Arab American University
Faculty of Graduate Studies

Genetic Association between Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) and IL-6 Polymorphisms and Recurrent Abortion among Palestinian Women

By
Sama Khalid Omar Darwish

Supervisor

Dr. Hisham Mohammed Ahmed Darwish

This thesis was submitted in partial fulfilment of the requirements for the Master's degree in

Molecular Genetics and Genetic Toxicology

June/ 2025

Thesis Approval

Genetic Association between Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) and IL-6 Polymorphisms and Recurrent Abortion among Palestinian Women

By
Sama Khalid Omar Darwish

This thesis was defended successfully on June 19th 2025 and approved by:

Committee members	Signature
1. Professor Hisham M. Darwish: Supervisor	
2. Dr. Mohannad Khader: Internal Examiner	
3. Dr. Mahmoud A. Srour: External Examiner	

Declaration

I Sama Darwish hereby declare that my MS thesis titled “Genetic Association between Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) and IL-6 Polymorphisms and Recurrent Abortion among Palestinian Women” is the result of my own research and was written independently with no other sources than those referenced.

Name: Sama Darwish
ID: 202216574

Signature:

Date: 6.8.2025

Dedication

This thesis is dedicated to my dear family and to every woman who has faced the heartbreak of pregnancy loss — may research continue to shed light and bring hope.

Acknowledgment

First and foremost, I would like to express my sincere gratitude to my supervisor, Dr. Hisham Darwish for his invaluable guidance, patience, and continuous support throughout the course of this research and whose insights and expertise have been instrumental in shaping this thesis.

I would like to extend my deepest gratitude to Dr. Randa Al Asie and Dr. Ayman Najjar as well as all the lab technicians at Horus Medical Labs. Your cooperation and contributions were essential and invaluable.

I am deeply thankful to all my colleagues and the lab technicians at AAUP for their continuous support and assistance throughout the duration of the study.

Abstract

Recurrent Pregnancy Loss (RPL) has been defined as a complicated pregnancy disorder involving repeated failure or loss of pregnancy. About 3% of women experience RPL. The prevalence of RPL in Palestine is yet to be determined. This study investigates the association of CTLA-4 (rs231775) and IL-6 (rs1800796) single nucleotide polymorphisms (SNPs) with RPL in Palestine. Restriction fragment length polymorphism (RFLP) polymerase chain reaction (PCR) was used to analyze the indicated polymorphisms in a total of 172 subjects; 88 cases and 84 healthy controls. forty-four patients experienced RPL in the first trimester of pregnancy and forty-four experienced this condition in the second trimester. Genotypic and allelic analysis reveal no significant association between the CTLA-4 rs231775 SNP and RPL. On the other hand, IL-6 rs1800796 polymorphism showed a significant association with RPL. The data revealed the C allele of this variant to have a protective effect against RPL ($p = 0.005$). Trimester-specific analysis indicated the strongest association between the indicated IL-6 polymorphism and first-trimester RPL cases. Haplotype analysis further supported the protective role of the C allele. These findings aligned with those of previous studies regarding the emphasis on the role of IL-6 in immune regulation during pregnancy. However, further functional studies are required to confirm the effects of IL-6 (rs1800796) SNP during pregnancy as well as to understand the biological and molecular mechanisms underlying the association. This study underscores the significance of genetic screening in identifying RPL susceptibility and exploring potential therapeutic strategies.

Keywords:

Recurrent pregnancy loss (RPL), CTLA-4 +49 A/G polymorphism, IL-6 -634 C/G polymorphism, Single Nucleotide Polymorphism (SNP), Restriction fragment length polymorphism (RFLP), Palestine

Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

Abbreviation	Term
APL	Antiphospholipid Syndrome
APS	Antiphospholipid syndrome
ART	Assisted Reproductive Technology
ASRM	American Society for Reproductive Medicine
CI	Confidence Interval
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
CyTOF	Cytometry by Time-Of-Flight
dNK	Decidual natural killer
ESHRE	European Society of Human Reproduction and Embryology
GWAS	Genome-Wide Association Study
HWE	Hardy-Weinberg Equilibrium
IFN-γ	Interferon Gamma
IL-6	Interleukin-6
IVF	In Vitro Fertilization
NK Cells	Natural Killer Cells
OR	Odds Ratio
PCR-RFLP	Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

RIF	Recurrent implantation failure
RPL	Recurrent pregnancy loss
SNP	Single Nucleotide Polymorphism
TGF-β	Transforming Growth Factor Beta
Th1	T Helper 1 Cells
Th2	T Helper 2 Cells
Tregs	Regulatory T Cells

Chapter 1
Introduction

1.1 Recurrent pregnancy loss (RPL)

Recurrent pregnancy loss **(**RPL) is a complicated pregnancy disorder which is experienced by approximately 2.5% of women attempting to conceive (Dimitriadis et al., 2020). This mode of pregnancy loss involves miscarriage before reaching 20 weeks (about 4 and a half months) of gestation (Moghbeli, 2019). The repeated failure or loss of pregnancy has been described to be distinct from other forms of infertility due to its spontaneous nature ("Definitions of Infertility and Recurrent Pregnancy Loss: A Committee Opinion," 2020).

According to guidelines from the European Society of Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM), RPL has been defined as the loss or failure of two or more clinically recognized pregnancies ("Endometriosis. Guideline of European Society of Human Reproduction and Embryology – 2022," 2022), (American Society for Reproductive Medecine, 2012). The Royal College of Obstetricians and Gynecologists, on the other hand, defines RPL as the loss of three or more consecutive pregnancies (Gynaecologists, 2011).

The reported incidence of recurrent pregnancy loss (RPL) varies significantly due to differences in definitions, criteria, and population characteristics. Primary RPL involves multiple pregnancy losses in a woman with no previous viable infants, while secondary RPL refers to multiple losses in a woman who has had at least one pregnancy that progressed beyond 20 weeks of gestation. Tertiary RPL is defined as multiple pregnancy losses occurring between successful pregnancies (El Hachem et al., 2017).

1.2 Epidemiology

The prevalence of women who have experienced two miscarriages is 1.9%; those who have experienced 3 or more making up 0.7% of the global population (Quenby et

al., 2021). Studies conducted on the Palestinian population have estimated the prevalence of recurrent miscarriages at 4-8% among women who attend antenatal care clinics, which is relatively high compared to the general population (Hussein et al., 2010). Such a high frequency is concerning as the social and psychological impact of early pregnancy failure can be very taxing and devastating for the parents (Jauniaux & Burton, 2005).

Early pregnancy loss is described as failed pregnancy which takes place earlier than the 10-week gestational mark. This form of pregnancy loss includes peri-implantation loss, ectopic pregnancy, pre-embryonic loss and embryonic loss (Moghbeli, 2019). Multiple factors including maternal immune system, and the genetics of the embryo have been reported to cause recurrent implantation failure (RIF), however, the mechanism by which this occurs is unknown (Kwon et al., 2023). Evidently, the most common cause of early spontaneous miscarriage involves genetic factors, making up about 50-60% of cases (Garrido-Gimenez & Alijotas-Reig, 2015), while other established risk factors in RPL include anatomical, endocrine and hemostatic alterations. The idiopathic nature of about 50% of cases has also led to the shifting of focus towards immunological risk factors (Vomstein et al., 2021).

1.3 Etiology

The etiology of RPL has been broadly classified into several categories including genetic, anatomic, endocrine, antiphospholipid antibody syndrome, immunological and environmental factors (Pillarisetty & Mahdy, 2024). Genetic causes account for 2-5% of cases, anatomic abnormalities are responsible for 10-15%, autoimmune disorders contribute to 20%, infections are implicated in 0.5-5%, and a significant proportion including, 40-50% of cases remains unexplained. The unexplained category includes non-APS (antiphospholipid syndrome) thrombophilias, among other factors (Ford & Schust, 2009).

1.4 Genetics

The genetic factors of RPL are often linked to chromosomal abnormalities, with fetal aneuploidy being a major cause. The frequency and types of chromosomal defects vary with gestational and maternal age. Couples with RPL may have higher rates of chromosomal abnormalities and a genetic predisposition. While higher aneuploidy rates are found in embryos from women with RPL, miscarriage tissues from these women show fewer chromosomal abnormalities compared to sporadic losses, suggesting non-cytogenetic factors may also play a role (Hyde & Schust, 2015).

1.4.1 Genetic polymorphisms indicated in RPL

Over the past two decades, various genetic polymorphisms have been investigated for their potential role in RPL. Among the most studied are genes involved in thrombophilia, folate metabolism and immune regulation. Factor V Leiden (FVL) and prothrombin (F2) G20210A mutations for example have been associated with increased thrombosis risk which potentially leads to placental insufficiency and fetal loss (Lund et al., 2010). Methylenetetrahydrofolate reductase (MTHFR) polymorphisms, p articularly C677T and A1298C, influence homocysteine levels and folate metabolism, potentially impairing endometrial receptivity and embryonic development (Nelen et al., 2000). Vitamin D receptor gene variants such as FokI, BsmI and TaqI have been linked to immunological imbalances and endometrial dysfunction in women with RPL (Moradkhani et al., 2024).

In a study by (Maysa, 2024), significant associations were observed in the VDR rs1544410 variant, where the G allele and GG genotype were more frequent in RPL cases and a dominant genetic model in this study also showed a significantly increased risk of RPL (p=0.006). This study by (Maysa, 2024) also reported a familial mutation in HLA-G (495delC), causing a frameshift and premature stop codon (Leu154fs60). It was found to be associated with impaired fetal-maternal immune interaction. This indicated

the HLA-G gene to be a central player in fetal immune tolerance and also emphasizes the role of immunogenetics in this field.

Cytokine-related genes such as TNF-α -308G/A, IL-10 -1082G/A and IL-1β +3954C/T, may alter cytokine expression profiles which could contribute to implantation failure and early pregnancy loss due to immune intolerance (Parveen et al., 2013). While it is true that individual studies might report varying outcomes as a result of population specific genetic backgrounds, the aforementioned findings show that the pathophysiology of RPL can be of multifactorial genetic predisposition.

1.5 Immune system

Pregnancy represents a unique immunological challenge, requiring maternal immune system to balance tolerance toward the semi-allogeneic fetus while maintaining effective immune surveillance. Key players in this immune orchestration include decidual natural killer (dNK) cells, decidual macrophages, and regulatory T cells (Tregs). These immune cells facilitate the critical processes of decidualization and placentation, promote fetal tolerance, and protect against infections (Ander et al., 2019). However, disruptions in these immune mechanisms can lead to adverse pregnancy outcomes, including RPL. Several studies have underscored the vital functions and overall involvement of the immune system during pregnancy. Where immune regulators, the presence of antiphospholipid antibodies (APL) has been shown to influence and interfere with normal pregnancy processes (Kirovakov et al., 2024).

Tolerance and immunity play very important roles in determining the fate of each pregnancy by preventing the fetus from being attacked by the maternal immune system (Nasiri & Rasti, 2016; Zenclussen, 2006). Emerging evidence shows that the innate and adaptive immune system cells play crucial roles in maternal tolerance towards the embryo and endometrial remodeling(Ticconi et al., 2019). Innate lymphoid cells (ILCs), along with myeloid cells, T cells, and B cells, play crucial roles in maintaining immune

tolerance during pregnancy. Among these, ILCs, which include both NK cells and non-NK ILCs, are the most prevalent immune cells in the pregnant uterus (Wu et al., 2021).

In the study by (Wu et al., 2021), CyTOF (Cytometry by Time-Of-Flight) was employed to examine immune cells in the decidua of RPL patients, uncovering differences related to embryonic chromosomal status. Key findings included an increase in pro-inflammatory CD11c high macrophages and a decrease in CD39high NK cells. Notably, RPL with normal chromosomes exhibited elevated levels of pro-inflammatory CD11c high NK and CD161highCD8+ T cells, while RPL with chromosomal abnormalities showed an enrichment of inactivated and naive CD8+/CD4+ T cells. These findings suggest that immune cell dysregulation, including regulatory T cells, may disrupt maternal tolerance and contribute to RPL, highlighting potential targets for therapeutic intervention to improve pregnancy outcomes.

1.5.1 Fetal T cell immunity

Fetal T cell development starts around week 8 of gestation, with γδ and αβ T cells present by 12–14 weeks. Tregs are more prevalent in the second trimester and are sensitive to TGFβ. Th1 cells, associated with inflammation, can be enriched in preterm infants, while the role of Th17 cells is less understood but shows increased differentiation capacity at term. Innate-like T cells, such as γδ T cells and iNKT cells, are present in the fetus and aid in early immune responses. Fetal CD8 T cells exhibit enhanced proliferation but reduced cytotoxic activity compared to adults and contribute to antiviral responses.

Fetal T cell regulation is influenced by both intrinsic properties and external factors, including maternal and fetal bacteria. Dysregulation of these immune responses can lead to complications like preterm birth and necrotizing enterocolitis (Rackaityte & Halkias, 2020). Figure 1.1 shows that in the fetal stage, the immune system prioritizes a regulatory environment rich in suppressive signals and tolerogenic factors including

TGF- β,estrogen and limited antigen exposure. This fosters a development of tolerant T cells along with several intrinsic and extrinsic mechanisms to suppress immune activation. After birth, this environment shifts, and the immune system becomes more pro-inflammatory due to increased exposure to antigens and withdrawal from tolerogenic factors to enable defense against infections. This shift from immune tolerance to immune activation reflects the changing needs from in utero development to postnatal life. Some immune mechanisms like CD71+ erythrocytes and Treg priming wane over time while others remain uncertain.

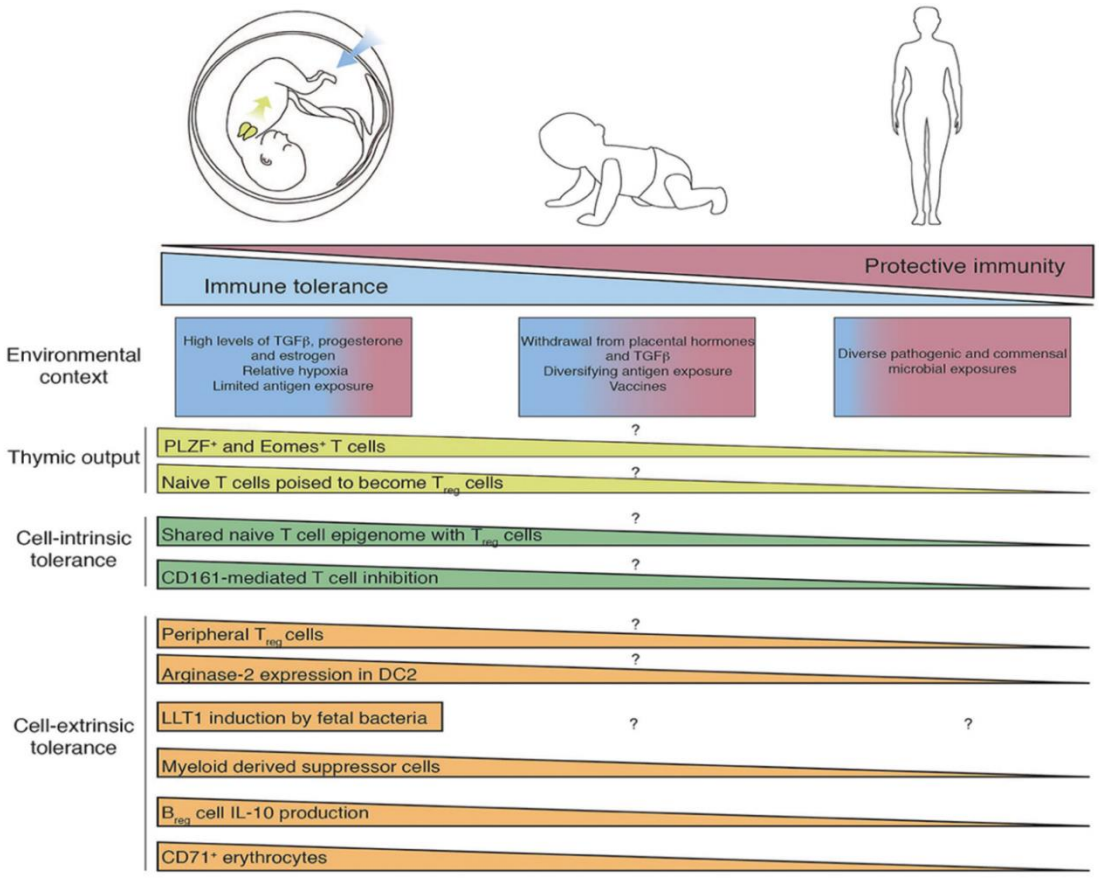

Figure 1.1: Fetal T cell immunity (as described by (Rackaityte & Halkias, 2020)

Recent studies have shown a concrete link between the occurrence of RPL and immunological factors. It was found that amongst several cases of lost embryos, 95% were shown to have a normal karyotype and alloimmune rejection by the maternal

humoral/cellular immunity accounted for most of these losses (Kniotek et al., 2021). Since the embryo is treated by the maternal body as a haploidentical allograft, several immune cells are found at the site of implantation. These cells include subpopulations of decidual Natural Killer cells (dNK), T cells (Th1, Th2, Th17 and Treg), Natural Killer T cells (NKT), macrophages and dendritic cells. A major proportion (70%) of the immune cells in the endometrium constitute dNKs and type 2 innate lymphoid cells (ILC2s) cells which produce IL-4, Il-5 and IL-13 cytokines. As a result, these cells are known to control angiogenesis and the implantation process (Moffett & Colucci, 2014).

Dysregulation of dNKs has been linked to termination of pregnancy, preeclampsia or gestational trophoblastic disease (Liu et al., 2021). Tregs by releasing large amounts of TGF-β, IL-10 and IL-35 (inhibitory cytokines) inactivate dNK thereby maintaining immune tolerance (Tanaka & Sakaguchi, 2017). The overexpression of pro-inflammatory cytokines on the other hand, such as (IL-1β and IL-6) blocks the development of Tregs instead promoting the differentiation of Th17 cells. This subsequently results in a disruption of immune tolerance at the feto-maternal interface.

One study by Kniotek et al., (2021) assessed the effects of erectile dysfunction and pulmonary arterial hypertension drug, Sildenafil, on the immune system; specifically, T regulatory cells (Tregs), T helper 17 cells (Th17) as well as the drug's effect on the production of cytokines associated with subsets of T helper cells. The results of this study revealed that SC significantly decreased the concentrations of IL-6 and IL-12, and improved TGF-β production.

Tregs have reportedly played a large role in tolerance towards fetal alloantigens (Zenclussen, 2006). Several studies have provided evidence supporting an augmentation in CD4 (+) CD25 (bright) Treg cells that takes place during pregnancy and

simultaneously a diminished activity in patients suffering from miscarriage (Sasaki et al., 2004).

1.6 Significance of the study

This is the first study on the association of CTLA-4 and IL-6 selected variants with RPL among Palestinian women with this condition. Considering the above average RPL incidence rates in the Palestinian population such studies should be more explored. In addition, the study focuses on investigating the potential link between selected immunological gene variants with genetics to diagnose conditions such as RPL is considered relatively new in the field and hence should be more thoroughly studied. The results of this study may provide useful information regarding RPL diagnosis and prognosis.

1.7 Study problem

Since RPL is of multifactorial origin (Vaiman, 2015) and about 50% of its cases are unexplained, this allowed diagnostic focus to be more shifted towards genetic and immunological risk factors (Garrido-Gimenez & Alijotas-Reig, 2015; Vomstein et al., 2021). The CTLA-4 rs231775and IL-6 rs1800796 polymorphisms have been suggested to play crucial roles in immune tolerance of the mother towards her fetus since both of these polymorphisms have been correlated with RPL risk (Messaoudi et al., 2014; Rasti et al., 2016). The G allele of the CTLA-4 +49 A/G polymorphism was said to play a protective role while that of the *IL-6* 634C/G polymorphism seems to increase RPL risk in other populations. In Palestine the role of these variants and the involvement of the immune system in the development of RPL in our population were not investigated.

1.8 Study objectives

In this study the association of CTLA-4 and IL-6 polymorphisms (rs231775 and rs1800796 respectively) will be investigated among Palestinian women suffering from RPL (≥ 3 consecutive losses). The association across first and second trimesters of

pregnancy will also be observed in order to evaluate whether the association between CTLA-4+49A/G and IL-6–634C/G polymorphisms and RPL differs across the two trimesters and to isolate the functional role of the two genes during particular pregnancy stages.

1.9 Study questions

The main focus of this study is to investigate whether there is an association between CTLA-4+49A/G and IL-6–634C/G polymorphisms and RPL. How do both alleles and genotype frequencies differ between women with recurrent abortion and healthy controls? Is there a combined functional effect of the two indicated variants on the risk of recurrent abortion? Can these variants serve as potential genetic markers for predicting recurrent abortion susceptibility?

1.10 Study limitations

Recurrent abortion is a multifactorial condition which can be influenced by several genes and environmental factors, therefore, focusing on two polymorphisms may not capture the full genetic contribution to this condition. In addition, since the study does not measure protein expression or plasma cytokine levels, this makes it difficult to determine the functional impact of these polymorphisms on immune regulation. Finally, since the study is conducted on Palestinian women, the study population may not be representative of diverse ethnic or geographical groups.

1.11 Study hypothesis

Several studies have shown that the CTLA-4+49A/G and IL-6–634C/G polymorphisms have influenced the RPL phenotype. The G allele of the CTLA-4+49A/G polymorphism was shown to play a protective role against the risk of RPL while the G allele of IL-6–634C/G increased the risk of RPL by >5 it is therefore hypothesized that these variants will be associated with RPL in Palestinian women experiencing recurrent miscarriage.

Chapter 2
Literature review

2.1 T-cell immune regulation: CTLA-4

Tregs are a subset of helper T cells that are important components of adaptive immunity as they mainly function in the regulation of the immune response (Sharma & Rudra, 2018). One of the two main subtypes of Tregs, CD4+ Tregs, have been reported to have a reduced population in women with RPL and gestational diabetes mellitus. The second main subtype of Tregs (CD8+) has conversely been shown to have regulatory functions in cancer, autoimmune diseases and infectious diseases; in other words, CD8+ relevance in pregnancy is yet to be elucidated. The functions of these Tregs are attributed to surface markers, including CTLA-4 (Wang et al., 2022). Both subtypes, however, are known to express high levels of surface CTLA-4 upon activation (Jarvis et al., 2008).

CTLA-4 is a CD28 homologue expressed on activated T cells and is involved in their immune suppressive function (Read et al., 2000; Takahashi et al., 2000). This antigenic system has been considered one of the most important so far (Vaziri et al., 2023). As seen in figure 2.1, T cells require two signals to become fully activated, the first being an antigen-specific receptor signal sent by T-cell receptors found on T cells and antigens in major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells.

The second signal which is co-stimulatory, involves the transfer of the signal by CD28 on T cells and B7 on APCs which results in complete activation of the T cells (Wang et al., 2005). Based on this method of signaling known as the CTLA-4 dependent pathway, CTLA-4 has been shown to play a very important role in the negative regulation of co-stimulation by responding to stimulus with anti-CD3 and anti-CD28 antibodies and inhibiting T cell proliferation and IL-2 synthesis. This, in turn, prevents

the interaction between CTLA-4 and B7 and enhances the T-cell responses (Frauwirth & Thompson, 2002).

Due to this mechanism of action, the CTLA-4 gene has been linked to autoimmune diseases and its inhibition has been proposed as a therapy to suppress T cells in such cases (Hosseini et al., 2020). In other words, CTLA-4 which is a costimulatory molecule belongs to the same family as CD28 and both have binding affinity for the same ligands B7.1 (CD80) and B7.2 (CD86). Between CTLA-4 and CD28, however, CTLA-4 has higher affinity for B7 (Van Coillie et al., 2020). As a result, the conclusion is that abnormally low expression of CTLA-4 may be linked to RPL.

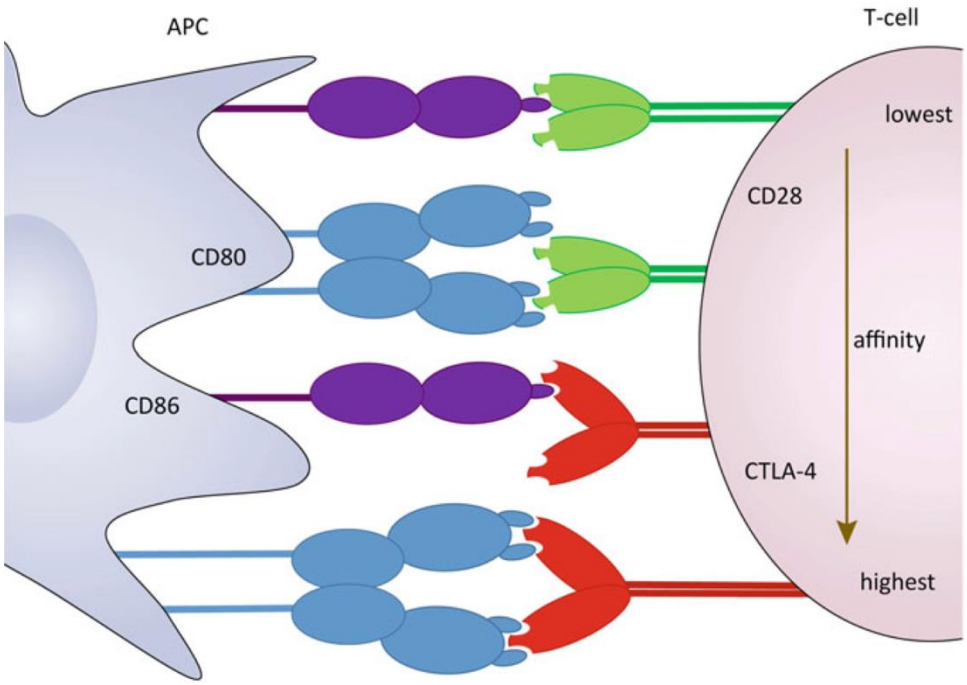

Figure 2.1: CTLA-4 and CD86 binding affinities (as described by Van Coillie et al., 2020)

2.2 CTLA-4 genetic mutations

Several autoimmune diseases have been associated with genetic mutations or post translational modifications of CTLA-4 and its promoter (Van Coillie et al., 2020). According to the (National Center for Biotechnology Information (NCBI), n.d.-a), the CTLA-4 gene is located on chromosome 2 and contains 4 exons (accession # NC_000002.12). The gene coding for CTLA-4 is translated into a 233 amino acid peptide (Nasiri & Rasti, 2016).

Previous studies have shown through comparative sequence analysis of CTLA-4 gene in mice and humans significant similarity in both the coding and non-coding regions such that the intracellular domain displayed complete amino acid conservation. This provides further evidence to support the vital role played by CTLA-4 in the regulation of the immune system. The fact that CTLA-4 gene has experienced limited change across various species corroborates its importance in immunological processes (Ling et al., 1999).

The CTLA-4 gene has been reported to contain over 100 polymorphic sites. The following distinct polymorphisms were shown to be associated with autoimmune and infectious diseases including CTLA-4 +49A/G, CTLA-4 -1722T/C and CTLA-4-318C/T (Dias et al., 2013). According to NCBI Clinvar the CTLA-4+49 A/G polymorphism (rs231775) results in a threonine to alanine substitution at position 17 (T17A). Given the high degree of conservation of the CTLA-4 gene, this amino acid change may adversely affect its function. Studies have shown that the G allele (encoding alanine)can affect CTLA-4 trafficking, expression levels on the surface of T cells and interaction with ligands which all may impair the inhibitory function of CTLA-4 (Ueda et al., 2003).

2.3 Interleukin 6

ILs are a group of immunomodulatory proteins which function as mediators of a variety of immune reactions that take place in the human body. Several IL

polymorphisms have been associated with RPL including IL-1β, IL-6, IL-10 and IL-18 (Zhang et al., 2017). Among these ILs, IL-6 has been closely linked with CTLA-4 gene polymorphisms and RPL association. Macrophages, fibroblasts, epithelial cells, and placental trophoblasts all synthesize of IL-6 which is responsible for the development of the placenta and maintaining the pregnancy making it crucial for this process. It functions in the regulation of spiral artery remodeling and trophoblast invasion (Günther et al., 2023). IL-6 is a multifunctional cytokine which functions in mediating the balance between T helper (Th)-17 and Treg cells. IL-6 inhibits TGF- β induced Treg differentiation by inducing the development of Th-17 cells from naïve T cells and TGF- β (Bettelli et al., 2006)

IL-6's role in the development of placenta and the pregnancy is mediated through the regulation of trophoblast invasion and spiral artery remodeling. It was established as an indispensable cytokine for early pregnancy development. Too high or low levels of IL-6 could be responsible for sporadic abortion or RPL (Günther et al., 2023). This explains the role played by IL-6 during embryonic implantation and the fact that the highest level of IL-6 mRNA is observed during the implantation and menstruation periods (van Mourik et al., 2009) which is also correlated with the presence of IL-6 receptors on the endometrium and trophoblast (Cork et al., 2002). According to (National Center for Biotechnology Information (NCBI), n.d.-b), the IL-6 gene is located on chromosome 7p.15.3 with an entire sequence length of 4799nt. Two polymorphic markers have been identified on this gene: –174G/C and –634C/G (Daher et al., 2012) and a single study identified the –634C/G variant in Japanese patients and found that the G allele was significantly lower among recurrent spontaneous abortion (RSA) patients (Saijo et al., 2004).

2.4 CTLA-4+49A/G polymorphism

The mechanism of Tregs action is achieved through cell-cell contact mediated by CTLA-4 and the secretion of key cytokines such as TGF- β and IL-10 (Abdollahi et al., 2020). U-regulation of TGF- β CTLA-4 suppresses IL-6 which causes naive CD4+ T cells to differentiate into Tregs (d'Hauterive et al., 2004; Moghbeli, 2019). The G allele in the CTLA-4+49A/G polymorphism has been reported to play a protective role against RPL in a group of Iranian cohort. Additionally, correlation was shown between the IL-6 634C/G polymorphism and RPL where the G allele was associated with a >5 increase in the risk of RPL. As a result, these variants were introduced as risk factors of RPL.

The pathogenicity of CTLA-4+49A/G (rs231775) polymorphism has been supported by multiple studies (Nasiri & Rasti, 2016; Wang et al., 2005). For instance, (Chen et al., 2018) associated the G allele with increased risk of Type 1 Diabetes. Moreover, homozygosity for the G allele has also been linked to autoimmune conditions such as Grave's disease, rheumatoid arthritis and systemic lupus erythematous. The heterozygosity of this variant was associated with autoimmune thyroiditis (Gough et al., 2005; Salzer et al., 2013). Furthermore, a study conducted on various ethnic subgroups in Asia including China, India and Iran showed significantly decreased association between the CTLA-4+49A/G polymorphism and overall population risk indicating that it may weakly decrease the risk of RPL for women of childbearing age (Song et al., 2019). Alternatively, other instances have corroborated the significant association between the G allele of this polymorphism and decreased risk of RSA (Rasti & Nasiri, 2016) .

2.5 IL-6 –634C/G polymorphism

Regarding IL-6 the –174G/C polymorphism has been more widely studied than the -634C/G polymorphism. In one study conducted on 121 women with RPL and 121 healthy women in the Molecular Genetics Laboratory of Arsanjan University, the results showed a significant association between the risk of RPL and the –634C/G

polymorphism where the mutant G allele predisposed women to miscarriage 1.5 times greater than the control subjects (Rasti et al., 2016). These results were corroborated in another study indicating the G allele to increase the risk of RPL (Nasiri & Rasti, 2016). On the contrary, a different study showed the G allele of the –634C/G polymorphism was associated with decreased risk of RPL in Egyptian females (Abo-alella et al., 2021)

.

CTLA-4 is involved in immune suppression and its activation inhibits the activation of T cells through the production of proinflammatory cytokines like IL-6 which when inhibited, causes naive CD4+ T cells to differentiate into Tregs (d'Hauterive et al., 2004; Moghbeli, 2019). Additionally, CTLA-4 functions in mediating the balance between T helper (Th)-17 and Treg cells (Bettelli et al., 2006). Th17 along with Th1 and Th2 may all produce IL-10 which functions in the suppression of proliferation and cytokine production. TGF-β can function as an immunosuppressive cytokine. That said, TGF-β can also be immunostimulatory (Corthay, 2009). Studies have shown that reduced abortion rates resulting from adoptive transfer of Tregs were accompanied by increased CTLA-4 gene expressions as well as TGF-β and IL-10 levels in mice (Mohammadi et al., 2021). The CTLA-4 +49 A/G and IL-6 634C/G polymorphisms have been briefly studied as potential risk factors of RPL. Further studies are definitely required to fully understand their effects on RPL and whether they are considered pathogenic variants.

Chapter 3
Materials and methods

3.1 Study Subjects

A case control genetic association study within the framework of observational, analytical research was conducted on 172 women all of whom attended prenatal care clinics in the West Bank. The case group consisted of 88 women who had experienced three plus primary consecutive idiopathic miscarriages 44 of whom experienced miscarriage within the first 12 weeks of pregnancy and another 44 whose miscarriages took place between the 12^{th} and 24^{th} weeks of pregnancy. All samples were retrieved in accordance with the ESHRE guidelines.

The control group consisted of 84 women who had experienced two plus successful pregnancies in addition to a regular menstrual cycle and no history of recurrent abortion or other complications during pregnancy. All information on patients' medical histories was obtained through a uniform questionnaire, personal interview with the participants and consultation with the medical team. Information on participating subjects is shown in table 3.1.

Table 3.1: Subjects' Information

Category	Description
Study type	Case-control genetic association study (observational, analytical)
Total participants	172 women
Age range	≤35 years
Recruitment site	Prenatal care clinics, West Bank

Case group	88 women with ≥3 consecutive idiopathic miscarriages
1st trimester loss subgroup	44 women (miscarriage within first 12 weeks)
2nd trimester loss subgroup	44 women (miscarriage between 12-24 weeks)
Control group	84 women with ≥ 2 successful pregnancies, no history of miscarriage

The exclusion criteria was as follows: women over the age of 35, women diagnosed with inherited thrombophilia (FV Leiden, F2 G20210A), chromosomal anomalies, cigarette smokers, consumers of alcohol, women suffering from autoimmune diseases (anti-phospholipid antibodies, anti-cardiolipin antibodies, lupus anticoagulant, anti-nuclear antibodies and B2 glycoprotein 1) women diagnosed with uterine abnormalities, infectious diseases (rubella, toxoplasma, HIV, HBV, HCV, and CMV), endocrine diseases (diabetes mellitus, thyroid diseases, and hyperprolactinemia) as well as those with Rh blood group incompatibility and women suffering from any pregnancy complications other than recurrent pregnancy loss, such as those with a history of preterm birth or preeclampsia.

3.2 DNA Extraction

After collecting three ml of whole EDTA blood from control and case groups, genomic DNA was extracted from whole blood using a leukocyte-rich buffy coat according to the MasterPure™DNA Purification Protocol (Epicentre Biotechnologies, Wisconsin, USA, Cat No. MG71100). DNA extraction was achieved via the following steps: first, 150µl of the buffy coat was transferred into an Eppendorf tube. Next, 600µl of lysis buffer was added to the tube, mixed gently and incubated at room temperature for 5 minutes after flicking the bottom of the tube. The tube was mixed, flicked and

incubated for additional 5 minutes at room temperature . at the end of the second incubation period, the tubes were mixed, flicked and the white blood cells were then pelleted by centrifugation at 14000 rpm for 25 seconds in a microcentrifuge The supernatant was removed, and the WBCs were resuspended in 20-50 µl of the remaining lysis 1 buffer and 300 µl of lysis buffer 2 were added and the cells were pipetted up and down 5-7 times. 250µl of precipitation solution were added to the tubes, vigorously vortexed for 30 seconds and the cellular debris was pelleted by centrifugation for 10 minutes at 10000 x g in a microcentrifuge.

The supernatant was then transferred to a new Eppendorf tube and 700µl of isopropanol was added to the tube. The tube was inverted a few times until the string-like precipitate of DNA became visible. The DNA was then precipitated by centrifugation at 4 °C at 14000 rpm for 10 minutes and the supernatant was carefully removed without dislodging the pellet. Next, 150µl of 75% ethanol was added to wash out the isopropanol and further purify the pellet and the tube was centrifuged again for one minute. The supernatent was then removed from the tube and the pellet was left to air dry at room temperature for about 10 minutes. The DNA was then resuspended in 100µl nuclease free water and stored at –30 °C.

3.3 DNA Quantitation and Qualification

The concentration and purity of the extracted genomic DNA were assessed with a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA). The average concentration for the control subjects was 52.48 ng/µL with an average 260/280 purity ratio of 1.98. The average concentration of the subjects who experienced RPL in the second trimester of pregnancy was 92.36 ng/µL with an average 260/280 purity of 2.02

and the average concentration of the subjects who experienced RPL in the first trimester of pregnancy was 34.27 ng/µL with an average 260/280 purity of 2.09.

3.4 Gel electrophoresis

Gel electrophoresis was done in order to check the quality of the prepared DNA using 1% (w/v) agarose gel (Standard Agarose Cat No. KI8100-500) was added to 100ml of diluted TAE working buffer (Tris Acetate EDTA (1X)). After heating the mixture for 90 seconds to ensure that the agarose had completely dissolved into the buffer, (10mg/ml) of Ethidium Bromide was added to the agarose mixture after cooling to ~70^0C in order to stain and enhance the visibility of DNA. 5µl genomic DNA was loaded into separate wells from each sample along with 5µl of ready-made DNA ladder (1KB) which was run alongside the samples to measure and determine the size of the bands. The gel was then run for 30-45 minutes at 80-120 volts for 35-40 minutes and then visualized using the Gel-Documentation machine under a UV transilluminator.

3.5 CTLA-4 and IL-6 Primer Design

The primers used throughout this study were designed using the Primer3Plus software. The primer pairs were adjusted to flank the regions around the two indicated SNPs (rs231775) and (rs1800796) in both genes. The product size for the amplified CTLA-4 gene product was 215bp, while that of the IL-6 gene was 201bp. For more details on the primers refer to Table 3.2.

Table 3.2: The primer sequences used for the amplification of CTLA-4 and IL-6 polymorphisms; DNA fragments encompassing (rs231775) and (rs1800796) respectively.

Gene	Polymorphism	Site of mutation	Primer sequence	Tm (°C)	Restriction enzyme
CTLA-4	rs231775 (+49A/G)	Exon 1	F: CTGAACACCGCTCCCATAAA R: CACTGCCTTTGACTGCTGAA	61.0	BSeX I
IL-6	rs1800796 (-634 C/G)	Promotor	F: GGCTGAAGCAGGTGAAGAAA R: CCAAGCCTGGGATTATGAAG	60.5	*Mbi I*

3.6 Polymerase Chain Reaction (PCR)

The segments of DNA containing the polymorphic sites in the CTLA-4, and IL-6 genes were amplified using PCR. The PCR reaction mixtures were as follows: 10µl Taq ^{R}PCR green master mix (2X), 1µl template DNA (100 ng) 1.0µl forward primer (10 µM), 1.0µl reverse primer (10 µM) and 7µl nuclease-free water in a 20µl final reaction volume. The thermal cycler amplification program for both genes was set as shown in Table 3.3.

Table 3.3: Optimized PCR conditions for CTLA-4 and IL-6 polymorphisms (rs231775) and (rs1800796) respectively.

PCR Conditions	**CTLA-4 SNP (rs231775)**		**IL-6 SNP (rs1800796)**	
Initial denaturation	**94°C, 5 min**		**94°C, 5 min**	
2nd Denaturation **Annealing** **Extension**	**94°C, 20 sec** **55.5°C, 30 sec** **72°C, 45 sec**	**32 cycles**	**94°C, 20 sec** **55.5°C, 30 sec** **72°C, 45 sec**	**32 cycles**
Final extension	**72°C, 5 min**		**72°C, 5 min**	

3.7 Genotyping

Genotyping of CTLA-4 +49A/G and IL-6 -634 C/G SNPs was done using restriction fragment length polymorphism (RFLP)-PCR. The indicated amplified DNA fragments were genotyped for the CTLA-4 SNP (rs231775) located on exon 1 of the CTLA-4 gene and IL-6 SNP (rs1800796) in the promoter of the IL-6 gene by digestion with MbiI and BSeXI restriction endonucleases. The digestion for both genes was performed in 15µl reaction volumes containing 10µl PCR product, 3.4µl H20, 1.5µl buffer (10x), 0.1µl Mbi I restriction enzyme (10000Unit/ml) for the IL-6 SNP and 10µl PCR product, 3.25µl H20, 1.5µl buffer (10x), 0.25µl BSeX I restriction enzyme (2000Unit/ml) for the CTLA-4 SNP. The digestion mixture was incubated at 37 ° C overnight. The following genotypes were obtained:

CTLA-4:

- Homozygous WT (AA), resulting in one band of 215bp.
- Heterozygous (AG), resulting in three bands: 215bp,~166bp &~49bp
- Homozygous MT (GG), resulting in two bands:~166bp &~49bp

IL-6:

- Homozygous WT (CC), resulting in one band of 201bp.
- Heterozygous (CG), resulting in three bands: 201bp,~135bp&~66bp
- Homozygous MT (GG), resulting in two bands: ~135bp&~66bp

The program used to analyze the data was SNPSTATS, an online statistical software used for the analysis of SNPs. After inputting the genotypes procured from observing RFLP-PCR samples using gel electrophoresis, into the software, it tested different inheritance models (dominant, recessive, etc.) to see how a SNP might

affect disease risk. The software was also used to measure allele frequency distribution, Hardy-Weinberg Equilibrium, linkage disequilibrium and perform haplotype analysis to identify whether there was any association between the two SNPs.

Chapter 4
Results

4.1 Study subjects

The study was conducted on 44 female participants who experienced RPL in the first trimester of pregnancy and 44 others, who experienced RPL in the second trimester. aged ≤35 years all of whom experienced a minimum of 3 consecutive miscarriages. The control group consisted of 84 women who had two previous successful pregnancies with no records of complications related to pregnancy and labor complications.

4.2 Detection of CTLA-4 and IL-6 SNPs

PCR was used to amplify a 215bp DNA fragment flanking the (rs231775) polymorphism in the CTLA-4 gene as shown in figure 4.1 and the 201bp DNA fragment flanking the (rs1800796) polymorphism in the IL-6 gene as shown in. figure 4.2 .both figures show only one DNA product with the expected specific size.

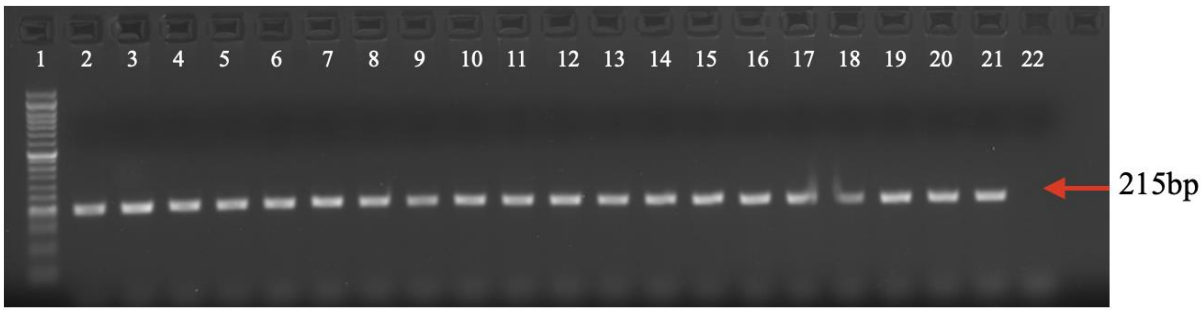

Figure 4.1: PCR amplification of the CTLA-4 gene fragment containing the +49A/G polymorphism, analyzed by agarose gel electrophoresis. Lane 1: 50bp DNA ladder; lanes (2-21) amplified PCR products; lane 22 negative control.

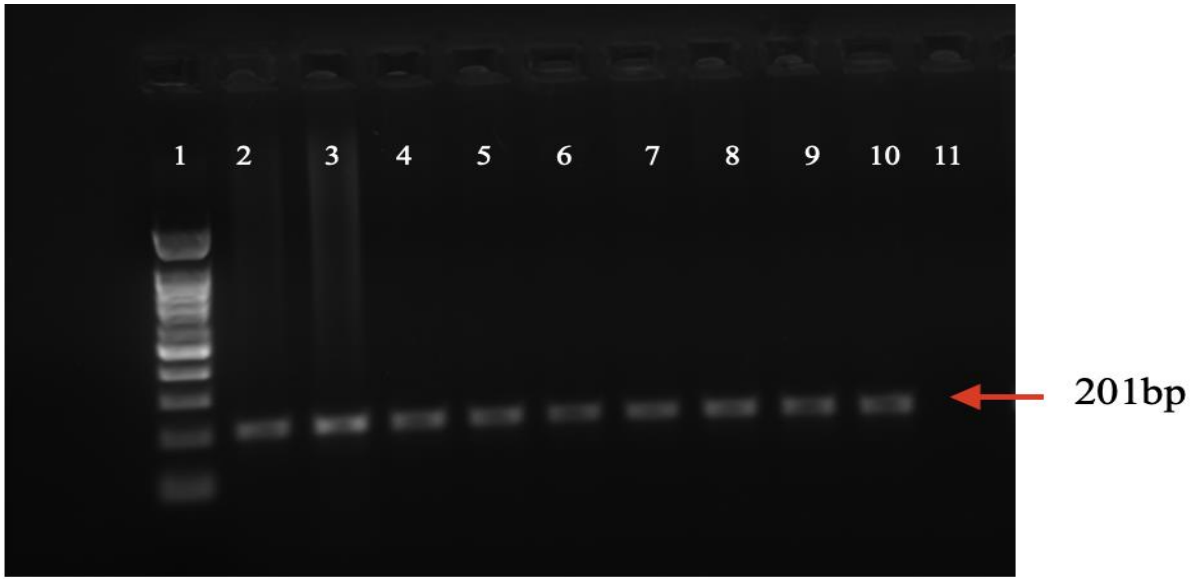

Figure 4.2: PCR amplification of the IL-6 gene fragment containing the -634 C/G Polymorphism, analyzed by agarose gel electrophoresis. Lane 1: 100bp DNA ladder; lanes (2-10) amplified PCR products; lane 11 negative control.

The exon1 amplified product of the CTLA-4 gene was digested using BSeX I restriction endonuclease resulting in the following genotypes: An uncut 215 bp fragment indicating the homozygous AA genotype , partially cut with 215bp, 166bp and 49bp fragments indicating the heterozygous AG genotype and finally complete digestion with 166bp and 49 bp fragments indicating the homozygous GG genotype as described in Figure 4.3. The IL-6 promotor amplified PCR product was digested using *Mbi I* restriction endonuclease allowing the following genotypes to be observed; An uncut 201 bp fragment indicating the homozygous CC genotype , partially cut with 201bp, 135bp and 66bp fragments indicating the heterozygous CG genotype and finally complete digestion with 135bp and 66 bp fragments indicating the homozygous GG genotype as described in Figure 4.4

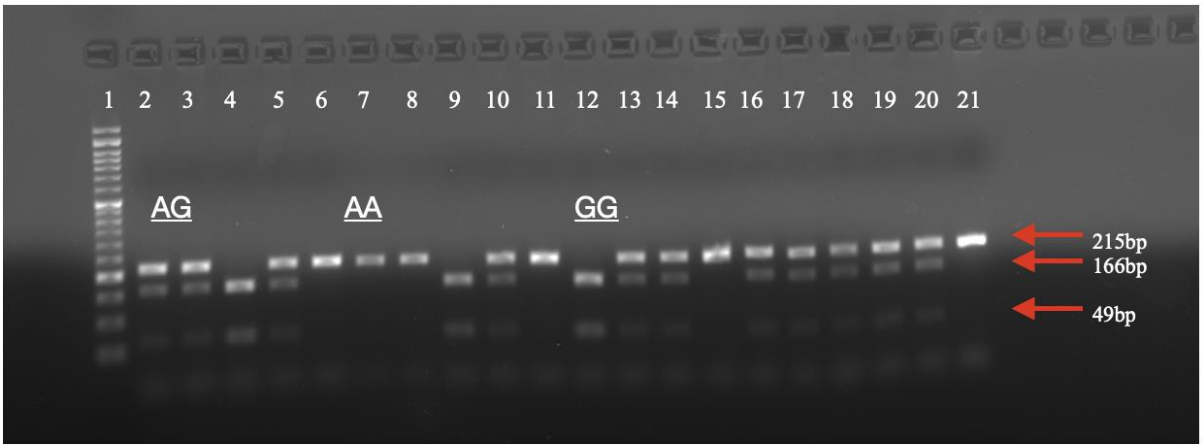

Figure 4.3: RFLP digestion results of the CTLA-4 +49A/G polymorphism after digestion with the BseXI restriction enzyme. Lane 1: 50bp ladder. Lanes 4,9&12: The presence of restriction sites in the G allele produces two fragments. Lanes 6,7,8,11,15& 21: The A allele remains undigested resulting in a single larger fragment. Lanes 2,3,5,10,13,14,16-20: Partial digestion results in three fragments showing both alleles.

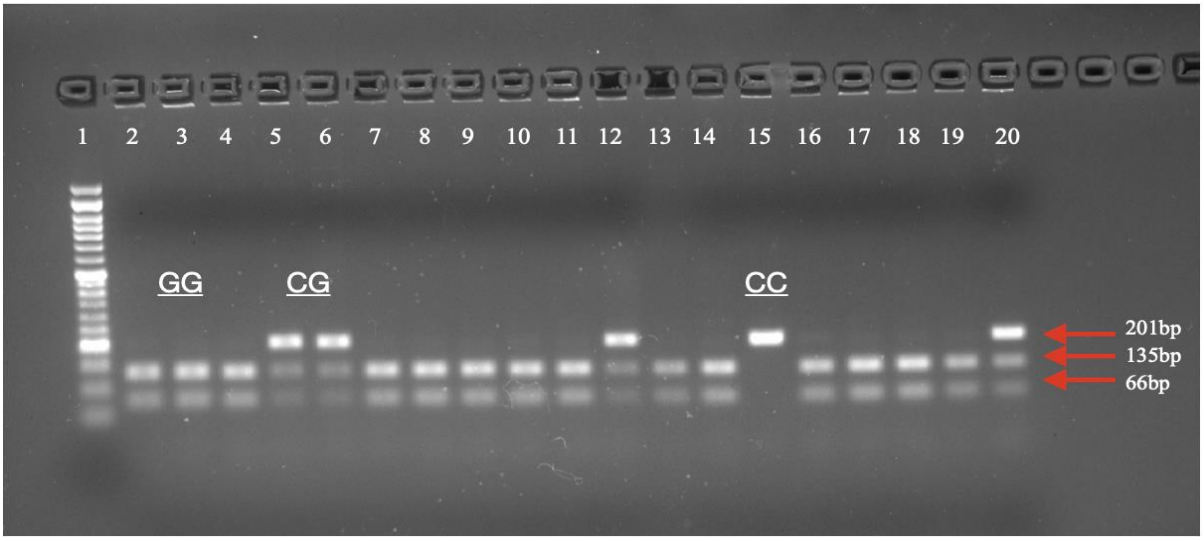

Figure 4.4: RFLP digestion results of the IL-6 -634 C/G polymorphism after digestion with the Mbi I restriction enzyme. lane 1: 50bp ladder. Lanes 2-4, 7-11, 13,14, 16-19: The presence of the G allele creates two fragments due to the restriction site. Lane 15 the

C allele produces a single undigested fragment. Lanes 5,6,12& 20: Partial digestion results in three fragments showing both alleles.

4.3 Hary-Weinberg equilibrium (HWE)

The hardy Weinberg equilibrium test was performed for SNP1 (CTLA-4) and SNP2 (IL-6) in the total population (n = 172) and the two subgroups (Control and Patients). CTLA-4 SNP1 showed no significant deviation from HWE. In the total population, the genotype distribution was 66 (AA), 79 (AG), and 27 (GG), with a p-value of 0.75. Similarly, both control group (p = 0.65) and patients group (p = 0.25) remained in equilibrium. These results suggest a stable allele distribution in this SNP.

Table 4.1: Analysis of HWE parameters for CTLA-4

SNP1.CTLA.4 exact test for Hardy-Weinberg equilibrium (n=172)						
	N11 AA	**N12 AG**	**N22 GG**	**N1 Total A**	**N2 Total G**	**P-value**
All subjects	66	79	27	211	133	**0.75**
Control Group	27	44	13	98	70	**0.65**
Patients Group	39	35	14	113	63	**0.25**

The IL-6 SNP genotypes significantly deviated from HWE. In the total population, the genotype counts were 141 (GG), 24 (CG), and 7 (CC), with a p-value of 0.0012. Both control group (p = 0.032) and patients group (p = 0.03) showed significant deviations. This suggests that external factors, such as selection or population substructure, may be influencing this SNP's distribution. Since CTLA-4 SNP follows Hardy-Weinberg expectations, while IL-6 does not, this indicates different genetic behaviors like natural selection or non-random mating, which may have implications for its role in the disease.

Table 4.2: Analysis of HWE Parameters for IL-6

SNP2.IL.6 Exact test for Hardy-Weinberg equilibrium (n=172)						
	N11 GG	**N12 CG**	**N22 CC**	**N1 Total G**	**N2 Total C**	**P-value**
All subjects	141	24	7	306	38	**0.0012**
Control Group	62	17	5	141	27	**0.032**
Patients Group	79	7	2	165	11	**0.03**

4.4 Allele frequency distribution and association of rs231775 (CTLA-4) and rs1800796 (IL-6) with recurrent pregnancy loss

After assessing the allele frequencies for the two variants: rs231775 (CTLA-4) and rs1800796 (IL-6) it was observed that regarding rs231775 (CTLA-4),the A allele was slightly more frequent in the RPL group (64%) than in controls (58%), while the G allele was more common in controls (42%) than in the patients group (36%). However, the association was not statistically significant (p = 0.27), suggesting no evidence of a link between this variant and RPL. As for the rs1800796 (IL-6), the G allele was significantly more frequent in the RPL group (94%) compared to controls (84%), whereas the C allele was more prevalent in controls (16%) compared to patients (6%). This difference was statistically significant (p = 0.005), suggesting a protective effect of the C allele against RPL. These findings indicated that the IL-6 polymorphism may play a role in RPL susceptibility, warranting further investigation.

Table 4.3: Allele Frequency Distribution of rs231775 (CTLA-4) and rs1800796 (IL-6) for All Subjects.

Variant	**Allele**	**Control group**	**RPL case**	**OR(95% CI)**	**P-value**
rs231775	A	98 (0.58)	113 (0.64)	1.28 (0.83 – 1.98)	0.27
	G	70 (0.42)	63 (0.36)		
rs1800796	G	141 (0.84)	165 (0.94)	0.35 (0.17 – 0.73)	0.005
	C	27 (0.16)	11 (0.06)		

4.4 Association of CTLA-4 and IL-6 polymorphisms with RPL

The genotype distributions and association analysis of the **CTLA-4** polymorphism in control subjects and RPL cases are summarized in Table 4.4.

The conclusion drawn from this information is that the CTLA-4 SNP showed an insignificant p-value when comparing the occurrence of the variant in patients and controls indicating that there is no link between this variant and RPL. However, the IL-6 SNP showed a statistically significant P-value indicating a link/association between this variant and the occurrence of RPL.

Table 4.4: Genotypic and Allelic Association of CTLA-4 Polymorphism with RPL Among All Subjects.

SNP	Model	Genotype	Control subjects	RPL Cases	OR (95%CI)	P-value
CTLA-4		AA	27 (32.1%)	39 (44.3%)	1.00	0.21
		AG	44(52.4%)	35(39.8%)	0.55 (0.28-1.07)	
		GG	13(15.5%)	14(15.9%)	1.39 (0.75-1.83)	
	Dominant	AA vs.	27 (32.1%)	39 (44.3%)	1.00	0.1
		AG+ GG	57 (67.9%)	49 (55.7%)	0.60 (0.32-1.11)	
	Recessive	AA +AG vs.	71 (84.5%)	74 (84.1%)	1.00	0.94
		GG	13 (15.5%)	14 (15.9%)	1.03 (0.45-2.35)	

As shown in the table in all models, the CTLA-4 SNP showed a statistically insignificant p-value once again showing that this SNP is not linked to the occurrence of RPL. The genotype distributions and association analysis of the **IL-6** polymorphism among control and RPL subjects are summarized in Table 4.5.

Table 4.5: Genotypic and Allelic Association of IL-6 Polymorphism among All RPL and Control Subjects

SNP	Model	Genotype	Control subjects	RPL Cases	OR (95%CI)	P-value
IL-6		GG	62 (73.8%)	79 (89.8%)	1.00	0.022
		CG	17 (20.2%)	7 (8%)	**0.32 (0.13-0.83)**	
		CC	5 (6%)	2 (2.3%)	0.31 (0.06-1.67)	
	Dominant	G/G vs.	62 (73.8%)	79 (89.8%)	1.00	0.0059
		C/G-C/C	22 (26.2%)	9 (10.2%)	**0.32 (0.14-0.75)**	
	Recessive	G/G-C/G vs.	79 (94%)	86 (97.7%)	1.00	0.22
		C/C	5 (6%)	2 (2.3%)	0.37 (0.07-1.95)	

The table above shows the analysis of the three genetic models of the IL-6 SNP, it is observed that the dominant model shows a statistically significant p-value which indicates a link between this variant and the occurrence of RPL. To further explore the genetic association, we performed a stratified analysis of both variants (rs231775 and rs1800796) on control and RPL subjects in the **second-trimester**. The results are presented in Tables 4.6 and 4.7.

Table 4.6: Genotypic and Allelic Association of CTLA-4 Polymorphism (rs231775) among Control and RPL Subjects in the Second Trimester

Second trimester patients						
SNP	**Model**	**Genotype**	**Control subjects**	**RPL Cases**	**OR (95%CI)**	**P-value**
CTLA-4		AA	27 (32.1%)	19 (43.2%)	1.00	0.41
		AG	44(52.4%)	18(40.9%)	0.58 (0.26-1.30)	
		GG	13(15.5%)	7(15.9%)	0.77	

					(0.26-2.28)	
	Dominant	AA vs.	27 (32.1%)	19 (43.2%)	1.00	0.22
		AG+ GG	57 (67.9%)	25 (56.8%)	0.62 (0.29-1.32)	
	Recessive	AA +AG vs.	71 (84.5%)	37 (84.1%)	1.00	0.95
		GG	13 (15.5%)	7 (15.9%)	1.03 (0.38-2.81)	

Table 4.7: Genotypic and Allelic Association of IL-6 Polymorphism with Second Trimester RPL

Second trimester patients						
SNP	**Model**	**Genotype**	**Control subjects**	**RPL Cases**	**OR (95%CI)**	**P-value**
IL-6		GG	62 (73.8%)	38 (86.4%)	1.00	0.23
		CG	17 (20.2%)	5 (11.4%)	0.48 (0.16-1.41)	
		CC	5 (6%)	1 (2.3%)	0.33 (0.04-2.90)	
	Dominant	G/G vs.	62 (73.8%)	38 (86.4%)	1.00	0.093
		C/G-C/C	22 (26.2%)	6 (13.6%)	0.44 (0.17-1.20)	
	Recessive	G/G-C/G vs.	79 (94%)	43 (97.7%)	1.00	0.32
		C/C	5 (6%)	1 (2.3%)	0.37 (0.04-3.25)	

The results indicated no association between CTLA-4 and IL-6 polymorphisms with RPL in second-trimester cases compared to all cases. This indicates that the observed difference of IL-6 variant (rs1800796) among the total subjects is not related to the second trimester. In response to the previous data, we performed a stratified analysis among both groups (Control and RPL) in the **first-trimester**. The results are presented in Tables 4.8 and 4.9.

Table 4.8: Genotypic and Allelic Association of CTLA-4 Polymorphism (rs231775) among Both Test Groups in the First Trimester

First trimester patients

SNP	Model	Genotype	Control subjects	RPL Cases	OR (95%CI)	P-value
CTLA-4		AA	27 (32.1%)	20 (45.5%)	1.00	0.28
		AG	44(52.4%)	17 (38.6%)	0.52 (0.23-1.17)	
		GG	13(15.5%)	7(15.9%)	0.73 (0.25-2.15)	
	Dominant	AA vs.	27 (32.1%)	20 (45.5%)	1.00	0.14
		AG+ GG	57 (67.9%)	24 (54.5%)	0.57 (0.27-1.20)	
	Recessive	AA +AG vs.	71 (84.5%)	37 (84.1%)	1.00	0.95
		GG	13 (15.5%)	7 (15.9%)	1.03 (0.38-2.81)	

Table 4.9: Genotypic and Allelic Association of IL-6 Polymorphism (rs1800796) among Both Test Groups in the First Trimester

First trimester patients						
SNP	Model	Genotype	Control subjects	RPL Cases	OR (95%CI)	P-value
IL-6		GG	62 (73.8%)	41 (93.2%)	1.00	0.018
		CG	17 (20.2%)	2 (4.5%)	0.18 (0.04-0.81)	
		CC	5 (6%)	1 (2.3%)	0.30 (0.03-2.68)	
	Dominant	G/G vs.	62 (73.8%)	41 (93.2%)	1.00	0.0049
		C/G-C/C	22 (26.2%)	3 (6.8%)	0.21 (0.06-0.73)	
	Recessive	G/G-C/G vs.	79 (94%)	43 (97.7%)	1.00	0.32
		C/C	5 (6%)	1 (2.3%)	0.37 (0.04-3.25)	

These results show a significant association between IL-6 polymorphisms and RPL in the first-trimester compared to second-trimester RPL. This confirmed that there was a trimester-specific genetic effect on RPL susceptibility.

4.5 Haplotype Association with Response

Haplotype analysis was done for SNP1 (CTLA-4) and SNP2 (IL-6) among all subjects (n=172) in relation to response (the likelihood of experiencing RPL). The data shows that the most common haplotype was, A-G (54.92%), which was used as the reference. It is apparent that none of the individual haplotypes reached statistical significance in the crude analysis, a trend toward a protective effect was observed for haplotypes containing the C allele at SNP2 (IL-6).

Table 4.10: Haplotype Analysis of CTLA-4 and IL-6 SNPs among All Subjects in Association with Response (Likelihood of experiencing RPL) (n = 172)

SNP1 (*CTLA-4*)	**SNP2 (*IL-6*)**	**Frequency (total)**	**OR (95% CI)**	**P-value**
A	G	0.5492	1.00	---
G	G	0.3403	0.79 (0.49 - 1.27)	0.33
A	C	0.0642	0.43 (0.17 - 1.13)	0.088
G	C	0.0463	0.34 (0.10 - 1.12)	0.079
Global haplotype association p-value: 0.047				

4.6 Linkage Disequilibrium Analysis

The table above shows that while the individual haplotype p-values are not statistically significant, the global haplotype p-value is significant. This indicates that there may be a trend toward protection regarding haplotypes containing the C allele. Any association observed is due to functional interaction and not genomic linkage.

Table 4.11: Linkage Disequilibrium Analysis of CTLA-4 and IL-6 SNPs among All Subjects

Measure	**SNP1.CTLA-4 vs. IL-6**
D	0.0036

D′	0.0524
r	0.0233
p-value	0.666

Linkage disequilibrium analysis between the CTLA-4 and IL-6 SNPs revealed a very weak LD where there was minimal disequilibrium between the D and D′ loci and the p-value was not significant. The results show that the two indicated SNPs are inherited independently in the population. Note that any association observed is due to functional interaction and not genomic linkage.

4.7 Screening for a Familial HLA-G 459delC Variant in the Study Cohort

In light of previous findings identifying the HLA-G 459delC variant as a potential contributor to RPL through its impact on fetal stability, immune tolerance and vascular remodelling at the maternal-fetal interface, we examined the frequency of this specific familial variant in our study cohort using sanger sequencing. However, our analysis did not detect the presence of this mutation among the participating subjects which indicated that while it may play a role in certain familial cases, it is not a common variant in the broader population studied.

Chapter 5
Discussion

RPL is a pregnancy disorder characterized by the repeated and unexplained loss of pregnancy where the mother experiences 2-3+ incidents of pregnancy loss consecutively which occur before the 24th week of pregnancy ("Endometriosis. Guideline of European Society of Human Reproduction and Embryology – 2022," 2022), (American Society for Reproductive Medecine, 2012). This condition is multifactorial and has been attributed to several causes whether they are anatomical, hormonal, immunological, etc. however; many of these cases remain idiopathic. Emerging evidence shows that genetic and immunogenetic factors may contribute significantly to the etiology of idiopathic RPL (Ford & Schust, 2009). Genes such as CTLA-4 and IL-6 have particularly gained attention in these studies due to their roles in modulating maternal immune tolerance during pregnancy.

In this study we investigated the association of CTLA-4 +49 A/G (rs231775) and IL-6 -634 C/G (rs1800796) SNPs with RPL. The target population of this study comprised Palestinian women of reproductive age ≤35 years who experienced idiopathic reccurent pregnancy loss, with the aim of investigating potential genetic associations involving CTLA-4 and IL-6 gene polymorphisms. The subjects were divided into two groups. The first group was comprised of women who experienced RPL in the first trimester of pregnancy and the second group was 44 women who experienced RPL in the second trimester. Our findings indicated a significant association of the IL-6 polymorphism with RPL susceptibility, while the CTLA-4 indicated variant did not show significant association. Even though the A allele was observed to be slightly more frequent among RPL cases (64%) compared to controls (58%), the difference was not significant ($p = 0.27$). When analyzing the different genotypes of the CTLA-4

(rs231775) variant using various genetic models including the dominant and recessive models, there was no statistically significant correlation between this polymorphism and RPL. This suggests that this CTLA-4 polymorphism does not seem to play a crucial role in the development of RPL within our study population.

These findings on CTLA-4 (rs231775) contrasted with the results of a recent meta-analysis, which suggested a decreased risk of RPL associated with the A allele approaches (Song et al., 2019). The meta-analysis included six case-control studies and over 5,000 individuals. It was found that the AA genotype significantly reduced RPL risk compared to the GG genotype, particularly in Chinese and non-Chinese populations. This finding suggested a protective effect of the A allele which may be population-specific or influenced by other genetic and/or other environmental factors, therefore, while the meta-analysis highlighted the potential role of CTLA-4 in immune tolerance during pregnancy, our study did not support the involvement of this pathway in our study population. This result is mostly attributed to differences in sample ethnic backgrounds between various populations.

Several previous studies have investigated the role of the CTLA-4 +49 A/G polymorphism in RPL yielding varied results across the different populations. In a study conducted on the Iranian Azeri Turkish ethnic group (Bonyadi et al., 2017), researchers found no significant association between CTLA-4 +49 A/G and RPL. The genotype distributions among women with RPL and healthy controls were comparable which suggested that in this population, this polymorphism does not seem to contribute to RPL susceptibility.

Another study conducted on Tunisian women identified the A/G heterozygous genotype along with specific haplotypes of the CTLA-4 gene as potential risk factors for

RPL (Messaoudi et al., 2014). Furthermore, a study conducted in southwest Iran reported significant association between the G allele and decreased risk of idiopathic recurrent spontaneous abortion indicating its potential protective role against RPL (Rasti & Nasiri, 2016). Moreover, a study among Finnish women linked the G allele to an increased risk of preeclampsia and placental abruption both which can lead to pregnancy complications (Jääskeläinen et al., 2008).

One case-control study conducted in Gaza studied the same CTLA-4 +49 A/G gene polymorphism in addition to three other CTLA-4 gene polymorphisms (-1661 A/G, -318C/T and -1772 T/C. The data that we observed in our study corroborated the results of this study as the CTLA-4 +49 A/G gene polymorphism was reported insignificant in terms of RPL association. Additionally, the other three polymorphisms in this gene were reportedly not associated with RPL.

These discrepancies suggest that there may be underlying factors influencing the impact of the CTLA-4 +49 A/G polymorphism on RPL including ethnic variation, genetic backgrounds, sample size and other potential environmental factors. This is clearly apparent since some studies indicate a protective effect of the G allele, others associate it with increased risk lack of correlation. As a result, there is currently no clear general consensus regarding the role of CTLA-4 +49 A/G polymorphism in RPL. Further research involving larger, multi-ethnic cohorts and functional comprehensive analyses is required to understand the precise relationship between this polymorphism and RPL.

More recently, a study was conducted on the role of this SNP among the Iranian population utilizing two groups of women including 120 RPL cases with a history of recurrent pregnancy loss (RPL) and 120 healthy controls study (Nezamdoust et al., 2023). The role of the rs3087243, rs231775, and rs5742909 polymorphisms of the

CTLA-4 gene were studied. Evidently, no significant difference between the case and control groups regarding the rs231775 variant ($p = 0.37$). Similar to the finding made in our present study.

Data on the IL-6 (rs1800796) variant showed the G allele was significantly more frequent among RPL cases (94%) compared to controls (84%), while the C allele was conversely more prevalent among controls (16%) compared to the RPL subjects (6%) ($P=0.005$) suggesting a protective role of the C allele against RPL. Similarly, a study conducted in the Chinese population reported the G allele of the indicated IL-6 variant increased the risk of RPL, and women carrying the GG + CG genotypes had a significantly higher risk of RPL compared to subjects with the CC genotype (Liu et al., 2022).

The IL-6 -634C/G polymorphism has also been the subject of multiple studies investigating its association with RPL. One study conducted in Iran reported that women carrying the GG or GC genotypes of IL-6 -634C/G were at a higher risk of RPL compared to those with the CC genotype and the mutant G allele predisposed women to miscarriage 1.5 times more than controls (Rasti et al., 2016). Research conducted on Chinese population conversely indicated a protective effect associated with the CG genotype which was associated with a decreased risk of RPL and the GG genotype showed even lower risk (Ma et al., 2017). In a meta-analysis that involved relevant data from various ethnic disparities, revealed the IL-6 -634C/G polymorphism is clearly associated with RPL susceptibility in non-Caucasian populations, specifically among Asians (Lee et al., 2015). The G allele was associated with an increased risk of RPL. Once again, the divergent finding s underscore the complexity of genetic factors in RPL and suggest that the impact on RPL susceptibility differs across various ethnic groups.

In this study we chose to analyse the indicated genetic data from the RPL cases stratified by the specific trimester stage of all tested variants. Evidently, a stronger association was observed between the rs1800796 variant of the IL-6 gene in the first trimester compared to the second-trimester subjects. The CG genotype showed a significant protective role in the first-trimester ($p = 0.018$) with no association in second-trimester cases ($p = 0.23$) indicating trimester-specific genetic influence, where IL-6 plays a critical role in early pregnancy loss. Haplotype analysis showed that none of the individual haplotypes reached statistical significance, however, a clear trend was observed toward a protective effect of the C allele of IL-6 (rs1800796) where the A-C and G-C haplotypes had lower odds ratios (0.43 and 0.34, respectively), suggesting a potential protective effect.

These results highlight the importance of IL-6 polymorphism in RPL susceptibility, particularly in early pregnancy loss. The observed deviation from HWE as well as the statistical significance regarding the association between the C allele and reduced RPL risk suggest a potential impact on biological processes. The IL-6 plays a crucial role in immune regulation and inflammation and both processes are critical for pregnancy maintenance. The observed association between IL-6 (rs1800796) SNP and RPL might have concrete biological or molecular consequences such as altering IL-6 expression levels thereby affecting immune function or influencing inflammation pathways that are crucial for maintaining pregnancy. A variant that influences IL-6 expression may impact maternal-fetal immune tolerance which would then lead to pregnancy complications.

In contrast, the lack of significant association observed with the indicated CTLA-4 variant suggests that its role in RPL may be limited or influenced by other genetic

factors. As a key immune checkpoint regulator, the CTLA-4 has been implicated in autoimmune disorders however, its involvement in RPL remains uncertain.

Given the role of IL-6 in inflammation and immune regulation, therapeutic strategies targeting its signaling have been investigated in various autoimmune and inflammatory conditions. Increased IL-6 levels have been linked to adverse pregnancy outcomes, including recurrent abortion, likely due to heightened inflammatory responses at the maternal-fetal interface. IL-6 inhibitors, such as tocilizumab and siltuximab, have been explored for their potential to regulate inflammatory pathways and restore immune balance (Nakajima et al., 2016). However, their use during pregnancy remains challenging due to concerns regarding fetal safety and the intricate immunological adaptations necessary for a successful pregnancy. Further research is required to determine whether modulating IL-6 signaling could be a viable therapeutic option for individuals experiencing recurrent pregnancy loss associated with immune dysregulation.

The present study is influenced by several limitations which may have important impacts on the generated results and their association with RPL. First, the sample size may not include enough participants to allow the detection of moderate genetic variation and lacked statistical power to identify weaker associations. Secondly, the results lack the presence of functional studies linking, the effect of the observed genetic variation on IL-6 expression and function. Functional studies will allow direct analysis of genetic variation on gene expression and cytokine levels which may be crucial to confirm the biological relevance of the indicated gene variant and its potential impact on immune regulation during pregnancy.

Additionally, we analyzed the potential effect of the HLA-G Leu154fs*60 variant, which was clearly identified in a family as a major genetic factor linked to RPL among affected members (Maysa, 2024), using sanger sequencing to investigate its potential involvement among the RPL study group. HLA-G is known to play a crucial role in maternal-fetal immune tolerance by inhibiting immune responses against the semi-allogenic fetus. HLA is primarily expressed in the placenta and interacts with immune cells like NKs and T cells to promote immune tolerance (Zhuang et al., 2021). Due to its essential function in establishing and maintaining pregnancy, we sought to determine whether the **Leu154fs*60** frameshift variant, as it could potentially alter HLA-G protein function. Our results however, showed that none of the samples exhibited significance for this variant suggesting that it may not be a contributing factor in the cohort that we studied.

Conclusions and Recommendations

This study investigated the association between CTLA-4 +49 A/G (rs231775) and IL-6 -634 C/G (rs1800796) SNPs and susceptibility to RPL among Palestinian women. The results indicate a significant association between the IL-6 -634 C/G (rs1800796) polymorphism and increased risk of RPL, specifically in first-trimester losses, which suggests a trimester specific genetic influence. The C allele and CG genotype of IL-6 were associated with a protective effect against RPL, supporting the role of inflammatory cytokine regulation in the maintenance of pregnancy.

In contrast, the between CTLA-4 +49 A/G (rs231775) polymorphism, showed no significant association with RPL in our study group. This may reflect population-specific genetic differences, varying sample sizes, or the multifactorial nature of RPL. These findings emphasize the importance of genetic screening and trimester-specific analysis in

imporoving our understanding of RPL etiology. IL-6 gene variants may potentially serve as genetic markers for identifying women at increased risk of RPL which would aid in early diagnosis and personalized care strategies

Reccomendations

1. Larger, multicenter studies across diverse populations are recommended to validate these findings and assess the consistency of IL-6 and CTLA-4 associations with RPL.
2. Functional studies are necessary to elucidate the biological mechanisms by which IL-6 polymorphisms influence pregnancy outcomes.
3. Inclusion of other cytokine and immune-regulatory genes may provide a more comprehensive genetic profile contributing to RPL susceptibility.
4. Consideration of environmental and lifestyle factors in future analyses is essential to understand gene-environment interactions in RPL.
5. The integration of genetic screening into clinical practice should be explored, particularly for high-risk groups of women with unexplained RPL, to guide targeted interventions or counseling

References

Abdollahi, E., Saghafi, N., Rezaee, S. A. R., Rastin, M., Jarahi, L., Clifton, V., & Rafatpanah, H. (2020). Evaluation of 1,25(Oh)2d3 effects on foxp3, ror-γt, gitr, and ctla-4 gene expression in the pbmcs of vitamin d-deficient women with unexplained recurrent pregnancy loss (urpl). *Iranian Biomedical Journal*, *24*(5). https://doi.org/10.29252/ibj.24.5.290

Abo-alella, D. A., Ouda, I. M., Abd Elhady, R. R., & El Shahawy, A. A. (2021). Interleukin-6-634 G/C gene polymorphisms in recurrent pregnancy loss among Egyptian women: Does it make a difference? *Meta Gene*, *30*. https://doi.org/10.1016/j.mgene.2021.100955

American Society for Reproductive Medecine. (2012). Practice Committee of American Society for Reproductive Medicine Evaluation and treatment of recurrent pregnancy loss: a committee opinion. *Fertility and Sterility*, *98*.

Ander, S. E., Diamond, M. S., & Coyne, C. B. (2019). Immune responses at the maternal-fetal interface. In *Science Immunology* (Vol. 4, Issue 31). https://doi.org/10.1126/sciimmunol.aat6114

Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T. B., Oukka, M., Weiner, H. L., & Kuchroo, V. K. (2006). Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature*, *441*(7090). https://doi.org/10.1038/nature04753

Bonyadi, M., Parsa, S., Taghavi, S., & Zeinalzadeh, N. (2017). Association study of CTLA-4 +49A/G gene polymorphism with recurrent pregnancy loss in the Iranian Azeri Turkish ethnic group. *Turkish Journal of Medical Sciences*, *47*(3), Article 9.

Chen, Y., Chen, S., Gu, Y., Feng, Y., Shi, Y., Fu, Q., Wang, Z., Cai, Y., Dai, H., Zheng, S., Sun, M., Zhang, M., Xu, X., Chen, H., Xu, K., & Yang, T. (2018). CTLA-4 +49 G/A, a functional T1D risk SNP, affects CTLA-4 level in Treg subsets and IA-2A

positivity, but not beta-cell function. *Scientific Reports*, *8*(1). https://doi.org/10.1038/s41598-018-28423-9

Cork, B. A., Tuckerman, E. M., Li, T. C., & Laird, S. M. (2002). Expression of interleukin (IL)-11 receptor by the human endometrium in vivo and effects of IL-11, IL-6 and LIF on the production of MMP and cytokines by human endometrial cells in vitro. *Molecular Human Reproduction*, *8*(9). https://doi.org/10.1093/molehr/8.9.841

Corthay, A. (2009). How do regulatory t cells work? In *Scandinavian Journal of Immunology* (Vol. 70, Issue 4). https://doi.org/10.1111/j.1365-3083.2009.02308.x

Daher, S., Mattar, R., Gueuvoghlanian-Silva, B. Y., & Torloni, M. R. (2012). Genetic Polymorphisms and Recurrent Spontaneous Abortions: An Overview of Current Knowledge. In *American Journal of Reproductive Immunology* (Vol. 67, Issue 4). https://doi.org/10.1111/j.1600-0897.2012.01123.x

Definitions of infertility and recurrent pregnancy loss: a committee opinion. (2020). *Fertility and Sterility*, *113*(3). https://doi.org/10.1016/j.fertnstert.2019.11.025

d'Hauterive, S. P., Charlet-Renard, C., Berndt, S., Dubois, M., Munaut, C., Goffin, F., Hagelstein, M. T., Noël, A., Hazout, A., Foidart, J. M., & Geenen, V. (2004). Human chorionic gonadotropin and growth factors at the embryonic-endometrial interface control leukemia inhibitory factor (LIF) and interleukin 6 (IL-6) secretion by human endometrial epithelium. *Human Reproduction*, *19*(11). https://doi.org/10.1093/humrep/deh450

Dias, F. C., Medina, T. da S., Mendes-Junior, C. T., Dantas, R. O., Pissetti, C. W., Rodrigues Junior, V., Dellalibera-Joviliano, R., Marin-Neto, J. A., Gutierrez, F. R. S., Moreau, P., Silva, J. S., & Donadi, E. A. (2013). Polymorphic Sites at the Immunoregulatory CTLA-4 Gene Are Associated with Chronic Chagas Disease and

Its Clinical Manifestations. *PLoS ONE*, *8*(10). https://doi.org/10.1371/journal.pone.0078367

Dimitriadis, E., Menkhorst, E., Saito, S., Kutteh, W., & Brosens, J. (2020). Recurrent pregnancy loss. *Nature Reviews Disease Primers* , *6*(98).

El Hachem, H., Crepaux, V., May-Panloup, P., Descamps, P., Legendre, G., & Bouet, P. E. (2017). Recurrent pregnancy loss: Current perspectives. In *International Journal of Women's Health* (Vol. 9). https://doi.org/10.2147/IJWH.S100817

Endometriosis. Guideline of European Society of Human Reproduction and Embryology – 2022. (2022). *REPRODUCTIVE ENDOCRINOLOGY*, *66*. https://doi.org/10.18370/2309-4117.2022.66.8-19

Ford, H. B., & Schust, D. J. (2009). Recurrent pregnancy loss: etiology, diagnosis, and therapy. *Rev Obstet Gynecol*, *2*(2), 76–83.

Frauwirth, K. A., & Thompson, C. B. (2002). Activation and inhibition of lymphocytes by costimulation. *Journal of Clinical Investigation*, *109*(3). https://doi.org/10.1172/jci14941

Garrido-Gimenez, C., & Alijotas-Reig, J. (2015). Recurrent miscarriage: Causes, evaluation and management. *Postgraduate Medical Journal*, *91*(1073). https://doi.org/10.1136/postgradmedj-2014-132672

Gough, S. C. L., Walker, L. S. K., & Sansom, D. M. (2005). CTLA4 gene polymorphism and autoimmunity. In *Immunological Reviews* (Vol. 204). https://doi.org/10.1111/j.0105-2896.2005.00249.x

Günther, V., Allahqoli, L., Deenadayal-Mettler, A., Maass, N., Mettler, L., Gitas, G., Andresen, K., Schubert, M., Ackermann, J., von Otte, S., & Alkatout, I. (2023). Molecular Determinants of Uterine Receptivity: Comparison of Successful Implantation, Recurrent Miscarriage, and Recurrent Implantation Failure. In

International Journal of Molecular Sciences (Vol. 24, Issue 24). https://doi.org/10.3390/ijms242417616

Gynaecologists, R. C. of O. and. (2011). The Investigation and Treatment of Couples with Recurrent First-trimester and Second-trimester Miscarriage. *RCOG Green-Top Guideline No. 17*.

Hosseini, A., Gharibi, T., Marofi, F., Babaloo, Z., & Baradaran, B. (2020). CTLA-4: From mechanism to autoimmune therapy. In *International Immunopharmacology* (Vol. 80). https://doi.org/10.1016/j.intimp.2020.106221

Hussein, A. S., Darwish, H., & Shelbayeh, K. (2010). Association between factor v Leiden mutation and poor pregnancy outcomes among Palestinian women. *Thrombosis Research*, *126*(2). https://doi.org/10.1016/j.thromres.2010.04.017

Hyde, K. J., & Schust, D. J. (2015). Genetic considerations in recurrent pregnancy loss. *Cold Spring Harbor Perspectives in Medicine*, *5*(3). https://doi.org/10.1101/cshperspect.a023119

Jääskeläinen, E., Toivonen, S., Keski-Nisula, L., Paattiniemi, E. L., Helisalmi, S., Punnonen, K., & Heinonen, S. (2008). CTLA-4 polymorphism 49A-G is associated with placental abruption and preeclampsia in Finnish women. *Clinical Chemistry and Laboratory Medicine : CCLM / FESCC*, *46*(2). https://doi.org/10.1515/CCLM.2008.034

Jarvis, L. B., Goodall, J. C., & Gaston, J. S. H. (2008). Human leukocyte antigen class I-restricted immunosuppression by human CD8+ regulatory T cells requires CTLA-4-mediated interaction with dendritic cells. *Human Immunology*, *69*(11). https://doi.org/10.1016/j.humimm.2008.08.277

Jauniaux, E., & Burton, G. J. (2005). Pathophysiology of histological changes in early pregnancy loss. *Placenta*, *26*(2–3). https://doi.org/10.1016/j.placenta.2004.05.011

Kirovakov, Z., Konova, E., Hinkova, N., Markova, S., & Penchev, P. (2024). Immunological Risk Factors in Recurrent Pregnancy Loss in Patients With Hereditary Thrombophilia. *Cureus*, *16*(3), e56555.

Kniotek, M., Zych, M., Roszczyk, A., Szafarowska, M., & Jerzak, M. M. (2021). Decreased production of tnf-α and il-6 inflammatory cytokines in non-pregnant idiopathic rpl women immunomodulatory effect of sildenafil citrate on the cellular response of idiopathic rpl women. *Journal of Clinical Medicine*, *10*(14). https://doi.org/10.3390/jcm10143115

Kwon, M. J., Kim, J. H., Kim, K. J., Ko, E. J., Lee, J. Y., Ryu, C. S., Ha, Y. H., Kim, Y. R., & Kim, N. K. (2023). Genetic Association between Inflammatory-Related Polymorphism in STAT3, IL-1β, IL-6, TNF-α and Idiopathic Recurrent Implantation Failure. *Genes*, *14*(8). https://doi.org/10.3390/genes14081588

Lee, Y. H., Choi, S. J., & Ji, J. D. (2015). Association between IL-6 −174 G/C, IL-6 −634 G/C, and IFN-γ +874 A/T polymorphisms and susceptibility to recurrent pregnancy loss: a meta-analysis. *Journal of Assisted Reproduction and Genetics*, *32*(9). https://doi.org/10.1007/s10815-015-0566-3

Ling, V., Wu, P. W., Finnerty, H. F., Sharpe, A. H., Gray, G. S., & Collins, M. (1999). Complete sequence determination of the mouse and human CTLA4 gene loci: Cross-species DNA sequence similarity beyond exon borders. *Genomics*, *60*(3). https://doi.org/10.1006/geno.1999.5930

Liu, Q., Li, Y., Li, J., Wang, Y., & Mao, B. (2022). Association of the IL-6 gene rs1800796 polymorphism with recurrent pregnancy loss in a population of women from northwestern China: a case-control study. *Journal of Obstetrics and Gynaecology*, *42*(7). https://doi.org/10.1080/01443615.2022.2109407

Liu, Y., Gao, S., Zhao, Y., Wang, H., Pan, Q., & Shao, Q. (2021). Decidual Natural

Killer Cells: A Good Nanny at the Maternal-Fetal Interface During Early Pregnancy. In *Frontiers in Immunology* (Vol. 12). https://doi.org/10.3389/fimmu.2021.663660

Lund, M., Nielsen, H. S., Hviid, T. V., Steffensen, R., Nyboe Andersen, A., & Christiansen, O. B. (2010). Hereditary thrombophilia and recurrent pregnancy loss: A retrospective cohort study of pregnancy outcome and obstetric complications. *Human Reproduction*, *25*(12). https://doi.org/10.1093/humrep/deq280

Ma, J., Zhang, X., He, G., & Yang, C. (2017). Association between TNF, IL1B, IL6, IL10 and IFNG polymorphisms and recurrent miscarriage: A case control study. *Reproductive Biology and Endocrinology*, *15*(1). https://doi.org/10.1186/s12958-017-0300-3

Maysa, S. (2024). Vitamin D and Recurrent Abortion: Inference through several Vitamin D Receptor (VDR) gene polymorphisms and vitamin D status among the inflicted Palestinian women. Thesis submitted to the *Arab American University*.

Messaoudi, S., Houas, I., Yaseen, K., Dandana, M., & Mahjoub, T. (2014). CTLA-4 gene polymorphisms and risk of idiopathic recurrent pregnancy loss in a Tunisian population. *BMC Genomics*, *15*(S2). https://doi.org/10.1186/1471-2164-15-s2-p11

Moffett, A., & Colucci, F. (2014). Uterine NK cells: Active regulators at the maternal-fetal interface. In *Journal of Clinical Investigation* (Vol. 124, Issue 5). https://doi.org/10.1172/JCI68107

Moghbeli, M. (2019). Genetics of recurrent pregnancy loss among Iranian population. In *Molecular Genetics and Genomic Medicine* (Vol. 7, Issue 9). https://doi.org/10.1002/mgg3.891

Mohammadi, S., Abdollahi, E., Nezamnia, M., Esmaeili, S. A., Tavasolian, F., Sathyapalan, T., & Sahebkar, A. (2021). Adoptive transfer of Tregs: A novel

strategy for cell-based immunotherapy in spontaneous abortion: Lessons from experimental models. In *International Immunopharmacology* (Vol. 90). https://doi.org/10.1016/j.intimp.2020.107195

Moradkhani, A.; Azami, M.; Assadi, S. . et al. (2024). Association of vitamin D receptor genetic polymorphisms with the risk of infertility: a systematic review and meta-analysis. *BMC Pregnancy and Childbirth*, *24*, 398.

Nakajima, K., Watanabe, O., Mochizuki, M., Nakasone, A., Ishizuka, N., & Murashima, A. (2016). Pregnancy outcomes after exposure to tocilizumab: A retrospective analysis of 61 patients in Japan. *Modern Rheumatology*, *26*(5). https://doi.org/10.3109/14397595.2016.1147405

Nasiri, M., & Rasti, Z. (2016). CTLA-4 and IL-6 gene polymorphisms: Risk factors for recurrent pregnancy loss. *Human Immunology*, *77*(12). https://doi.org/10.1016/j.humimm.2016.07.236

National Center for Biotechnology Information (NCBI). (n.d.-a). *CTLA-4cytotoxic T-lymphocite associated protein 4 [Homo sapiens (human)]*. Retrieved June 23, 2025, from https://www.ncbi.nlm.nih.gov/gene/1493

National Center for Biotechnology Information (NCBI). (n.d.-b). *IL6 interleukin 6 [Homo sapiens (human)]*. Retrieved June 23, 2025, from https://www.ncbi.nlm.nih.gov/gene/3569

Nelen, W. L. D. M., Blom, H. J., Steegers, E. A. P., Den Heijer, M., & Eskes, T. K. A. B. (2000). Hyperhomocysteinemia and recurrent early pregnancy loss: A meta-analysis. *Fertility and Sterility*, *74*(6). https://doi.org/10.1016/S0015-0282(00)01595-8

Nezamdoust, F. V., Hadinedoushan, H., & Ghasemi, N. (2023). Association of cytotoxic T-lymphocyte-associated protein 4 polymorphisms with recurrent pregnancy loss: A case-control study. *International Journal of Reproductive BioMedicine*, *21*(1). https://doi.org/10.18502/ijrm.v21i1.12664

Parveen, F., Shukla, A., & Agarwal, S. (2013). Cytokine gene polymorphisms in

northern Indian women with recurrent miscarriages. *Fertility and Sterility*, *99*(2). https://doi.org/10.1016/j.fertnstert.2012.09.025

Pillarisetty, L. S., & Mahdy, H. (2024). Recurrent Pregnancy Loss. In *StatPearls [Internet]*. StatPearls Publishing.

Quenby, S., Gallos, I. D., Dhillon-Smith, R. K., Podesek, M., Stephenson, M. D., Fisher, J., Brosens, J. J., Brewin, J., Ramhorst, R., Lucas, E. S., McCoy, R. C., Anderson, R., Daher, S., Regan, L., Al-Memar, M., Bourne, T., MacIntyre, D. A., Rai, R., Christiansen, O. B., … Coomarasamy, A. (2021). Miscarriage matters: the epidemiological, physical, psychological, and economic costs of early pregnancy loss. In *The Lancet* (Vol. 397, Issue 10285). https://doi.org/10.1016/S0140-6736(21)00682-6

Rackaityte, E., & Halkias, J. (2020). Mechanisms of Fetal T Cell Tolerance and Immune Regulation. In *Frontiers in Immunology* (Vol. 11). https://doi.org/10.3389/fimmu.2020.00588

Rasti, Z., & Nasiri, M. (2016). Association of the +49 A/G polymorphism of CTLA4 gene with idiopathic recurrent spontaneous abortion in women in southwest of Iran. *Journal of Reproduction and Infertility*, *17*(3).

Rasti, Z., Nasiri, M., & Kohan, L. (2016). The IL-6 -634C/G polymorphism: A candidate genetic marker for the prediction of idiopathic recurrent pregnancy loss. *International Journal of Reproductive BioMedicine*, *14*(2). https://doi.org/10.29252/ijrm.14.2.103

Read, S., Malmström, V., & Powrie, F. (2000). Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25+CD4+ regulatory cells that control intestinal inflammation. *Journal of Experimental Medicine*, *192*(2). https://doi.org/10.1084/jem.192.2.295

Saijo, Y., Sata, F., Yamada, H., Kondo, T., Kato, E. H., & Kishi, R. (2004). Single nucleotide polymorphisms in the promoter region of the interleukin-6 gene and the risk of recurrent pregnancy loss in japanese women. *Fertility and Sterility*, *81*(2). https://doi.org/10.1016/j.fertnstert.2003.06.023

Salzer, E., Santos-Valente, E., Klaver, S., Ban, S. A., Emminger, W., Prengemann, N. K., Garncarz, W., Müllauer, L., Kain, R., Boztug, H., Heitger, A., Arbeiter, K., Eitelberger, F., Seidel, M. G., Holter, W., Pollak, A., Pickl, W. F., Förster-Waldl, E., & Boztug, K. (2013). B-cell deficiency and severe autoimmunity caused by deficiency of protein kinase C δ. *Blood*, *121*(16). https://doi.org/10.1182/blood-2012-10-460741

Sasaki, Y., Sakai, M., Miyazaki, S., Higuma, S., Shiozaki, A., & Saito, S. (2004). Decidual and peripheral blood CD4 +CD25 + regulatory T cells in early pregnancy subjects and spontaneous abortion cases. *Molecular Human Reproduction*, *10*(5). https://doi.org/10.1093/molehr/gah044

Sharma, A., & Rudra, D. (2018). Emerging functions of regulatory T cells in tissue homeostasis. In *Frontiers in Immunology* (Vol. 9, Issue APR). https://doi.org/10.3389/fimmu.2018.00883

Song, Y., Chen, Y., & Xu, Q. (2019). Association among cytotoxic T-lymphocyte antigen 4 gene, rs231775 polymorphism, and recurrent pregnancy loss risk. *Bioscience Reports*, *39*(2). https://doi.org/10.1042/BSR20181760

Takahashi, T., Tagami, T., Yamazaki, S., Uede, T., Shimizu, J., Sakaguchi, N., Mak, T. W., & Sakaguchi, S. (2000). Immunologic self-tolerance maintained by CD25+CD4+ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *Journal of Experimental Medicine*, *192*(2). https://doi.org/10.1084/jem.192.2.303

Tanaka, A., & Sakaguchi, S. (2017). Regulatory T cells in cancer immunotherapy. In *Cell Research* (Vol. 27, Issue 1). https://doi.org/10.1038/cr.2016.151

Ticconi, C., Pietropolli, A., Di Simone, N., Piccione, E., & Fazleabas, A. (2019). Endometrial immune dysfunction in recurrent pregnancy loss. In *International Journal of Molecular Sciences* (Vol. 20, Issue 21). https://doi.org/10.3390/ijms20215332

Ueda, H., Howson, J. M. M., Esposito, L., Heward, J., Snook, H., Chamberlain, G., Rainbow, D. B., Hunter, K. M. D., Smith, A. N., Di Genova, G., Herr, M. H., Dahlman, I., Payne, F., Smyth, D., Lowe, C., Twells, R. C. J., Howlett, S., Healy, B., Nutland, S., … Gough, S. C. L. (2003). Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. *Nature*, *423*(6939). https://doi.org/10.1038/nature01621

Vaiman, D. (2015). Genetic regulation of recurrent spontaneous abortion in humans. In *Biomedical Journal* (Vol. 38, Issue 1). https://doi.org/10.4103/2319-4170.133777

Van Coillie, S., Wiernicki, B., & Xu, J. (2020). Molecular and Cellular Functions of CTLA-4. In *Advances in Experimental Medicine and Biology* (Vol. 1248). https://doi.org/10.1007/978-981-15-3266-5_2

van Mourik, M. S. M., Macklon, N. S., & Heijnen, C. J. (2009). Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment. *Journal of Leukocyte Biology*, *85*(1). https://doi.org/10.1189/jlb.0708395

Vomstein, K., Feil, K., Strobel, L., Aulitzky, A., Hofer-Tollinger, S., Kuon, R. J., & Toth, B. (2021). Immunological risk factors in recurrent pregnancy loss: Guidelines versus current state of the art. In *Journal of Clinical Medicine* (Vol. 10, Issue 4). https://doi.org/10.3390/jcm10040869

Wang, X., Lin, Q., Ma, Z., Hong, Y., Zhao, A., Di, W., & Lu, P. (2005). Association of the A/G polymorphism at position 49 in exon 1 of CTLA-4 with the susceptibility to unexplained recurrent spontaneous abortion in the Chinese population. *American Journal of Reproductive Immunology*, *53*(2). https://doi.org/10.1111/j.1600-0897.2004.00251.x

Wu, Z., Wang, M., Liang, G., Jin, P., Wang, P., Xu, Y., Qian, Y., Jiang, X., Qian, J., & Dong, M. (2021). Pro-Inflammatory Signature in Decidua of Recurrent Pregnancy Loss Regardless of Embryonic Chromosomal Abnormalities. *Frontiers in Immunology*, *12*. https://doi.org/10.3389/fimmu.2021.772729

Zenclussen, A. C. (2006). Regulatory T cells in pregnancy. *Springer Seminars in Immunopathology*, *28*(1), 31–39.

Zhuang, B., Shang, J., & Yao, Y. (2021). HLA-G: An Important Mediator of Maternal-Fetal Immune-Tolerance. In *Frontiers in Immunology* (Vol. 12). https://doi.org/10.3389/fimmu.2021.744324

Zhang, M., Xu, J., Bao, X., Niu, W., Wang, L., Du, L., Zhang, N., & Sun, Y. (2017). Association between genetic polymorphisms in interleukin genes and recurrent pregnancy loss - A systematic review and meta-analysis. *PLoS ONE*, *12*(1). https://doi.org/10.1371/journal.pone.0169891

Appendices

1. Consent form

موافقة للمشاركة في دراسة بحثية

تهدف هذه الدراسة لتحديد الطفرات الوراثية في مورثتي (جينات) CTLA-4 and IL-6 المرتبطة بحالات الاجهاض المتكرر لدى السيدات الفلسطينيات. هذه الدراسة يُجريها فريق البحث تحت إشراف أ.د. هشام درويش في الجامعة العربية الأمريكية كجزء من رسالة الماجستير للطالبة سما درويش في برنامج الوراثة الجزيئية والسمية الجينية.

ما الهدف من هذه الدراسة؟

تجميع عينات الدم من المرضى لمساعدة الباحثين لمحاولة فهم سبب الأمراض وتطوير فحوصات جديدة لكشف ومراقبة هذه الأمراض، للوقاية منها وعلاج بعضها أو كلها في المستقبل، وعمل التوصيات اللازمة للتعامل معها. ومن المتوقع مشاركة 100 سيده تعاني من الاجهاض المتكررفي المجتمع الفلسطيني, ولن يكون هناك أية تكاليف عليك عند مشاركتك بهذه الدراسة.

ما المطلوب مني في هذه الدراسة؟

سنقوم بأخذ عينة دم (3-5 ml) من أحد أوردة ذراعك. كما وسنطلب منك بعض المعلومات التي ستساعدنا في تقييم نتائج البحث. وسيتم ذلك مع المحافظة على سرية وخصوصية هذه المعلومات. وجميع عينات الدم التي سيتم جمعها ستحفظ في مختبر الوراثة الجزيئية للأبحاث في حرم الجامعة الأمريكية في حي الريحان قرب رام الله. لذا فإن اسمك أو أية معلومات قد تقوم بالتعريف بك ستزال عن عينة الدم ومن كل المعلومات عن وضعك الطبي وسيرتك المرضية التي ترافق عينتك، وستحفظ عن طريق إعطاء رمز خاص بك. وهذا الرمز سيكون معروفا فقط لدى المسؤولين عن جمع العينات. وسيتم ترتيب عملية سحب الدم في وقت يناسبك ، علما بأنه يمكنك اختيار التوقف عن المشاركة في أي وقت مستقبلا بعد إعلام الباحثين شخصيا بذلك. لن نقوم بالدفع لك لمشاركتك بهذه الدراسة.

بمن أتصل في حال كانت لدي أسئلة

للسؤال عن الدراسة أو إذا كانت أو أسئلة يرجى الاتصال ب أ.د. هشام درويش أو الطالبة سما درويش على العنوان الالكتروني التالي: hisham.darwish@aaup.edu
s.darwish2@student.aaup.edu

نص الموافقة

تم شرح الهدف من هذه الدراسة لي، والخطوات التي سيتم اتباعها. وسمح لي بسؤال أسئلة، وتمت الإجابة عن تساؤلاتي لحدٍ يرضيني. لقد تم إخباري بمن أتصل إذا كانت لدي تساؤلات، أو للحصول على معلومات، أو إعطاء أية إضافات حول البحث. لقد قمت بقراءة وثيقة الموافقة هذه وأوافق على المشاركة بهذه الدراسة، مع العلم أنه بإمكاني الانسحاب متى شئت.

☐ أوافق على أن يتم أخذ عينة دم مني لفحص المادة الوراثية.

الاسم الثلاثي للمشارك _____

توقيع المشارك _____ التاريخ _____

الاسم الثلاثي للباحث/ة: سما خالد درويش

توقيع الباحث/ة _____ التاريخ _____

2. Questionnaire

Case study

Patient's Name: _____ Age:_____

Patient's Number: _____

Please answer the following questions

- How many times she had experienced recurrent pregnancy loss? _____
- In which trimester? _____

Please clearly state if the patient suffers from any of the following conditions:

- Factor V mutations Yes◯ No◯ NA ◯
- Factor 2 prothrombin gene (PT G20210A) mutation Yes ◯ No◯ NA ◯
- Protein S deficiency Yes ◯ No ◯ NA ◯
- Protein C deficiency Yes ◯ No◯ NA ◯
- Anti prothrombin antibody Yes ◯ No◯ NA ◯
- Chromosomal anomalies Yes ◯ No◯ NA ◯
- Cigarette smoking Yes ◯ No◯ NA ◯
- Alcohol consumption Yes ◯ No◯ NA ◯
- Uterine abnormalities Yes ◯ No◯ NA ◯
- Autoimmune diseases Yes◯ No◯ NA◯
- Infectious diseases Yes◯ No◯ NA◯
- endocrine diseases (diabetes mellitus, thyroid diseases) Yes ◯ No◯ NA◯
- Rh blood group incompatibility Yes◯ No◯ NA◯
- Any history of preterm birth or preeclampsia Yes ◯ No◯ NA ◯
- Successful pregnancy Yes◯ No◯ NA ◯
  -If yes, how many times? _____

الملخص

الإجهاض المتكرر (RPL) يُعرّف بأنه اضطراب حملي معقد يتضمن الفشل أو الفقدان المتكرر للحمل. حوالي 3% من النساء يعانين من فقدان الحمل المتكرر. لم يتم بعد تحديد معدل انتشار هذا الاضطراب في فلسطين. وتهدف هذه الدراسة إلى فحص العلاقة بين تعدد الأشكال أحادي النوكليوتيد (SNP) في جيني CTLA-4 (rs231775) و IL-6 (rs1800796) مع فقدان الحمل المتكرر في فلسطين. تم استخدام تقنية (RFLP) لتحليل هذه التعددات الجينية في مجموعة مكونة من 172 شخصاً؛ 88 مريضة و84 من الأصحاء كعينة ضابطة. 44 من المريضات تعرضن للإجهاض المتكرر في الثلث الأول من الحمل، و44 منهن في الثلث الثاني. أظهرت التحليلات الجينية والأليلية عدم وجود ارتباط ذي دلالة إحصائية بين تعدد أشكال CTLA-4 وRPL. من ناحية أخرى، أظهر تعدد أشكال IL-6 ارتباطًا ذا دلالة إحصائية، حيث يبدو أن الأليل C له تأثير وقائي ضد فقدان الحمل المتكرر ($p = 0.005$). أشارت التحليلات الخاصة بالثلث الأول من الحمل إلى وجود ارتباط أقوى بين تعدد أشكال IL-6 وحالات فقدان الحمل في الثلث الأول. كما دعمت تحليلات الأنماط الجينية (haplotype) الدور الوقائي للأليل C. تتماشى هذه النتائج مع نتائج دراسات سابقة أكدت دور IL-6 في تنظيم المناعة خلال الحمل. ومع ذلك، هناك حاجة إلى مزيد من الدراسات الوظيفية لتأكيد تأثير SNP الخاص بـ IL-6 (rs1800796) خلال الحمل وفهم الآليات البيولوجية التي تكمن وراء هذا الارتباط. تؤكد هذه الدراسة على أهمية الفحص الجيني في تحديد القابلية للإصابة بـ RPL واستكشاف استراتيجيات علاجية محتملة.