACGTCACTGTGACCTAGG

Sequence A8: sequence of IL-10-592 A/C rs1800872 (uncut)

GGTGAGCACTACCTGACTAGCATATAAGAAGCTTTCAGCAAGTGCAGACTACTCTTACCCACTTCCCCC
AAGCACAGTTGGGGTGGGGGACAGCTGAAGAGGTGGAAACATGTGCCTGAGAATCCTAATGAAATCGG
GGTAAAGGAGCCTGGAACACATCCTGTGACCCCGCCTGT**C**CTGTAGGAAGCCAGTCTCTGGAAAGTAA
AATGGAAGGGCTGCTTGGGAACTTTGAGGATATTTAGCCCACCCCCTCATTTTTACTTGGGGAAACTAA
GGCCCAGAGACCTAAGGTGACTGCCTAAGTTAGCAAGGAGAAGTCTTGGGTATTCATCCCAGGTTGGG
GGGACCCAATTATTTCTCAATCCCATTGTATTCTGGAATGGGCAATTTGTCCACGTCACTGTGACCTAGG

Sequence A9: sequence of normal genotype of IL-10-819 C/T, (uncut)

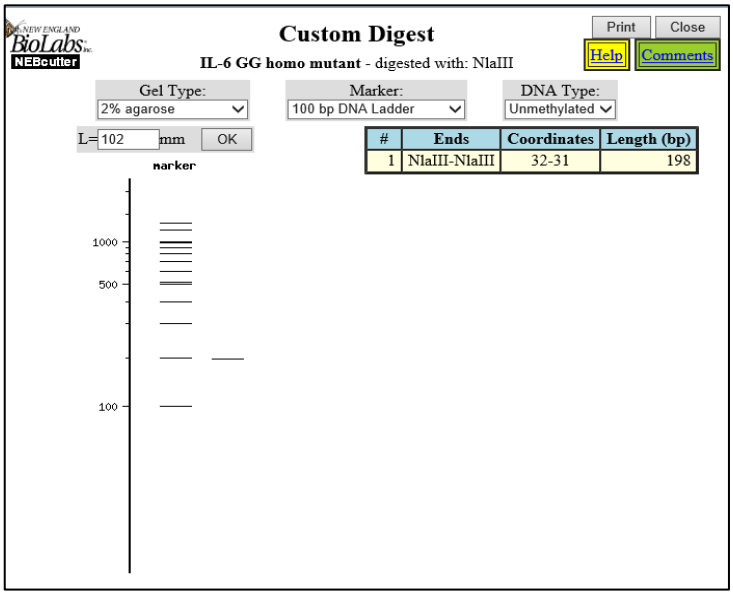
TCATTCTATGTGCTGGAGATGGTGTACAGTAGGGTGAGGAAACCAAATTCTCAGTTGGCACTGGTGTAC
CCTTGTACAGGTGATGTAA**T**ATCTCTGTGCCTCAGTTTGCTCACTATAAAATAGAGACGGTAGGGGTCA
TGGTGAGCACTACCTGACTAGCATATAAGAAGCTTTCAGCAAGTGCAGACTACTCTTACCCACTTCCC
CCA

Sequence A10: sequence of IL-10-819 C/T rs1800871, digested by Mea III.

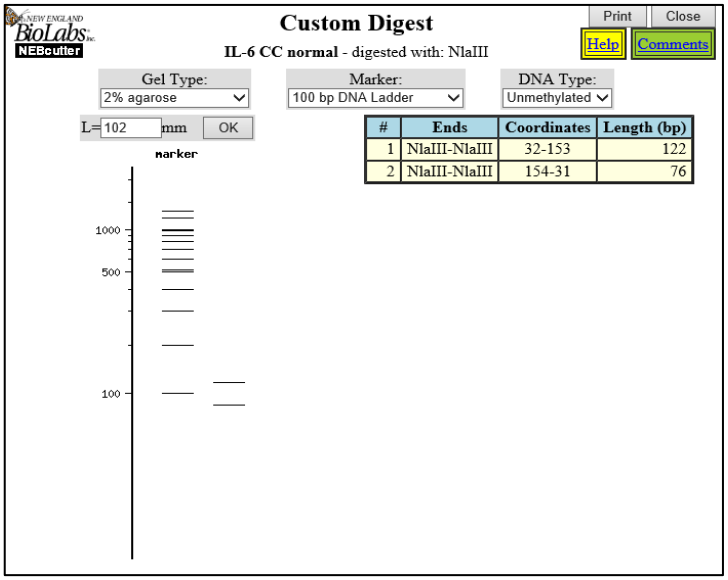
TCATTCTATGTGCTGGAGATGGTGTACAGTAGGGTGAGGAAACCAAATTCTCAGTTGGCACTGGTGTAC
CCTTGTACAGGTGATGTAA**C**ATCTCTGTGCCTCAGTTTGCTCACTATAAAATAGAGACGGTAGGGGTC
ATGGTGAGCACTACCTGACTAGCATATAAGAAGCTTTCAGCAAGTGCAGACTACTCTTACCCACTTCCC
CCA

Appendix B

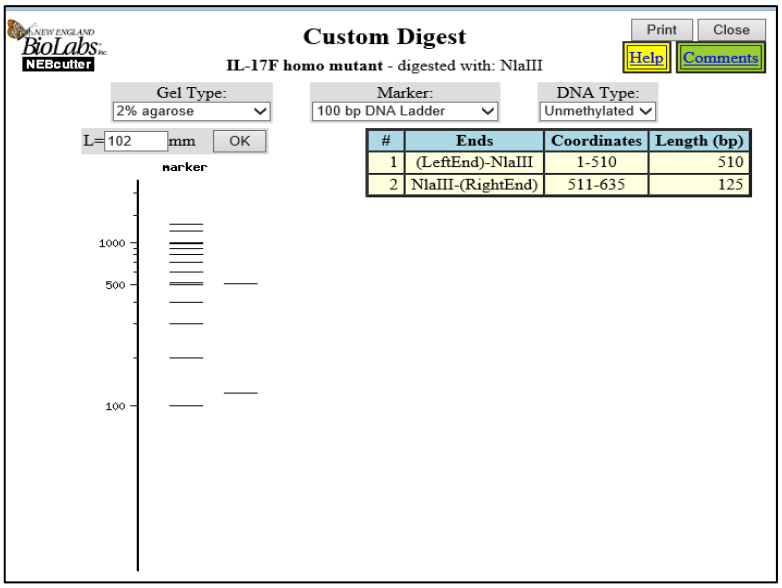
Virtual cuts of Interleukins genes sequences by different enzymes determined by NEBcutter software (http://nc2.neb.com/NEBcutter2/).



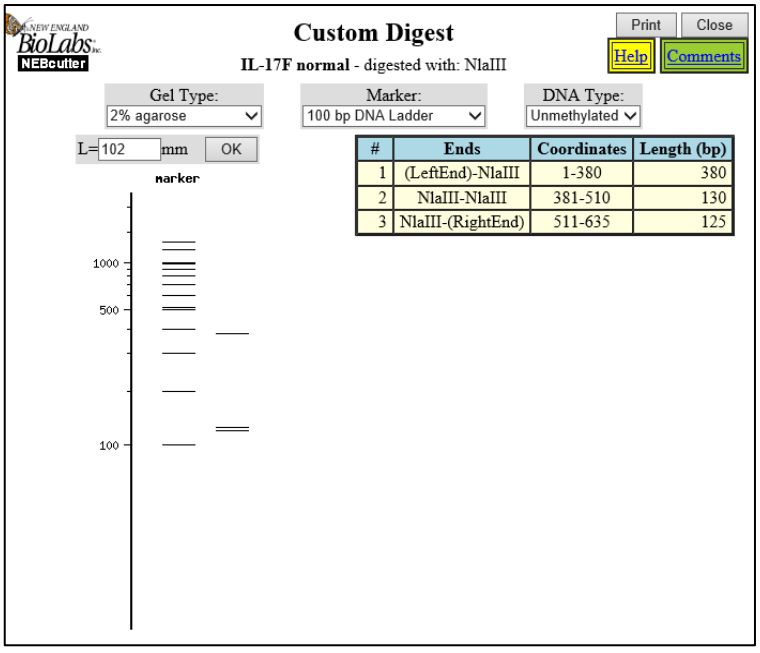
B1: IL-6 (GG) uncutt



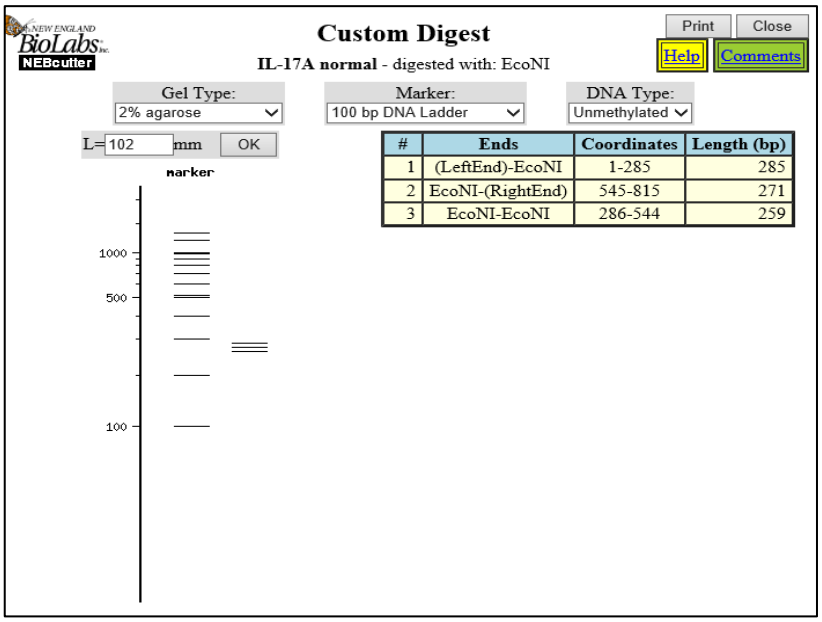
B2: IL-6 (CC)



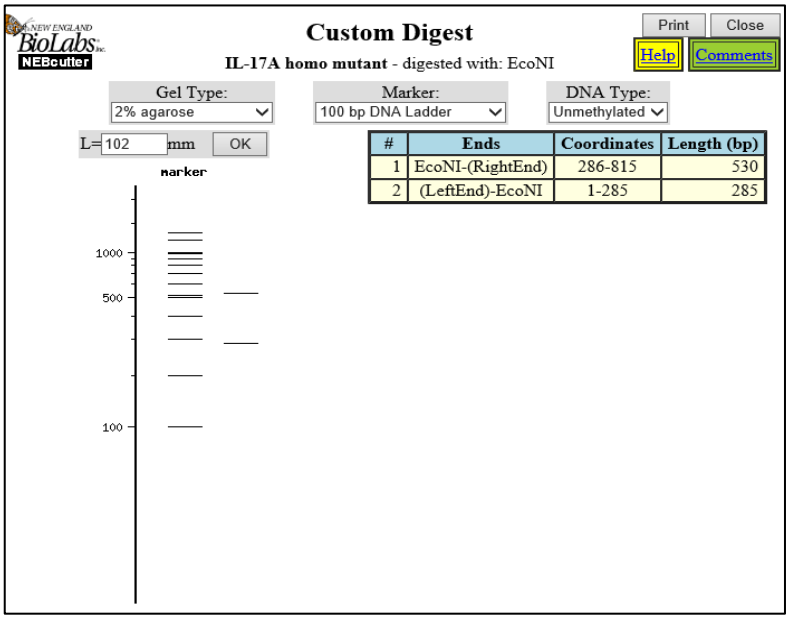
B3: IL-17F (CC)



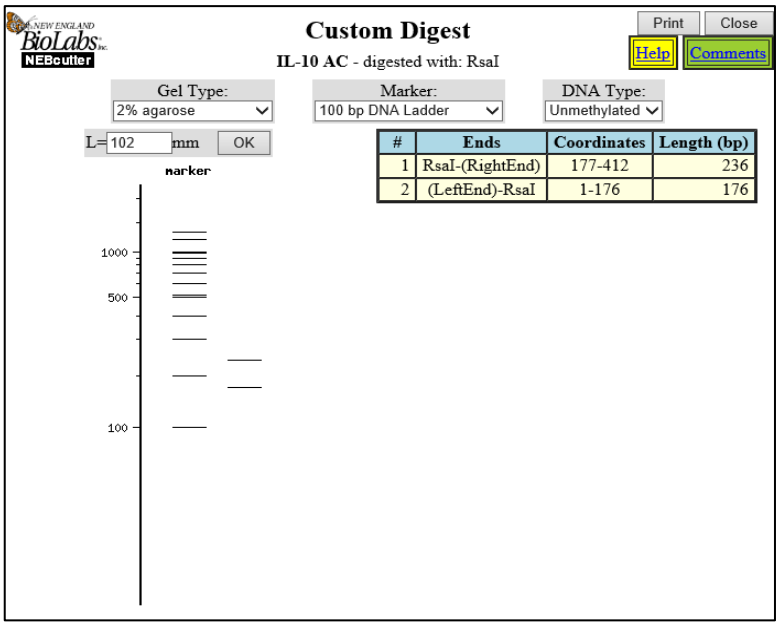
B4: IL-17F (TT)



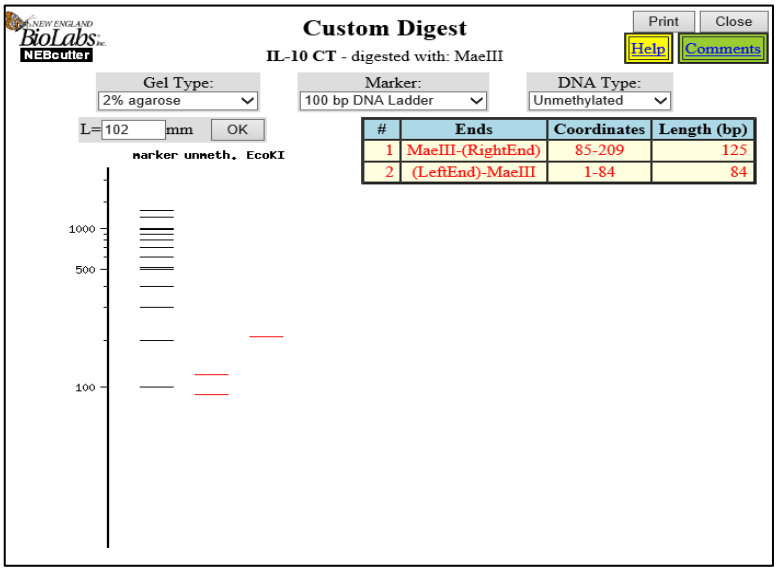
B5: IL-17A (GG)



B6: IL-17A (AA)



B7: IL-10 (AA) normal



B8: IL-10 (C/T)

Appendix C

Study Questionnaire/ English

Risk factors of miscarriage: a case-control study

Name: (Last, First, M.I.):		**Date of Birth:** (month/day/year)		**Today's Date:** (month/day/year)	
Address:		**Telephone:**			
Interviewee code: Status:		1.Case		0.Control	

1. At what age your menses began? _____

2. Are your menstrual cycle spaced irregularly? _____
□ Yes □ No

3. How many live births did you have?_____

4. How many miscarriages did you have?_____

5. Please mention the gestational age/ages when you had miscarriage. _____

6. How many stillbirths did you have? _____

7. Please mention the gestational age/ages when you had stillbirths. _____

8. Have you had any D&C and when? _____
□ Yes □ No

9. How many total pregnancies (including live births, abortions, miscarriages, stillbirths) did you ever have? _____

10. Have you ever smoked cigarettes? (At least one cigarette)? _____
□ Yes □ No

11. How often did you smoke before your last pregnancy?
□1. Never
□2.Once a month or less
□3. Several days a month
□4. Several days a week
□5. Every day

12. How often did you smoke during your last pregnancy?
□1. Never
□2.Once a month or less
□3. Several days a month
□4. Several days a week
□5. Every day

13. How many of your household members smoked during your last pregnancy?_____

14. How often people smoked in the same room where you were during your last pregnancy?
□1. Every day
□2. Several days a week
□3. Once a week
□4. Several days a month
□5. Once a month or less
□6. Never

15. Did you ever drink coffee?
□ Yes □ No

16. How many cups of coffee per day did you drink before your last pregnancy?
□1.None
□2.Less than 1 cup per day
□3. 1-2 cups
□4. 3-4 cups
□5. More than 4

17. How many cups of coffee per day did you drink during your last pregnancy?
□1.None
□2. Less than 1 cup per day
□3. 1-2 cups
□4. 3-4 cups
□5. More than 4

18. Have you ever been diagnosed with Chlamydia infection? _____
□ Yes □ No

19. Have you ever had pelvic inflammatory disease? _____
□ Yes □ No

20. Have you ever been diagnosed with uterine fibroids or polyps? _____
□ Yes □ No

21. Have you ever been diagnosed with endometriosis? _____
□ Yes □ No

22. Hormone laboratory tests and ultra sound scan results? _____
□ Normal □ Abnormal

23. Your partner's semen tests result? _____
□ Normal □ Abnormal
24. Have you taken contraceptive pills? For how long? _____
□ Yes □ No

25. Are you complaining of any medical condition? _____

□ Yes □ No

(Diabetes, High Blood Pressure, Bleeding or blood clotting disorder, Blood transfusion..etc)

26. Are you presently taking any medication? _____

□ Yes □ No

27. Has anybody in your family had any medical condition? _____

□ Yes □ No

28. Have you been screened for Antiphospholipids, Anticardiolipin, and Rubella titer?

□ Yes □ No (**if yes**: □ Normal □ Abnormal)

29. On average how many hours did you sleep before your last pregnancy during 24 hours?

□1. ≤ 8 hours □2. ≥8 hours

30. On average how many hours did you sleep during your last pregnancy during 24 hours?

□1. ≤ 8 hours □2. ≥8 hours

31. Did you have traumas/accidents during your last pregnancy?

□ Yes □ No

32. What was your weight before last pregnancy? _____ (kg)

□. Don't know □. Refused to answer

33. What is your height?_____ (cm)

□. Don't know □. Refused to answer

34. Education:

□1.School (less than 10 years)
□2. School (10-12 years)
□3.Institute/University
□4.Postgraduate

35. Are you employed?

□ Yes □ No

THANK YOU
for your participation!!!

Study Questionnaire/ Arabic

تحية طيبة وبعد,,,

في البداية نود أن نشكركم على الوقت والجهد الذي سوف تبذلونه في ملئ هذا الاستبيان. كما أن اهتمامكم وحرصكم على تعبئة هذا الاستبيان بموضوعية وشفافية هو محل شكرنا وتقديرنا, فنتائج هذا الاستبيان ستساعد الباحثين على استكمال بحثهم وحل مشكلة البحث الذي سيدور حول "بعض عوامل خطر الاجهاض "

ملاحظة: المعلومات التي سيتم جمعها, والإطلاع عليها لأغراض البحث العلمي فقط. ولن يتم إساءة استخدامها وسيتم حفظها جيداً وهي معلومات سرية لن يتم الافصاح عن اسم المريضه او اي معلومات تخص المشاركين في البحث ..

نرجو منكم الإجابة على الأسئلة التالية, وشاكرين لكم تعاونكم.

استبيان :من عوامل خطر الاجهاض

الاسم الثلاثي :_ _ _ _ _ _ _ _ _ _ _ _ _ _ **تاريخ الميلاد:** _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _

(اليوم، الشهر، السنه)

تاريخ اليوم: _ _ _ _ _ _ _ _ _ _ _ _ _ _ **رقم التلفون:**_ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _

العنوان:_ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ **وضع الحالة :**_ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _

1- في اي سن بدات عندك الدورة الشهرية ؟ _ _ _ _ _ _ _ _ _ _ _ _ _

2- هل الدورة الشهرية غير منتظمه او متباعدة؟ _ _ _ _ _ _ _ _ _ _ _ _ _ _

☐ نعم ☐ لا

3- كم عدد المواليد لديك؟ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _

4- كم عدد حالات الجهاض لديك؟ _ _ _ _ _ _ _ _ _ _ _ _ _ _

5- يرجى ذكر سن الحمل في كل مرة حصل فيها الإجهاض ؟ _ _ _ _ _ _ _ _ _ _ _ _ _ _

6- كم عدد حالات موت المواليد داخل الرحم لديك؟ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _

7- يرجى ذكر سن الحمل في كل مرة عندما كان لديك موت الجنين داخل الرحم ؟_ _ _ _ _ _ _ _

8- هل كان لديك اي عملية جراحية في الرحم, متى كان ذلك ؟ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _

☐ نعم ☐ لا

9- كم عدد مجموع حالات الحمل (بما في ذلك ولادة حية، الإجهاض، موت الجنين داخل الرحم) لديك ؟ _ _ _ _ _ _ _ _ _ _ _ _ _ _

10-هل سبق لك أن دخنت السجائر؟ (سيجارة واحدة على الأقل) ؟ _ _ _ _ _ _ _ _ _ _

☐ نعم ☐ لا

11- كم عدد المرات التي دخنتي بها قبل حملك الاخير؟

☐ ابدا ☐ مرة واحده في الشهر او اقل

☐ عدة مرات في الشهر ☐ عدة مرات في الاسبوع

☐ كل يوم

12- كم عدد مرات التدخين لديك اثناء حملك الاخير؟

☐ ابدا ☐ مرة واحده في الشهر او اقل

☐ عدة مرات في الشهر ☐ عدة مرات في الاسبوع

□ كل يوم

13- كم عدد أفراد عائلتك الذين يدخنون خلال فترة الحمل الأخير ؟ _ _ _ _ _ _ _ _ _ _ _ _ _

14- عدد المرات التي يدخن بها اشخاص في نفس الغرفة التي تكونين بها خلال فترة حملك الاخير ؟

□ ابدا
□ مرة واحده في الشهر او اقل
□ عدة مرات في الشهر
□ عدة مرات في الاسبوع
□ كل يوم

15-هل تتناولين الفهوة ؟

□ نعم
□ لا

16- كم عدد فناجين القهوة التي كنت تشربينها في اليوم الواحد قبل حملك الاخير؟

□ صفر
□ اقل من فنجان في اليوم الواحد
□ 2-1 فناجين
□ 4-3 فناجين
□ اكثر من 4

17- كم عدد فناجين القهوة التي كنت تشربينها في اليوم الواحد خلال فترة حملك الاخير ؟

□ صفر
□ اقل من فنجان في اليوم الواحد
□ 2-1 فناجين
□ 4-3 فناجين
□ اكثر من 4

18- هل سبق أن تم تشخيصك بعدوى الكلاميديا؟ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _

□ نعم
□ لا

19- هل سبق و كنت تعانين من التهاب في الحوض؟ _ _ _ _ _ _ _ _ _ _ _ _ _ _

□ نعم
□ لا

20-هل سبق لك أن تم تشخيصك بالأورام الليفية الرحمية أو الاورام الحميدة ؟ _ _ _ _ _ _ _ _ _

□ نعم
□ لا

21-هل سبق لك أن تم تشخيصك بالتهاب بطانة الرحم ؟ _ _ _ _ _ _ _ _ _ _ _ _ _

□ نعم
□ لا

22- نتائج الفحوصات المخبرية للهرمونات و مسح الصوت (الالتراساوند)؟ _ _ _ _ _ _ _ _ _

☐ طبيعية ☐ غير طبيعية

23- نتيجة الفحص المخبري للسائل المنوي للزوج ؟ _ _ _ _ _ _ _ _ _ _ _ _ _

☐ طبيعي ☐ غير طبيعي

24- هل استعملت حبوب منع الحمل ؟الى متى ؟ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _

☐ نعم ☐ لا

25- هل تشكتكين من اي حالة مرضية ؟ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _

(مرض السكري، وارتفاع ضغط الدم، نزيف أو اضطراب في الدم, تخثر الدم, نقل دم, الخ)

☐ نعم ☐ لا

26-هل تاخذين اي نوع من انواع الادوية في الفترة الحالية ؟ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _

☐ نعم ☐ لا

27-هل يعاني اي من افراد عائلتك من حالة مرضية ؟_ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _

☐ نعم ☐ لا

28- هل سبق و خضعتي لفحص الحصبة الالمانية؟ antiphospholoipids, anticardiolipin

☐ نعم ☐ لا

☒ اذا كانت الاجابة نعم:

☐ طبيعي ☐ غير طبيعي

29- في المتوسط كم عدد ساعات نومك خلال ال 24 ساعه قبل بداية حملك الاخير ؟

☐ اقل من 8 ساعات ☐ اكثر من 8 ساعات

30- في المتوسط كم عدد ساعات النوم خلال الحمل الاخير في ال 24 ساعة ؟

☐ اقل من 8 ساعات ☐ اكثر من 8 ساعات

31. هل تعرضت لصدمات / حوادث أثناء الحمل الأخير?

☐ نعم ☐ لا

32- كم كان وزنك قبل الحمل الأخير (كغم) ؟ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _

☐ لا اعرف ☐ رفض الاجابة

33- ما هو طولك (سم) ؟_ _ _ _ _ _ _ _ _ _

□ لا اعرف □ رفض الاجابة

34- ما المستوى التعليمي :

□ مدرسة (اقل من 10 سنوات) □ مدرسة (10-12 سنه)

□ جامعة\معهد □ دراسات عليا

35-هل انت موظفة ؟

□ نعم □ لا

شكرا لمشاركتكم

Consent form:

Here I certify that I have voluntarily participated in this study by filling up this questionnaire and donating a blood sample and authorize the researcher to use the above data and blood sample for scientific research only.

Signature: Date:

العلاقه بين تعدد الاشكال الجيني للانترلوكينات (Interleukins) و الاجهاض المتكرر غير المبرر بين نساء الضفه الغربيه.

اعداد: نيفين نظام عرفات صب لبن

اشراف: د. رسمي ابو حلو

الملخص:

مقدمه: الإجهاض المتكرر (RM) هو فقدان الحمل مرتين أو أكثر قبل الأسبوع العشرين من الحمل، وهو أحد أكثر مضاعفات الحمل شيوعًا والتي تصيب ما يصل إلى 2-4٪ من النساء في سن الإنجاب. تم الكشف عن العديد من عوامل مساهمه في خطر الاصابه بالاجهاض المتكرر ، مثل العيوب الوراثية والجهاز المناعي والغدد الصماء والتشريحية . إن المسببات المرضية في حوالي 50٪ من حالات الاجهاض المتكرر مجهولة الاسباب ، ولكن تم الافتراض بأن نسبة من هذه الخسائر المتكررة للحمل قد تكون ناجمة عن أسباب مناعية.

أثناء الحمل الطبيعي ، هناك تفاعل تعاوني بين نظام المناعة الأمومي ومستضد الجنين ، ويؤدي فشل هذا النظام المناعي إلى رفض الجسم للجنين مما يؤدي إلى فقدان الحمل.

بعد مواجهة المستضد ، تتمايز خلايا تي المساعدة (Th) إلى Th1 و Th2 ، مع أنماط فريدة من إنتاج السيتوكينات. تخضع مستويات إنتاج السيتوكينات جزئيًا لسيطرة جينية ، ويمكن تغيير تعبير الجينات باختلاف النوكليوتيد. وقد أظهرت العديد من الدراسات أن السيتوكينات تلعب دوراً رئيسياً في الظواهر التناسلية ، حيث ارتبطت الاستجابة السائدة لل Th2 مع الحمل الطبيعي ، وكانت استجابة Th1 مرتبطة بفشل الحمل.

وقد ارتبط تعبير السيتوكينات المؤيدة للالتهابات في الرحم مثل TNF,IL-17 و IL-6 بفقدان الأجنة. أما السيتوكينات المضادة للالتهاب مثل IL-10 تحمي من الإجهاض الناجم عن الالتهاب.

الاهداف: البحث في علاقة تعدد الأشكال الجيني ل IL-10 المضادة للالتهابات (-592 C / A ، 819- C / T) ، و ثنان من الجينات السيتوكينية IL-17A و IL-17F و (174 G / C) IL-6 المواليه للالتهابات عند النساء الفلسطينيات اللواتي يعانين من الاجهاض المتكرر مقارنة بالنساء الاصحاء

الطرق والمواد: شملت هذه الدراسة 107 امرأه من مناطق مختلفه من الضفة الغربيه, 55 امرأه يعانين من الاجهاضات المتكرره بدون سبب محدد، و 52 امرأه لا يعانين من اي مشاكل متعلقه بالحمل. حيث تم استخلاص الحمض النووي(DNA) من عينات دم النساء المتطوعات, ثم تصنيف هذه العينات بتحليل جينات الدراسه (5 جينات), باستخدام تفاعل سلسلة البوليمرية (PCR) و(RFLP)، باستخدام انزيمات القطع المتخصصه لكل جين منهم.

النتائج: أثبتت هذه الدراسه وجود ارتباط عال لتعدد الأشكال في الجين IL10- C / T 819وزيادة تواتر الإجهاض المتكرر بين النساء الفلسطينيات وعدم وجود ارتباط بين تعدد الأشكال في الجينات IL10-592C / A و IL6-174G/C و IL-17 ومرض الإجهاض في مجموعة الدراسة.

الاستنتاجات: لا يوجد عامل جيني واحد فقط، وانما العديد من العوامل المرضية المسببة للإجهاض المتكرر. هناك حوالي 50% منها مجهولة السبب. إذا تم توضيح العلاقة بين العوامل الوراثية واضطرابات الجهاز المناعي، فإن الأشكال المتعددة للجينات الوراثية للسايتوكاينات قد تمثل علامات لاختيار الخيارات العلاجية ولتقديم المشورة للمرضى الذين يعانون من الإجهاض التلقائي المتكرر.

هناك اختلافات كبيرة في نتائج الدراسات السابقة التي أجريت في مناطق مختلفه من العالم حول هذا الموضوع, يمكن تفسير ذلك بسبب الاختلافات العرقية لمجموعات الدراسة, بالاضافة الى عدد المتغيرات المختلفة التي يتضمنها كل بحث.

بحدود معرفتي هذه الدراسة هي اول دراسة في الضفة الغربية تبحث في العلاقة بين مرض الاجهاض المتكرر التلقائي وتعدد الاشكال الجيني للانترلوكينات المذكورة، و يمكن اعتبارها داعمة لدراسات سابقة في نفس المجال, و مع ذلك تبقى الالية الحقيقيه لهذه العلاقة غير معروفة وتحتاج الى المزيد من البحث.