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CTLA-4 Gene Polymorphisms in Women with Idiopathic Recurrent Pregnancy Loss

التعدد الشكلي لجين مستضدات الخلايا الليمفاوية التائية السامة-4 عند النساء اللاتي تعاني من الاجهاض المتكرر مجهول السبب

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Supervised by

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إقـــــرار

أنا الموقع أدناه مقدم الرسالة التي تحمل العنوان:

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نتيجة الحكم على أطروحة ماجستير

بناءً على موافقة شئون البحث العلمي والدراسات العليا بالجامعة الإسلامية بغزة على تشكيل لجنة الحكم على أطروحة الباحثة/ **ايمان نايف سليمان حلس** لنيل درجة الماجستير في **كلية *العلوم*** قسم **العلوم الحياتية– تحاليل طبية** وموضوعها:

التعدد الشكلي لجين مستضدات الخلايا الليمفاوية التائية السامة –4 عند النساء اللاتي تعاني من الاجهاض المتكرر مجهول السبب

CTLA-4 gene polymorphisms in women with idiopathic recurrent pregnancy loss

وبعد المناقشة التي تمت اليوم الاثنين 06 شوال 1437هـ، الموافق 2016/07/11الساعة الحادية عشر صباحاً، اجتمعت لجنة الحكم على الأطروحة والمكونة من:

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وبعد المداولة أوصت اللجنة بمنح الباحث درجة الماجستير في كلية *العلوم*/ قسم **العلوم الحياتية – تحاليل طبية**.

واللجنة إذ تمنحه هذه الدرجة فإنها توصيه بتقوى الله ولزوم طاعته وأن يسخر علمه في خدمة دينه ووطنه.

والله ولي التوفيق،،،

نائب الرئيس لشئون البحث العلمي والدراسات العليا

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Abstract

CTLA-4 Gene Polymorphisms in Women with Idiopathic Recurrent Pregnancy Loss

Background:

Recurrent pregnancy loss (RPL) is the miscarriage of two or more consecutive pregnancies before 20^{th} gestational week. We suggest that dysregulated immune-tolerance contributes to idiopathic RPL. Cytotoxic T lymphocyte associated antigen-4 (CTLA-4) is considered as a negative regulator of T cell activation and its role in maintaining immune-tolerance is well established.

Objective:

The present study aimed to investigate the *CTLA-4* +49 A/G , -1661 A/G, -318 C/T and -1722 T/C single nucleotide polymorphisms (SNPs) and predisposition to RPL in Gaza Strip - Palestine.

Methods:

This case-control study was performed on DNA samples from 200 women with a history of two or more pregnancy losses (case group) and 200 control women with at least two live births and without any previous history of RPL. PCR-based restriction fragment length polymorphism (RFLP-PCR) method was used for genotyping *CTLA-4* polymorphisms.

Results:

Our study results revealed that there is no significant association between the allele/genotype frequencies of the investigated *CTLA-4* SNPs and RPL. This relation remained true under dominant, co-dominant and recessive models. The A\G genotype of -1661 A\G polymorphism was higher in patient (45%) as compared to controls (39.5%) but without statistical significance. The minor allele frequencies (MAFs) of the *CTLA-4* gene polymorphisms in the control group were as follows: +49A\G: 0.22, 318 C\T: 0.11, -1661 A\G: 0.26 and -1722T\C: 0.08.

Conclusion:

The study showed that there is no significant association between the four investigated *CTLA-4* polymorphisms and the risk of RPL in the study population. Testing other *CTLA-4* gene polymorphisms and the level of *CTLA-4* expression in RPL patients is recommended.

Key words:

Recurrent pregnancy loss, *CTLA-4*, Gene Polymorphism, PCR-RFLP.

Abstract in Arabic

الملخص

التعدد الشكلي لجين مستضدات الخلايا الليمفاوية التائية السامة -4 عند النساء اللاتي تعاني من الاجهاض المتكرر مجهول السبب

خلفية البحث:

يعرف فقدان الحمل المتكرر على انه إجهاض تلقائي يحدث لمرتين متكررتين أو اكثر قبل الأسبوع العشرين من الحمل. و حيث أن 50% من هذه الحالات لا يوجد لها أسباب معروفة فإننا نعتقد أن أسباب عدم تنظيم في التحمل المناعي تساهم في حدوث ذلك. يعتبر المستضد المرافق للمفاويات التائية السامة للخلايا رقم 4 (CTLA-4) هو المنظم السلبي لتفعيل الخلايا التائية و له دور في المحافظة على إيجاد التحمل المناعي.

أهداف البحث:

تهدف الدراسة الحالية لدراسة أربع أنماط من الجين *CTLA-4* وهي- 318 C/T ,-1661 A/G , +49 A/G , 1722 T/C و علاقتها بالتسبب بفقدان الحمل المتكرر مجهول السبب في قطاع غزة – فلسطين.

منهجية البحث:

تم دراسة الحالات و الشواهد لهذه الدراسة على عينات من الحمض النووي ل 200 امرأة لهن تاريخ من فقدان الحمل لمرتين متكررتين فاكثر (مجموعة الدراسة) و كذلك دراسة مجموعة ضابطة تكونت من 200 امرأة انجبن أطفالا أحياء مرتين على الأقل و ليس لهن تاريخ مرضي.
تم استخدام طريقة تعدد شكل طول جزء الحصر المعتمد على تفاعل البوليميراز المتسلسل (RFLP-PCR) لفحص تعدد أشكال النمط الجيني للمستضد المرافق للمفاويات التائية السامة للخلايا رقم 4 (CTLA-4) .

نتائج البحث:

أظهرت النتائج عدم وجود علاقة ذات دلالة إحصائية مهمة بين الأنماط الجينية المفحوصة وبين فقدان الحمل المتكرر في مجتمع الدراسة, كما ظهرت نفس النتيجة في كل من النموذج السائد والسائد المشترك والمتنحي. النمط الجيني A\G ل "1661 A\G-" كان بنسبة أعلى في المرضى (45%) مقارنة بالمجموعة الضابطة (39.5%) إلا انها لم تكن ذات دلالة احصائية. تكرار الاليلات الثانوية للتغيرات الجينية الاربعة في المجموعة الضابطة كالاتي : .1722T\C: 0.08 , 1661 A\G: 0.26, 318 C\T: 0.11, +49A\G: 0.22

خلاصة البحث:

أشارت الدراسة إلى عدم وجود علاقة ذات أهمية بين الأنماط الجينية الأربعة قيد الاختبار CTLA-4 وبين مخاطر حدوث الفقدان المتكرر للحمل عند مجتمع الدراسة. وينصح بدراسة التغير في التعبير الجيني في حالات فقدان الحمل المتكرر، والبحث عن أنماط أخرى لهذا الجين قد يكون لها صلة بفقدان الحمل المتكرر.

كلمات مفتاحية: فقدان الحمل المتكرر، جين مستضد الخلايا اللمفاوية التائية السامة للخلايا-4، *تعدد أشكال الجين، تقنية* PCR-RFLP

Dedication

To all of them I dedicate this work, fulfillment and recognition:

My great parents, who enlightened my way all the time, may Allah protect them.

All my wonderful brothers and sisters, for their endless love and support.

My teachers, friends and great family.

The souls of the martyrs of Palestine.

All Palestinian people all over the world.

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ACA	Anticardiolipin antibodies
ANA	Antinuclear antibody
APC	Antigen presenting cell
aPL	Antiphospholipid autoantibodies
APS	Anti phospholipid syndrome
ASRM	American society of Recurrent miscarrige
BCL-XL	B-cell lymphoma-extra large
BTLA	B- and T-lymphocyte attenuator
CD	Cluster of differentiation
CTLA-4	Cytotoxic T-lymphocyte associated antigen-4
FMI	Fetomaternal interface
FOXP3	Forkhead box P3 transcription factor.
GdA	Glycodelin-A
HLA	Human leukocyte antigen
hnRNA	Heteronuclear RNA.
HSV	Herpes simplex virus
ICOS	Inducible costimulator
IDO	Indoleamine 2,3 di-oxygenase
IRM	Idiopathic Recurrent Miscarriage
ITAM	Immunoreceptor tyrosine based activation motif
KIR	Killer inhibitory receptor
LA	Lupus anticoagulant
LEF-1	Lymphoid enhancing factor-1
MHC II	Major histocompatibility class II
mt/mt	Homozygous mutant type
MTHFR	Methyl tetrahydrofolate reductase
NCBI	The National Center for Biotechnology Information
NF-1	Nuclear factor-1
PCOS	polycystic ovarian syndrome
PCR-RFLP	Polymerase chain reaction-Restriction fragment length polymorphism

PCR-SSCP	Polymerase chain reaction- Single strand confirmation polymorphism
PD1	Programmed death 1
PDL1	Programmed death ligand 1
PI3K	Phosphoinositide 3-kinase
PTK	Protein tyrosine kinase
RCOG	Royal college of obstetric and Gynacologist
RPL	Recurrent Pregnancy Loss
RSA	Recurrent Spontaneous Abortion
sCTLA-4	Soluble Cytotoxic T lymphocyte associated antigen-4
SNP	Single nucleotide polymorhism
STAT-6	Signal transducer and activator of transcription-6
T reg	Regulatory T cell
TCR	T cell receptor
TGF-β	Transforming growth factor-beta
Th 2	Helper T cells type 2
TNF-α	Tumor necrosis factor-alpha
uNK	Uterine natural killer cells
URSA	Unexplained Recurrent Spontaneous Abortion
WHEC	Women Health and Education Center
WHO	World health organization
wt/wt	Homozygous wild type
ZAP-70	ζ-chain-associated protein kinase of 70 kDa

Chapter 1

Introduction

Chapter 1
Introduction

1.1 Overview

Human pregnancy may be interrupted at various stages, leading to abortion, and this process occurs repetitively in some patients. Repetitive interruptions of pregnancy are variously referred to as recurrent spontaneous abortion (RSA), recurrent pregnancy loss (RPL), recurrent miscarriage (RM), idiopathic recurrent miscarriage (iRM), or unexplained recurrent spontaneous abortion (URSA)**.** In most definitions of this syndrome, those different terms indicate termination of pregnancy occurring more than 2 times for a given patient, before 24 weeks of gestation. In humans, it appears that at least 15% of the recognized pregnancies end with miscarriage (Vaiman, 2015).

Recurrent pregnancy loss (RPL), is a prevalent health problem that affects ~1–2% of couples who are trying to establish a family (Kelachayeh, Salehi, and Aleyasin, 2014). It is a distressing problem, particularly to Palestinian families who are fond of having large families (Sharif, 2012).

A spectrum of factors is known to be involved in RPL including genetic, endocrine, anatomic, immunologic, infectious, environmental factors and thrombophilic disorders. Even after a thorough evaluation, however, the potential cause remains unexplained in about one third to one half of cases (Kelachayeh et al., 2014; Nagirnaja et al., 2014).

In the face of unknown etiological factor(s), dysregulated immunity is proposed as a potential mechanism underlying unexplained RPL. This reportedly includes autoimmune abnormalities (e.g., positive anti-phospholipid, anti-nuclear and anti-microsomal antibodies), increased cell-mediated immunity and altered T helper Th1-Th2-Th17- regulatory T cells (Treg) balance (Zenclussen et al., 2006; Saito, Shima, Nakashima and Lin, 2010). In this regard, normal pregnancy has been shown to be associated with predominance of anti-inflammatory Th2 cytokines whereas, unregulated and excessive pro-inflammatory Th1 cytokines are harmful to the fetus (Makhseed et al., 2001; Bansal, 2010).

Maternal immune tolerance imposed on the developing semi-allogeneic fetus is a crucial aspect for successful pregnancies. Establishment of the maternal immune tolerance requires the interplay of various immune cells such as antigen presenting cells (e.g., dendritic cells and macrophages), natural killer cells, Th1, Th2, Th17 and (Tregs) cells. Regulation of number and activity of those cells in turn, is controlled by an intricate web of cell-cell interactions, cytokines, transcription factors and other signaling molecules. Imbalance in the quantities of immune-regulatory molecules produced by immune tolerance-related genes is believed to breach the immune tolerance and lead to pregnancy complications including RPL. On this subject, an increase in circulatory Tregs has been reported during early pregnancy, which peaks at the second trimester and declines postpartum. Fetal antigens are also recognized by the maternal B cells and tolerance to the fetus is achieved by deletion of alloantigen-specific B cells during gestation (Tripathi and Guleria, 2015).

Negative co-stimulatory signaling pathways, like CTLA-4/B7 and PD1/PDL1, expressed on the Tregs and involved in the regulation of effector T cell activation and proliferation are known to contribute toward the tolerance of the fetus (Dehaghani, Doroudchi, Kalantari, Pezeshki and Ghaderi, 2005). These signaling molecules act through suppressing immune-reactive cells and decreased expression of *CTLA-4* has been shown to contribute to fetal loss in humans (Tripathi and Guleria, 2015).

CTLA-4 gene is located on the chromosomal region 2q33 and contains more than 100 polymorphic sites. Distinct single nucleotide polymorphisms (SNPs) such as +49A/G (rs231775), -318C/T (rs5742909), -1661A/G (rs4553808) and -1722T/C (rs733618) have been associated with autoimmune diseases and preeclampsia (Dias et al., 2013).

Inter-individual genetic variation, in terms of SNPs in the immune tolerance-related genes may influence the rate of transcription and thereby the quantity of immune-regulatory molecules. Indeed, some promoter SNPs have been shown to affect *CTLA-4* mRNA and protein levels (Wang, Priskanen, Giscombe and Lefvert, 2008).

The present study was designed in order to investigate the association between four *CTLA-4* gene polymorphisms namely, +49A/G, -318C/T, -1661A/G and -1722T/C and RPL in a group of Palestinian women.

1.2. Problem

RPL is encountered in about 1-2% of pregnant women and in around 50% of those cases the underlying etiology remains unclear. This study will address the contribution of +49A/G, -1722T/C, -318C/T and -1661 A/G *CTLA-4* gene polymorphisms to the risk of RPL in the Palestinian population.

1.3. Objectives.

1.3.1. General objective

To study the relation between +49A/G, -1722T/C , -318C/T and -1661 A/G *CTLA-4* gene polymorphisms and unexplained recurrent pregnancy loss in Palestinian women.

1.3.2. Specific objectives

1. To employ PCR-RFLP technique for the detection of *CTLA-4* gene polymorphisms.
2. To determine genotype, major and minor allele frequencies of +49A/G, -1722T/C, -318C/T and -1661 A/G polymorphisms in *CTLA-4* gene among women with idiopathic RPL as compared to healthy controls.
3. To compare our study results with those reported in other populations.
4. To suggest appropriate recommendations regarding the relation between the investigated polymorphisms and RPL.

1.4. Significance

1. This study is the first to investigate the relationship between *CTLA-4* gene polymorphisms and RPL in women in Gaza strip.
2. This investigation may help elucidate one of the causes of idiopathic RPL.
3. This study may help in understanding the role of immunologic factors and related genes in feto-maternal tolerance and successful pregnancy.

Chapter 2

Literature Review

Chapter 2

Literature Review

2.1. Recurrent pregnancy loss definition

Recurrent pregnancy loss (RPL) defined as the spontaneous loss of pregnancy before 24 weeks of gestation, and sometimes it is considered before 20 weeks due to advances in neonatal care. It is referred to the miscarriage of two or more consecutive pregnancies in the first or early second trimester of gestation (Gaboon, 2013).

The Practice Committee of the American Society for Reproductive Medicine defines it as a disease distinct from infertility, defined by two or more failed clinical pregnancies, which have been documented by either ultrasound or histopathological examination (ASRM, 2012).

However, the Royal College of Obstetricians and Gynaecologists (RCOG) Green-top Guideline No. 17, defines RPL as the spontaneous loss of a pregnancy before the fetus has reached viability at 24 weeks (Zegars-Hochschild et al., 2009; Van Niekerk, Siebert and Kruger, 2013). This includes all pregnancy losses from the time of conception until 23 completed weeks of gestation (ASRM, 2008).

The ASRM recommends that couples who have 2 or more consecutive spontaneous abortions warrant an evaluation to identify any factor that may be associated with their poor reproductive history (Kutteh and Odom, 2012).

2.2. RPL Incidence

Approximately 15 percent of pregnant women experience sporadic loss of a clinically recognized pregnancy (Naz, Yasmin and Taj, 2014). Just 2 percent of pregnant women experience two consecutive pregnancy losses and only 0.4 to 1 percent have three consecutive pregnancy losses. The observed frequency of three consecutive pregnancy losses is slightly higher than that expected by chance alone (Tulandi and Al-Fonzan, 2013).

2.3. Risk factors and etiology of RPL

Although RPL is an important problem in women's health, there are many unsolved questions regarding its etiology, evaluation, and management. Unfortunately, the cause of RPL can be determined in only 50 percent of patients. General etiological categories of RPL include anatomic, immunological, genetic, endocrine, infectious, thrombophilic, and environmental factors.

The prevalence of miscarriage is higher with increasing maternal age and at very early gestational ages. Additionally, previous pregnancy outcome can positively or negatively affect the risk in the next pregnancy (Tulandi and Al-Fonzan, 2013).

2.3.1. Anatomical disorders

Anatomic uterine defects are present in 15% of women evaluated for three or more consecutive pregnancy loss (Devi, Pham and Arici, 2006). Congenital uterine abnormalities are associated with second trimester pregnancy loss. Potentially relevant congenital Mullerian tract anomalies include unicornuate, didelphic, bicornuate, septate, or arcuate uteri.

The septate uterus is the most common uterine abnormality associated with RPL and is associated with the poorest reproductive outcome, with a miscarriage rate of more than 60% (Van Niekerk et al., 2013).

2.3.2. Infectious etiologies

Certain infections, including *Listeria monocytogenes*, *Toxoplasma gondii*, *Rubella*, *Herpes simplex virus* (*HSV), Measles, Cytomegalovirus*, and *Coxsackie viruses*, are known or suspected to play a role in the sporadic spontaneous pregnancy loss. However, the role of infectious agents in recurrent loss is less clear and not proven, with a proposed incidence of 0.5% to 5%. Those particular infections speculated to play a role in RPL include *Mycoplasma, Ureaplasma, Chlamydia trachomatis, L monocytogenes*, and *HSV*. The most pertinent risk for RPL secondary to infection is chronic infection in an immunocompromised patient (Ford and Schust, 2009).

2.3.3. Lifestyle, Environmental, Occupational Factors

Heavy alcohol consumption has been reported to be associated with an increased risk of miscarriage though other studies have not shown the same.

Smoking has also been associated with RPL, which is positively related to the number of cigarettes. Caffeine and cocaine also have been linked to pregnancy loss. Stress has also been shown to be associated with the higher pregnancy loss rate. Body weight both high and low are associated with adverse pregnancy outcome. The risk of pregnancy loss is high in women with certain chronic maternal diseases, e.g. liver, renal and autoimmune diseases. Some medications such as nonsteroidal anti-inflammatory drugs and aspirin are linked to pregnancy loss. Certain environmental effects like radiation and environmental toxins can also influence the outcome of pregnancy (Begum et al., 2011).

2.3.4. Endocrine dysfunction

Endocrine disorders play a major role in approximately 8% to 12% of recurrent pregnancy loss (RPL). Indeed, the local hormonal milieu is crucial in both embryo attachment and early pregnancy.

Endocrine abnormalities, including thyroid disorders and disorders related to inadequate progesterone secretion by the corpus luteum, luteal phase defects, hyperprolactinaemia, diabetes mellitus and polycystic ovarian syndrome (PCOS) are some examples of endocrine disorders affecting pregnancy and have to be evaluated in any case of RPL. Moreover, elevated androgen levels and some endocrinological aspects of endometriosis are also factors contributing to RPL (Smith and Schust, 2011; Pluchino et al., 2014).

2.3.5. Haematological disorders

Thrombophilia is defined as a disorder of hemostasis that predisposes a person to a thrombotic event. Data suggest that at least 50% of cases of venous thromboembolism in pregnant women are associated with an inherited or acquired thrombophilia (Carbone and Rampersad, 2010). It is suggested that the association is caused by an increased risk of thrombus formation in the nascent placental vessels resulting in placenta infarctions (Larsen, Christiansen, Kolte and Macklon, 2013).

Inherited thrombophilias that are associated with recurrent miscarriage include factor V Leiden mutations, prothrombin gene mutation (PT G20210A) and deficiencies of natural anticoagulant protein C, protein S and antithrombin (Middeldrop, 2011).

Acquired factors include the presence of anti-phospholipid antibodies, lupus anticoagulant or anti-cardiolipin antibodies, which are deemed to be present when identified in repeated samples taken 3 months apart without pregnancy (Lassere and Empson, 2004).

Antiphospholipid syndrome (APS) is an autoimmune disease with the presence of antiphospholipid autoantibodies (aPL) formed against the person's own tissues. These autoantibodies interfere with coagulation. Although it is generally agreed that between 5% to 20% of RPL patients will test positive for antiphospholipid antibodies (aPLs), the actual reported range varies between 8% and 42% (ACOG practice Bulletin No. 118, 2011).

Hyperhomocysteinemia can be both hereditary and acquired, and are commonly associated with *MTHFR* gene polymorphisms. Thus hyperhomocysteinemia is considered as a risk factor for neural tube defects and recurrent embryo loss (Jeve and Davies, 2014).

2.3.6. Genetic causes

Chromosomal abnormality in one partner is found in 3% to 6% of RPL couples, which is ten times higher than the background population (Branch, Gibson and Silver, 2010). The most commonly encountered abnormalities include balanced reciprocal or Robertsonian translocations. Translocations and inversions do not have any consequences for the phenotype of the carrier, but in pregnancy there is a 50% risk of a fetus with an unbalanced chromosomal abnormality that can result in a miscarriage (Franssen et al., 2006). The exact risk depends on the specific chromosomes involved, the parent of origin and the size of the segment(s) involved in the rearrangement (Gaboon, 2013).

Embryonic chromosomal abnormalities (structural and numerical) may account for 30 - 57% of miscarriages (Van Niekerk et al., 2013).

A majority of miscarriages that occur before 10 weeks gestation are due to chromosomal aneuploidies arising from non-inherited non-disjunctional events (Sierra and Stephenson, 2006).

Old maternal age (≥35 years) is more prone to RPL, this is likely due to age-related chromosome abnormalities (non-disjunctions), mainly trisomy, rather than to structural translocation (Pokale, 2015).

Trisomy is the most common cytogenetic abnormality followed by polyploidy and monosomy X with the incidence of about 50% to 60% in miscarriages (Pokale, 2015).

Maternal diseases associated with increased fetal wastage include hematologic abnormalities like sickle cell anemia due to increased risk of placental vessels microinfarcts (Hsu et al., 2007). While fetal causes of recurrent miscarriage include autosomal recessive disorders (for example, alpha thalassemia major) (Chui and Waye, 1998), and X-linked disorders that are lethal in males may cause recurrent pregnancy loss (Allison and Schust, 2009).

2.3.7. Immune causes

A significant proportion of RPL is associated with immune etiologies (Saito, Nakashima and Shima, 2011).

Traditionally, it is thought that about 5% of couples suffer from RPL of immunological origin, and this number increases to 40% after four pregnancy losses (Beaman et al., 2012).

Both autoimmune and alloimmune mechanisms are implicated as causes of RPL. Autoimmune disorders involve an immune response directed against a specific part of the host or self (WHEC, 2014).

Autoimmune disorders that affect reproductive processes are often subclinical and most women who present with repeated miscarriage are otherwise well (Kwak-Kim, Kim and Gilman-Sachs, 2006). Numerous studies have attempted to identify specific autoantibodies associated with pregnancy loss (Kutteh and Odom, 2012). Organ-specific antibodies such as anti-thyroid antibodies, and organ-nonspecific antibodies, including antiphospholipid antibody, lupus anticoagulant (LA), anticardiolipin antibodies (ACA), antinuclear antibody (ANA), anti-ssDNA, anti-dsDNA, and anti-histone antibody has been reported to be associated with pregnancy losses or

obstetrical complications (Horne and Alexander, 2005; Kwak-Kim, Park, Ahu, Kim and Gilman-Sachs, 2010).

Alloimmune disorders involve human leukocyte antigens (HLAs) compatibility of couples and an abnormal maternal immune response to fetal or placental antigens, including absence of maternal leukocytotoxic antibodies, or the absence of maternal blocking antibodies (WHEC, 2014).

However, successful pregnancies have been reported among women without blocking antibodies and also among women with agammaglobulinemia (Wong, Porter and Scott, 2014).
Defects in molecular immunosuppressive factors (cytokines and growth factors) at the local decidual/trophoblast level have been implicated as causes of RPL (Wong, Porter and Scott, 2014).

On the other hand, overactivity of T helper-1 (Th-1) cytokines and natural killer (NK) cells have been also reported to be the major alloimmune cause of idiopathic RPL (Kwak-Kim et al., 2006).

Uterine NK cells have long been considered the most important cell type for the success of pregnancy due to their abundance in the decidua. Unlike peripheral blood NK cells, uNK cells are the only weakly cytotoxic *in vitro*, and don't kill trophoblast *in vivo* (Ruocco, Chaouat, Florez, Bensussan and Klatzmann, 2015).

While, NK cells are thought to alter the humoral response to pregnancy and induce the Th1 dominance that would lead to pregnancy loss (Peeva, 2010). Uterine NK cells secrete cytokines and angiogenic factors, important for placental development and establishment of pregnancy.

Aberrant presentations of uterine and peripheral blood NK cells have been associated with various reproductive failures, such as RPL, multiple implantation failures or pre-eclampsia (Lee et al., 2013).

2.4. Immunology of normal pregnancy

A successful pregnancy belongs to the interaction between mother and fetus (Rull, Nagirnaja and Laan, 2012). This interaction is vital since the fetus is not genetically the same as the mother. Several mechanisms have been found to establish this feto-maternal interaction. Among these, immunological mechanisms are one of the important factors involved in tolerance toward the semi-allogeneic fetus by maternal immune system **(**Christiansen, 2013; Molazadeh, Karimazadeh and Azizi, 2014).

Many mechanisms are suggested to be involved in maternal immune tolerance and immunologic acceptance of the semi-allogeneic fetus during pregnancy (Tilburgs et al., 2008).

Placenta provide an immunological barrier between the fetus and the maternal immune system, it produce progesterone, estrogen and human chorionic gonadotropin (hCG), all of which redirect maternal immunity from damaging Th1 cells toward Th2 subset mediated immunosuppression (Dealtry, O'Farrell and Fernandez, 2000).

Fetal trophoblasts play a crucial role in circumventing a destructive maternal immune response in different ways. Fetal tissue can inhibit allogeneic immune responses by expressing indoleamine 2,3 dioxygenase "IDO" (that inhibits rapid proliferation of cells), FAS ligand (that can cause apoptosis of activated cells that express FAS), and complement inhibitory proteins to prevent complement activation as described in **Figure 2.1.** These mechanisms can inhibit immune responses at the fetal-maternal interface in an antigen-nonspecific manner (Tilburgs et al., 2008).

Another regulatory glycoprotein called glycodelin-A (GdA) is also secreted by the trophoblast and endometrial cells in response to progesterone, which selectively inhibits Th1 type of cells and shifts the cytokine milieu toward a Th2 type (Tripathi and Guleria, 2015).

Trophoblast cells lack the expression of major histocompatibility complex (MHC) class II molecules on their surface, and thereby prevent the MHC class II-mediated T cell response. On the other hand, the trophoblast cells do express low levels of classical MHC class I molecules that makes the fetus prone to attack by the natural killer (NK) cells. The NK cell-mediated lysis of trophoblast cells is prevented by the

interactions of killer inhibitory receptor (KIR) of NK cells with non-classical MHC class I molecules like HLA-G expressed on the trophoblast as illustrated in **Figure 2.1.** (Tripathi and Guleria, 2015).

Distinct KIR receptor-ligand combinations have been associated with increased risk of early pregnancy loss and preeclampsia (Hiby et al., 2008; Faridi and Agrawal, 2011).

Decidual immune cells, mainly uterine natural killer cells (uNK), dendritic cells, TH1, Treg cells and T helper type 17 (Th17) play an eminent role not only in mediating immune tolerance, but also in vascular remodeling and decidual development (Chaouat, 2013). Both the Th17 and Treg populations have been shown to play a role in modulating the tolerance at the feto-maternal interface (Saito et al., 2010).

During pregnancy, the composition of decidual immune cells and cytokine profile gradually undergoes extensive modification and new functional synapses between fetal trophoblast and maternal immune cells are formed, supporting the fourth piece of conception. Plodding predominance of Th2 cytokines, stepwise decline in uNK cell and increase of Tregs population are among the hallmarks of immune adaptation during pregnancy. Such complex modifications synergistically create a state of tolerance (Erlebacher, 2013). Transcription factors play an important role in the maintenance of balance between these cells like STAT-3 which is highly expressed and activated in decidual cells and play a crucial role in embryo attachment and implantation (Lee et al., 2013).

Many cytokines that modify the immune response are produced during pregnancy. Some of these favor pregnancy e.g., Th2 cytokines, (Il-3, Il-4, Il-5, IL-10- Il-13), and others are toxic to it e.g., Th1 cytokines, (TNF-alpha, TNF-beta, gamma-interferon, Il-2). A shift to Th1 dominance increases the risk of pregnancy loss. Th1 cytokines may directly damage the placenta or possibly activate immune cells that induce rejection (Begum et al., 2011).

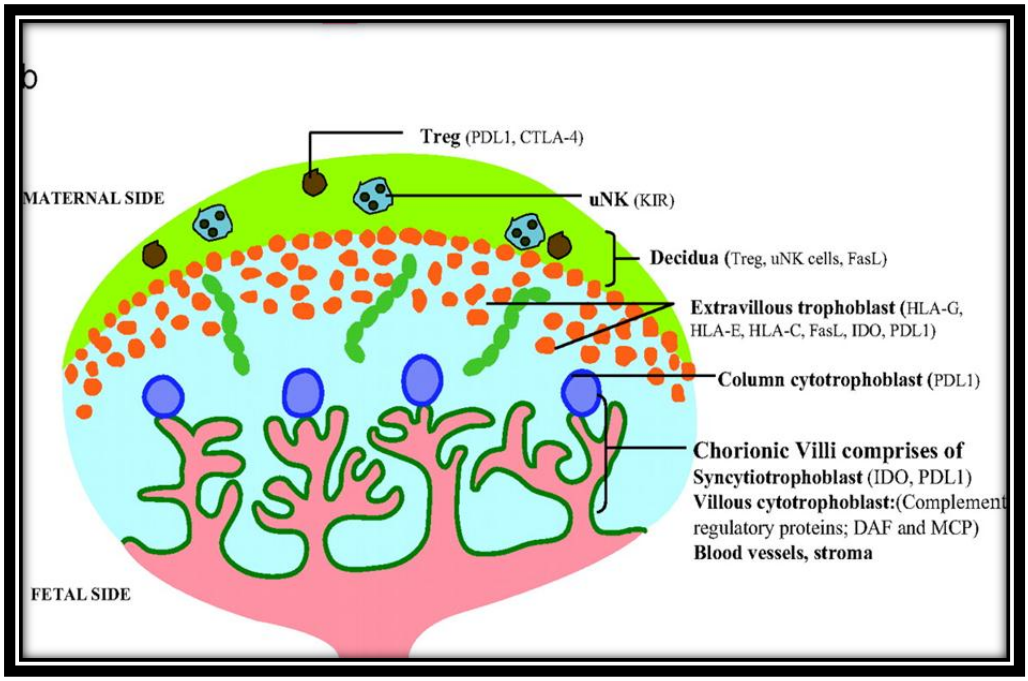

Figure (2.1): Cells and molecules involved in fetomaternal tolerance in human placenta. **(Adopted from:** Guleria and Sayegh, 2007**)**

Seminal plasma contains large amounts of transforming growth factor β (TGF- β) and prostaglandin E capable of induction of regulatory T cells (Treg). Seminal fluid initiates a series of events leading to induction of tolerogenic dendritic cells competent to prime Tregs (Robertson, Prins, Sharkey and Moldenhauer, 2013).

2.5. CD28 family of costimulatory molecules and their role in maintaining immune tolerance

To initiate an adaptive immune response naïve T cells requires two distinct signals. The first one is mediated by the association of the T cell receptor (TCR) with the Major Histocompatibility Complex (MCH) molecules, known in humans as the Human Leukocyte Antigen (HLA) (Thomas, 2007) and expressed on the antigen presenting cells (APCs). This interaction mediates the specificity of a T cell response by the recognition of specific epitopes of the presented antigen. The second signal is delivered by co-stimulatory molecules on APCs and accentuates or attenuates the first signal depending on the type of co-stimulation (Wing, Yamaguchi and Sakaguchi, 2011). The combination of the two signals determine whether a T cell becomes activated or anergic (Sakaguchi, Wing and Miyara, 2007).

Positive and negative costimulation by members of the CD28 family is critical for the development of protective immune responses against foreign pathogens and their proper termination to prevent inflammation-induced tissue damage. In addition, costimulatory signals are critical for the establishment and maintenance of peripheral tolerance (Bour-Jordan et al., 2011).

Five molecules of the CD28 family are expressed at different stages of differentiation of T lymphocytes: CD28, CTLA-4, Inducible costimulator (ICOS), Programmed death-1 (PD1), B- and T-lymphocyte attenuator (BTLA). Additionally, CD80, the ligand for CD28 and CTLA-4 normally present on APCs, is also expressed on T cells (Wakamatsu et al., 2013).

The positive signals induced by CD28 and ICOS molecules are counterbalanced by other co-inhibitory members of the CD28 family, including CTLA-4, PD1 and BTLA. These coinhibitory receptors act as threshold setters, modulators, check points and feedback mechanisms that can potentially fine tune the quality and the magnitude of the T cell immune response (Thaventhiran et al., 2013).

CD28 family molecules are also expressed on FoxP3+ Treg cells, key regulators of lymphoid homeostasis. CD28 and CTLA-4 control Treg differentiation and homeostasis, and there is strong evidence that CTLA-4 and PD1 are important players in Treg function (Wakamatsu et al., 2013).

The B7-1/B7-2:CD28/CTLA-4 pathway is involved in controlling the fate decision of naive T cells between activation and anergy. Sharpe has shown that this pathway not only regulates the responses of naive T cells, but also is key for the development and function of regulatory T cells (Tregs) (Sharpe, 2009).

CD28 is considered the primary costimulatory molecule, through its interactions with CD80 and CD86 on APCs, complement TCR-mediated signals and promote T-cell activation, proliferation and survival. Initial activation of naïve T cells requires CD28 signal to enhance the signal derived by TCR and the production of IL2 for the robust expansion of antigen-specific T cells (Wakamatsu et al., 2013).

When CTLA-4 is upregulated, CD28 expression is subsequently downregulated by endocytosis, such down-regulation of T lymphocytes proliferation may induce immune tolerance, which is fundamental for allograft acceptance. Thus, the outcome of an immune response involves a balance between CD28-mediated T cell activation and CTLA-4-mediated inhibition (Krichen et al., 2011).

2.5.1. CD28 family of costimulatory molecules and pregnancy

Accurate expression of costimulatory molecules at the maternal-fetal interface may ensure that the decidual cells do not elicit a 'danger' signal to the maternal immune system, perhaps instead contributing to the establishment of immune tolerance *in vivo* (Jin, Fan and Li, 2011).

CTLA-4 is one of the major negative costimulatory molecules along with PD-1, these molecules are suggested to play an important role in fetomaternal tolerance (Tripathi and Guleria, 2015). Decreased expression of CTLA-4 or low ratio of CTLA-4\CD28 is reported in fetal loss in humans (Wang, Ma, Hong, Lu and Lin, 2006; Jin, Chen, Zhang, Guo and Li, 2009).

While in mice, blockade of CD80 (B7.1) and CD86 (B7.2), the ligands for both CD28 and CTLA-4 is shown to be protective from abortion and increase fetal viability (Jin, Zhou, Wang, Zhu and Li, 2005).

Another member of these inhibitory molecules is PD-L1, the expression of which is prevalent at the uteroplacental interface (Riella et al., 2013).

PD-1 promotes type 2 helper T cell (Th2) bias and pregnancy maintenance by regulating CD4+ T cell function at the maternal–fetal interface (Wang et al., 2016). Another costimulatory pathway, called the ICOS/B7h, is also reported to play a critical role in the maintenance of a tolerogenic environment at the feto-maternal interface. Blockade of this pathway results in increase in fetal loss by reducing IDO and transforming growth factor beta (TGF-β) locally at the FMI and increasing CD8+ T effector cell response in the periphery (Riella et al., 2013).

2.6. Expression and functions of CTLA-4 at the maternal fetal interface

A pregnancy is associated with modifications in the immune status of the mother, but the mechanisms are not well understood. Several studies suggest that up-regulation of B7-2 and/or CD28 and/or down-regulation of CTLA-4 are correlated with the occurrence of pregnancy loss (Jin, Fan and Li, 2011).

CTLA-4 seems to be critical for function of regulatory T cells (Tregs), which are powerful suppressors of T cells (Petroff and Perchellet, 2010), its surface expression on Treg cells is a marker for activated and functional Treg cells (Saito, Nakashima, Shima and Ito, 2010). It appears to play an indispensable role in regulating homeostatic T-cell proliferation. Its regulatory functions are illustrated in CTLA-4-deficient mice in which rapid, polyclonal expansion of T cells occurs, which is ultimately fatal to the animals. The functions of CTLA-4 are thus critical in controlling immune responses to both foreign and self-antigens (Petroff and Perchellet, 2010).

Interestingly, surface CTLA-4 expression on Treg cells increases in decidual Tregs but not in peripheral blood Treg cells of early pregnancy subjects (Munn, Sharma and Mellor, 2004). Moreover, its expression by placental fibroblasts confirms its role in maintaining the immunetolerance (Rasiti and Nasiri, 2016).

Reverse signaling through B7-1/-2 after ligation by a soluble form of CTLA-4 was shown to upregulate the tryptophan catabolic enzyme, indoleamine-2,3-dioxygenase (IDO). The potent immunosuppressive activity of IDO was first identified in pregnancy, in which chemical inhibition of IDO activity abolished allogeneic pregnancy (Petroff and Perchellet, 2010).

The up-regulated IDO enzyme, induced by CTLA-4, depletes tryptophan at the materno-fetal interface, thereby preventing T cell, and NK cell activation (Saito et al., 2007).

The protective role for CTLA-4 in pregnancy is supported by the fact that both the number of Tregs and the level of CTLA-4 on Tregs are lower in the decidua in cases of spontaneous abortion (Sasaki et al., 2004), suggesting that functional Treg cells might induce alloantigen-specific tolerance, resulting in maintenance of pregnancy (Saito et al., 2010).

2.6.1. CTLA-4 and Pregnancy complications

There have been several studies that couple CTLA-4 deficiency to adverse pregnancy outcomes such as recurrent pregnancy loss (RPL), placental abrubtion, and pre-eclampsia (PE). Moreover, polymorphisms in the *CTLA-4* gene have been associated with dysregulated CTLA-4 production and function.

Heterozygosity of the *CTLA-4* A49G allele, has been reported as a predisposing factor to severe PE and placental abruption in some populations (Dehaghani et al., 2005; Jääskeläinen et al., 2008).

In addition, stretches of AT repeats in the 3'- untranslated region of the *CTLA-4* gene has been suggested to affect mRNA stability and fetal survival in humans (Dehaghani et al., 2005).

2.6.2. Discovery of CTLA-4

In 1987, French researchers hunting for cytotoxic cell surface molecules isolated a cDNA from activated CD8+ T cells and called it cytotoxic T cell antigen (CTLA)-4. It was first identified in 1991 as a second receptor for the T cell costimulation ligand B7. Uncertainties about its biological function plagued the early years after its discovery until 1995, when it was confirmed to be an inhibitor of T cell responses (Bashyam, 2007).

2.6.3. Molecular structure of CTLA-4 receptor

CTLA-4 (CD152) is a member of a class of cell surface molecules capable of terminating early events in the receptor-mediated signaling cascade. It is a 41–43 kDa, type 1 transmembrane glycoprotein of the immunoglobin superfamily, 223 amino acids in length. The extracellular architecture of CTLA-4 is characterized by a single immunoglobulin variable (IgV)-like domain containing the B7-1 (CD80)/B7-2 (CD86) ligand-binding site (Wang, Zuo, Sarker and Fisher, 2011). It is composed of four domain including a signal peptide, extracellular ligand binding domain, transmembrane domain and a short cytoplasmic tail (Intlekofer and Thompson, 2013).

CTLA-4 shares ~30% of homology with CD28 at the protein level and binds the same ligands CD80\CD86 that are expressed on APC, with a stronger binding affinity than CD28 in particular for CD80 (Wing et al., 2011).

2.6.4. The *CTLA-4* Gene

The *CTLA-4* gene is about 6.2 KB long and comprises 4 exons. The gene is located on the long (q) arm of chromosome 2 at position 33 (2q33) of human chromosome **(**Geng et al., 2014). The first exon of *CTLA-4* gene, encodes the leader sequence peptide while the second one encodes the extracellular immunoglobulin like domain containing the binding site. Exon 3 and 4 are responsible for producing the transmembrane and cytoplasmic domains, respectively (Teft, Kirchhof & Madrenas, 2006). *CTLA-4* hnRNA transcript undergoes alternative splicing, resulting into three final mRNA isoformes in human; (a) the surface full-length CTLA-4 (flCTLA-4) including exons 1, 2, 3 and 4, (b) the soluble CTLA-4 (sCTLA-4) lacking exon 3 (transmembrane domain) and (c) a transcript lacking both exons 2 and 3 (binding as well as transmembrane domains) (Oaks and Hallett, 2000). The specific contributions of each of the soluble splice isoforms to the overall biologic functions of CTLA-4 remain unknown (Intlekofer and Thompson, 2013).

2.6.5. *CTLA-4* expression pattern

CTLA-4 (CD152) is an inhibitory receptor expressed transiently on activated $CD4^+$ and $CD8^+$ T-lymphocytes and constitutively on $CD4^+CD25^+$ regulatory T cells (Treg) and few non lymphoid cells (Lozano et al., 2011).

In contrast to CD28, which is expressed on the surface of resting and activated T cells, CTLA-4 exhibits minimal expression in resting T cells. *CTLA-4* is induced at the mRNA and protein level in response to TCR activation (Intlekofer and Thompson, 2013), and it is used as an early activation marker. mRNA for CTLA-4 can be detected at 1 hour post-activation with a maximum expression between 24 and 36 hr the time when CTLA-4 is detectable on the cell surface (Shneider and Rud, 2014).

Expression of *CTLA-4* is enhanced by costimulation through CD28 and/or IL-2 (Alegre et al., 1996). Such that greater stimulation produces more CTLA-4 at the cell plasma membrane (Teft et al., 2006; Jain, Nguyen, Chamber and Kang, 2010).

Accumulation of CD28 occurs during T-Cell activation and has also been shown to induce expression of CTLA-4 and increase stability of CTLA-4 mRNA (Rudd, Taylor and Schneider, 2009).

Of note, although $CD4^+$ and $CD8^+$ T cells express CTLA-4, the inhibitory functions of CTLA-4 on $CD4^+$ T cells appear to be relatively more important for the prevention of autoimmune pathology (Gattinoni et al., 2006).

2.6.6. Cellular localization

CTLA-4 is primarily an intracellular protein found in transport vesicles whose surface expression is highly regulated. Only during immune stimulation does surface expression slightly increase, followed by rapid internalization. Tight regulation of CTLA-4 surface expression is critical for maintaing a controlled but effective immune response (Valk, Rudd and Schneider, 2008).

2.6.7. *CTLA-4* Gene Polymorphism

The *CTLA-4* gene, contains more than 100 polymorphic sites, and distinct polymorphisms have been associated with autoimmune and infectious diseases. The *CTLA-4* +49A/G single nucleotide polymorphism (SNP) is located in exon 1 and promotes a Threonine (A) to Alanine (G) substitution in the protein leader sequence at amino acid position 17 (Sun, Hu, Shen and Lin, 2009).

Threonine in this position results in a stronger interaction of CTLA-4 with B7.1 molecules, inducing a higher inhibitory effect on T-cell activation when compared to the presence of Alanine (Sun, Zhou, Yang, Hu and Tan, 2008). Moreover, the presence of Alanine at position 17 of the *CTLA-4* gene results in inefficient glycosylation and decreased molecule expression on the cell surface (Anjos, Nguyen, Ouniss, Tessier and Polychronakos, 2002). Some workers have shown association between the G allele of 49A/G and reduced control of T-cell proliferation (Du, Ma and Wang, 2014).

The *CTLA-4* -1722T/C and *CTLA-4* -318C/T SNPs were identified at the promoter region of the *CTLA-4* gene (Dias et al., 2013). The single nucleotide polymorphism (SNP) at position (-318) C/T in the promoter region (rs5742909) influences promoter activity and the expression of both *CTLA-4* mRNA in un-stimulated cells and cell-

surface CTLA-4 on activated cells (Kusztal, Kościelska-Kasprzak and Drulis-Fajdasz, 2010).

On the molecular level, -318 C/T SNP may influence CTLA-4 levels by changing the binding of a transcription factor LEF-1 (lymphoid enhancing factor 1) (Pastuszak-Lewandoska et al., 2013).

The *CTLA-4* -1722 polymorphism has been associated with an increased expression of *CTLA-4*, it is possible that the increased expression of CTLA-4 on the Treg surface may ameliorate its function in controlling disease-induced damage.

The *CTLA-4* -1661A>G (rs4553808) SNP, also located in the promoter, appears to be involved in the transcription-associated binding activity of nuclear factor (NF-1) and C/EBPα and might cause abnormal alternative splicing and affect the expression of CTLA-4 (Wang et al., 2008).

2.7. CTLA-4 function

CTLA-4 regulates T cell function through several ways, but the actual mechanism responsible for its function are unknown (Shneider and Rudd, 2014).

Much debate has focused on whether CTLA-4 inhibits T cell response by cell-extrinsic or intrinsic mechanisms.

Cell intrinsic mechanisms would reflect direct effects of the co-receptor on the expressing cells (signal transduction). while cell extrinsic effect relate the regulation of function by a distal cell or cytokine (Ise et al., 2010).

Cell intrinsic (cell autonomous) and cell non-autonomous (i.e. cell extrinsic) actions of CTLA-4 have been reported to operate and maintain T-cell tolerance to self antigen (Ise et al., 2010).

2.7.1. Cell intrinsic function of CTLA-4

In cell intrinsic model CTLA-4 signals seem to interfere with IL-2 production, as well as IL-2 receptor expression, cell cycle progression and therefore, T cell proliferation (Intlekofer and Thompson, 2013). **Figure 2.1** below presents an intrinsic model of CTLA-4 action.

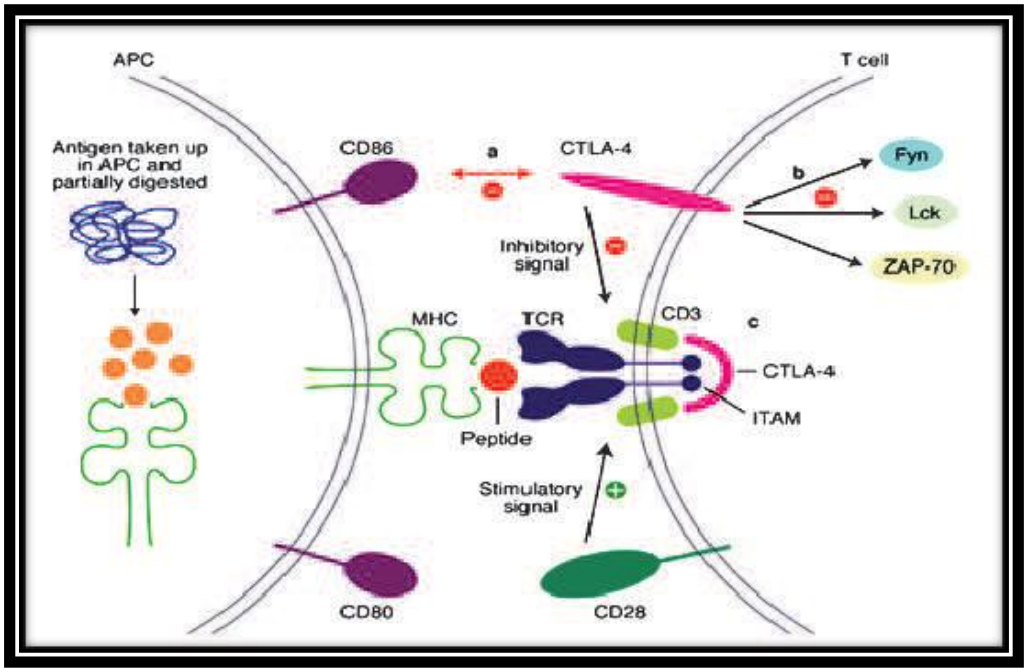

Figure (2.2): Proposed mechanisms by which CTLA-4 inhibits T-cell activation (**Adopted from:** Krichen et al., 2011)

(a) CTLA-4 might successfully compete with CD28 for CD80/86 ligands and thereby inhibit the costimulatory effect of CD28 (Thaventhiran et al., 2013).

(b) CTLA-4 cytoplasmic tail might interact with protein tyrosine kinases (PTKs) Lck, Fyn and microclusters containing ζ-chain-associated protein kinase of 70 kDa (ZAP-70) through protein tyrosine phosphatase SHP1 to prevent constitutive expression of downstream signaling of TCR\CD28 pathway (Krichen et al., 2011).

(c) CTLA-4 cytolasmic tail might directly interact with the TCR–CD3 complex at the immunological synapse to disrupt T-cell activation by binding and blocking the immunoreceptor tyrosine-based activation motif (ITAM) of the TCR-CD3 essentially disrupts the cascade of biochemical signals that lead to activation of T cells (Krichen et al., 2011).

In fact, cytoplasmic tail of CTLA-4 may also interact directly with and activate PI3K (Shneider H, 2008), and this activation permits induction of the antiapoptotic factor Bcl-XL which facilitate mitochondrial dependent T cell survival of the anergic T

cells and potentially ensuring T cell tolerance (parry et al., 2005; Shneider, Valk, Leung and Rudd, 2008; Thaventhiran et al., 2013).

2.7.2. Cell extrinsic function of CTLA-4

Tregs play a major role in cell-extrinsic regulation through engagement of its surface CTLA-4 with CD80/CD86 on dendritic cells (DCs) that can induce the release of IDO (Shneider and Rudd, 2014). A model for the extrinsic action of CTLA-4 is described in **Figure 2.3.**

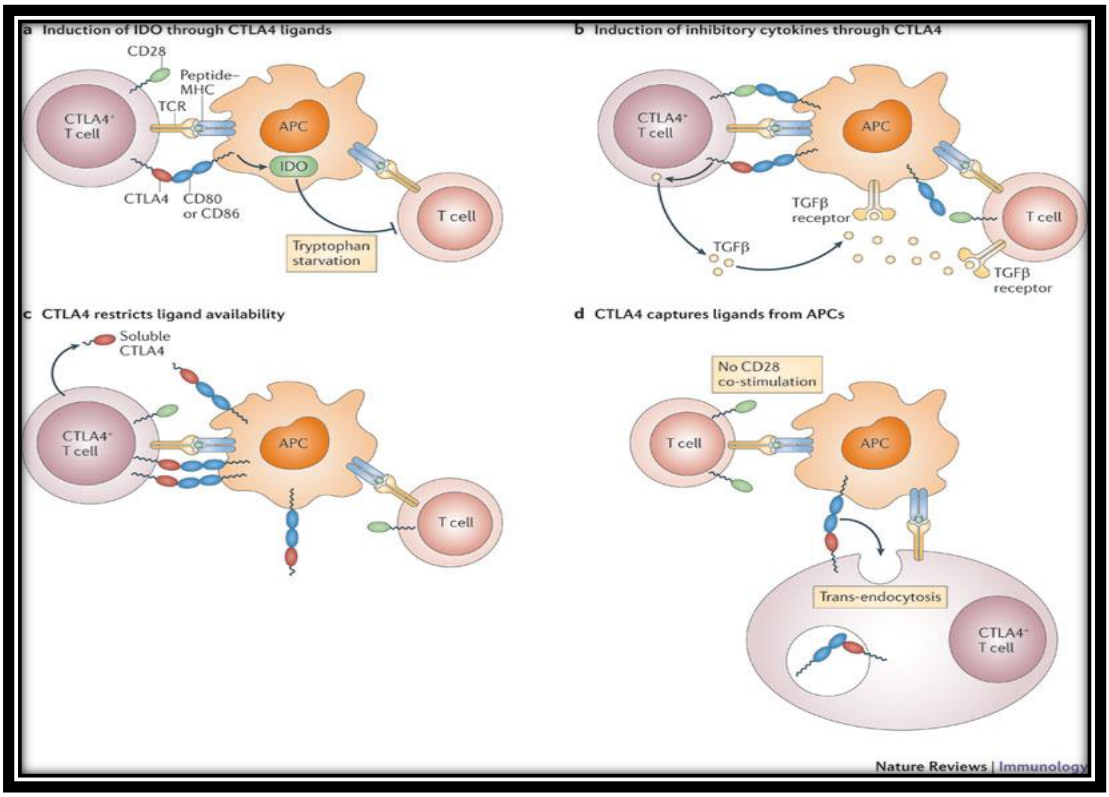

Figure (2.3): T cell-extrinsic models of CTLA-4 function.

(Adopted from: Walker and Sansom, 2011)

(a) CTLA-4 initiates reverse signals through CD80 and CD86 on APCs, resulting in the activation of the tryptophan-degrading enzyme indoleamine 2,3-dioxygenase (IDO). T cell inhibition might consequently be achieved by tryptophan depletion or local accumulation of inhibitory metabolites.

Moreover, IDO-expressing DCs may favor the emergence of CD4+CD25+Foxp3+ Tregs by the expansion/conversion from naive CD25−Foxp3− T cells (Curti, Trabanelli, Salvestrini, Baccarani and Lemoli, 2009).

(b) CTLA-4 signals stimulate the production of regulatory cytokines (such as transforming growth factor-β (TGF-β), which can act to inhibit downstream APC or T cell function (Oida, Yu, Weiner, Kitani and Strober, 2006).

(c) Inhibition of CD80 or CD86 availability by CTLA-4 prevents other T cells from receiving CD28 signals from the APC. Such an effect could be achieved by ligand sequestration by the regulating cells at the cell surface or by soluble splice variants of CTLA-4, both of which would result in decreased ligand availability and thereby modulate APC capacity to prime effector T cells, which leads to suppression and immune tolerance (Curti et al., 2009; Wing et al., 2011).

(d) It has been demonstrated that CD80 and CD86 on APCs can be captured and degraded by CTLA-4 via aprocess of transendocytosis thereby resulting in impaired T cell responses (Qureshi et al., 2011).

CTLA-4 ligation can also affect the transcriptional machinery of APCs by inducing nuclear translocation of FOXO3, which down regulates IL-6 expression of DCs (Wing et al., 2011) as described in **Figure 2.4.**

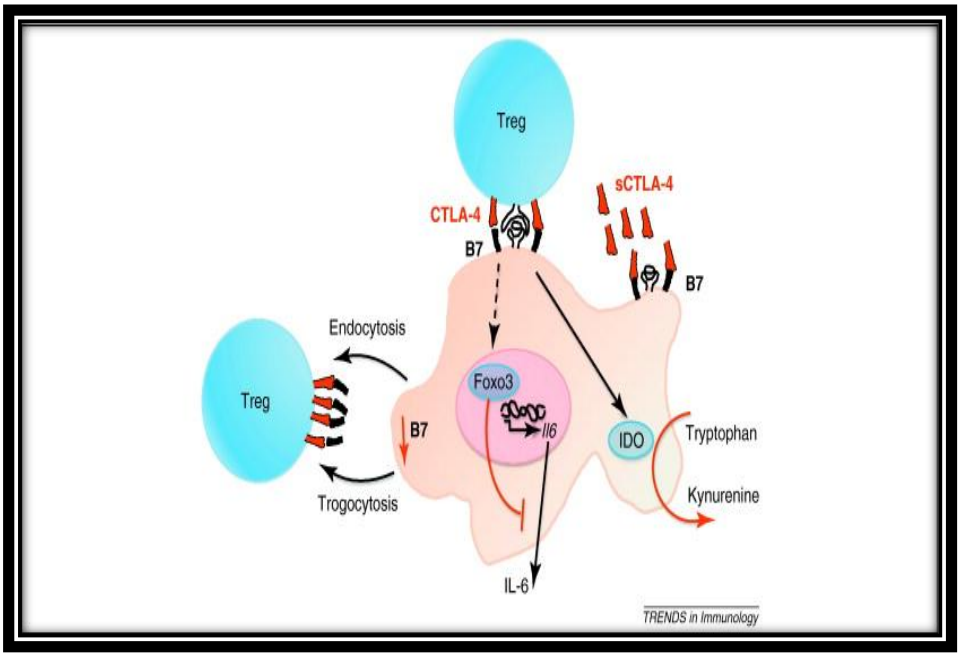

Figure (2.4): CTLA-4 can modulate APC function by several different mechanisms.

(**Adopted from:** Wing K et al., 2011)

The ability of these models to predict CTLA-4 functional behavior is variable. For example, despite popular perceptions of CTLA-4 as an inhibitory signal for T cell activation a consistent body of literature indicates that the major function of CTLA-4 *in vivo* is via a cell-extrinsic pathway, i.e. CTLA-4 influences the cells around it rather than the cell expressing it (Hou et al., 2015).

Additionally, CTLA-4 signals influence CD4+ T-cell differentiation. Indeed, in CTLA-4-deficient mice, T cells were shown to be strongly skewed towards a Th2 phenotype, even in the absence of the Th2 lineage transcription factor signal transducer and activator of transcription-6 (STAT-6). CTLA-4 was also recently shown to influence Th17 responses, as blockade of CTLA-4 resulted in increased Th17 differentiation and IL-17 production both *in vitro* and *in vivo*. These data imply that CTLA-4 signals inhibit Th17 responses (Bour-Jordan et al., 2011).

2.8. Disease relevance of CTLA-4

In humans, *CTLA-4* gene has been reported to contribute to a general susceptibility for autoimmune diseases, especially for endocrine disorders like Diabetes type 1 but also systemic lupus erythematosus, multiple sclerosis, and rheumatoid arthritis (Gough, Walker and Sansom, 2005; Ghaderi, 2011). It is an important target for cancer immunotherapy, as CTLA-4 blocking antibody may help protect against some cancers (Intlekofer and Thompson, 2013).

2.9. Previous Studies

A study on 60 couples including 59 Caucasian and one African American couple with a history of 3 or more unexplained spontaneous abortion showed that there is an association between the transmission of longer repeats of (AT)n alleles of *CTLA-4* gene among women undergoing repeated pregnancy losses. The authors indicated that G allele in exon-1 of *CTLA-4* 49 A/G and the common long (AT)n allele (i.e., 106 bp allele) are in LD among RPL cases and also observed almost three fold increased risk for 106 (AT)n allele (Tsai et al., 1998).

Genotyping to investigate the relationship between susceptibility to RPL and *CTLA-4* A49G polymorphism was performed in 168 Chinese women with unexplained RPL

and 117 women with normal pregnancy history by using PCR–RFLP method, showed that this SNP is associated with the immunopathogenesis of RPL (Wang et al., 2005).

In Iran, a study was performed to investigate the frequency of *CTLA-4* A49G polymorphism in severe PE on 36 pregnant women with severe preeclampsia and 151 healthy women by using polymerase chain reaction–single-strand conformation polymorphism (PCR–SSCP) method for genotyping. The results suggests that heterozygosity in the *CTLA-4* A49G allele might be a predisposing factor for severe preeclampsia (Dehaghani et al., 2005).

In Finland, a study was performed to investigate the *CTLA-4* A49G polymorphism and individual susceptibility to the development of preeclampsia or placental abruption on 361 women (132 with preeclampsia, 117 with placental abruption and 112 healthy controls). The results showed that the 49A-G polymorphism is associated with the development of placental abruption and preeclampsia, with women having the G allele being at risk (Jääskeläinen et al., 2008).

A study on 242 Ningxia Han women with RPL and 228 women with normal pregnancy history was conducted to investigate the relationship between *CTLA-4* gene SNPs (+49A/G, -318 C/T, -1661 A/G and -1722 T/C) and RPL by using PCR–RFLP method showed that genotype and allele frequencies of -1661 A/G, -1722 T/C and +49 A/G SNPs were not statistically significant, while in (-318 C/T) the CC and CT genotypes may be predisposing factors to RPL (Chaili, 2010).

In Brazil, a case control study was performed on 130 patients with preeclampsia (PE) and 261 control women without any obstetric or systemic disorders to evaluate the *CTLA-4* (+49 A/G), *CD28* (+17 T/C) and *ICOS* (−1564 T/C) gene polymorphisms in Brazilian women with PE by using PCR–RFLP method. The authors found a significantly lower frequency of the *ICOS* (−1564) T allele in women with mild PE as compared to the controls and no differences in the *CTLA-4* (+49 A/G) and *CD28*

(+17 T/C) genotypes and allelic frequencies between the PE patients and controls (Pendeloski et al., 2011).

In north India, a retrospective case control study was performed on 300 patients with RPL and 500 age and ethnically matched controls to investigate the role of *CTLA-4* +49 A/G, *CTLA-4* (AT)n 3`UTR, *TNF-a*-308G/A and *TNF-a*-238G/A polymorphisms as susceptibility markers for RPL by using the PCR–RFLP method. While gene sequencing method was adopted for studying the *CTLA-4* (AT)n 3`UTR polymorphism. The study reported that the mutant homozygous genotype GG of *CTLA-4* +49A/G, AA genotype and A allele of *TNF-a*-308, G allele of *TNF-a*-238 play predisposing roles in recurrent miscarriage patients (Gupta, Prakash, Parveen and Agrawal, 2012).

In Iran, 60 women with the history of two or more pregnancy losses were selected and considered as the case group. A group of healthy women (n=60) with at least two live births without any previous history of pregnancy loss were taken as control group. The *CTLA-4* +49 A/G genotypes were detected using (PCR-RFLP) assay. The results showed that *CTLA-4* +49 A/G polymorphism was not significantly different between the two groups (Bonyadi, Parsa, Taghavi and Zeinalzadeh, 2014).

In a case-control study, to evaluate the association between *CTLA-4* gene SNPs and unexplained RPL in an Iranian population. Patients (n= 195) with histories of at least three consecutive miscarriages with unexplained etiology before 20th week of gestation and 102 healthy women with at least two normal pregnancies were included. The genotypes for two SNPs in the *CTLA-4* gene a C/T transition at position -318 and A/G transition at position +49, were determined by using PCR-RFLP analysis. The results showed that the - 318C/T polymorphism in *CTLA-4* gene promoter region differed significantly between unexplained RPL patients and controls, while no association was found between +49A/G in exon 1 polymorphism and unexplained RPL (Naderi et al., 2014).

In Tunisia, a study was performed to investigate the relationship between unexplained RPL and *CTLA-4* gene SNPs -318 C/T (rs5742909), +49A/G (rs231775) and CT60 A/G (rs3087243), by Taq-Man assays. The study sample consisted of 470 Tunisian women comprising 235 RPL cases and 235 multiparous controls. Their results showed a significant association between rs231775 AG genotype and CGA and CAG haplotypes and RPL susceptibility and no significant association with other investigated SNPs (Messaoudi, Houas, Yaseen, Dandana and Mahjoub, 2014).

In Japan, a case control study was performed to investigate the relation between *PD-1* and *CTLA-4* gene polymorphisms and RPL by using Taqman PCR assay on 264 patients who were diagnosed with unexplained RPL and 181 fertile controls and the subsequent live birth rate in the 264 patients was compared with and without the risk allele. The study found that there was a significant difference in frequency of the SNP of *PD-1* gene of the RPL group however, there was no difference in the SNP of *CTLA-4* gene. From this study, the live birth rates of patients with the risk allele of *PD-1* gene were determined to be 84.6% and those without to be 81.7%, with no significant difference after excluding cases with an abnormal embryonic karyotype. Product of conception (decidua and villus tissue) was tested by immunohistochemistry on cases resulting in pregnancy loss and having a normal embryo in the next pregnancy and showed little difference in the expression of *PD-L1* with or without risk allele of *PD-1* gene (Hayashi et al., 2015).

A study on 120 women with RPL and 120 control group in Southwest of Iran was performed to investigate the relationship between RPL and *CTLA-4* (+49 A/G) polymorphism by using PCR-RFLP method. The results showed a significant association between the minor alleles (G) with the decreased risk of RPL. The frequency of the G allele in controls and patients was 25% and 12%, respectively. A GG genotype in the co-dominant model and in the dominant model for allele G (GG+AG vs. AA) showed significant associations with RPL (Rasti and Nasiri, 2016).

Chapter 3

Methodology

Chapter 3

Methodology

3.1. Materials

3.1.1. Equipment

The present work was carried out in the Genetics lab at the Islamic University of Gaza. The major equipment used in the study are listed in **Table 3-1**.

Table (3.1): The major equipment used in this study

	Item	**Manufacturer**
1.	Thermal Cycler	BioRad, USA Biometra
2.	Horizontal electrophoresis chambers/tanks	BioRad, Germany
3.	Electrophoresis power supply	BioRad, Germany
4.	Digital balance	AE adam, USA
5.	Vortex mixer	BioRad, Germany
6.	Gel documentation system	Vision, Scie-Plas Ltd, UK
7.	Safety cabinet	Heraeus, Germany
8.	Microcentrifuge	BioRad, Germany
9.	Freezer, refrigerator	ORSO, pharml-spain
10.	Micropipettes 0.1-2.5 µl 0.5-10 µl 5-50 µl 20-200 µl 100-1000 µl	Dragon-lab, USA
11.	Microwave Oven	L.G, Korea

3.1.2. Chemicals, Kits and Disposables

Chemicals, kits and disposables used in this study are listed in **Table 3-2.**

Table (3.2): Chemicals, kits and disposables used in this study

	Item	**Manufacturer**
1.	Wizard ® Genomic DNA Purification Kit	Promega (Madison, USA)
2.	PCR Go Taq® Green Master Mix	Promega (Madison, USA)
3.	Agarose	Promega (Madison, USA)
4.	PCR primers	Promega (Madison, USA)
5.	Nuclease free water	Promega (Madison, USA)
6.	Ethidium Bromide (EtBr) 10mg/ml	Promega (Madison, USA)
7.	DNA molecular size marker (Ladder).	BioLab, New England (UK)
8.	Restriction enzymes	BioLab, New England (UK)
9.	Absolute Isopropanol and Ethanol 70%	(Sigma USA)
10.	Filter tips 0.1-10 µl 5-50 µl 20-200 µl 100-1000 µl	Labcon, USA
11.	Microfuge tubes for PCR - thin wall 0.2 mL capacity	Labcon, USA
12.	Microfuge tubes - 1.5 mL capacity	Labcon, USA
13.	EDTA tubes	Hy. Labs. Israel
14.	Disposable tips	Labcon, USA

3.2. Study sample

3.2.1. Study design

The current study is a case-control study, in which women with RPL were compared to women without any record of abortion.

3.2.2. Study location

Genetics lab-Islamic University, Gaza strip.

3.2.3. Study subjects

The study group (n=200) included women aged 20-35 years who had experienced at least two spontaneous abortion before 20th week of gestation. The control group (n=200) consisted of women who had delivered at least one healthy child and had no previous history of pregnancy loss. Controls were matched with study subjects for all other possible characteristics. None of the individuals included in the study population used oral contraceptives, hormonal, or any serious medication affecting body vital functions. Individuals with known causes of RPL were excluded from the study group.

All study samples were recruited from the Genetics lab of the Islamic university of Gaza.

3.2.4. Ethical considerations

Informed consent was taken from all the subjects who participated in the study. The objective of the study was fully explained to all participants and their consent was taken. The study was approved by the Ministry of Health (MOH) in Gaza Strip.

3.3. Genotyping:

3.3.1. DNA extraction and polymorphism determination

About 2.0 ml of venous blood were drawn into sterile EDTA tubes under quality control and safety procedures. Genomic DNA was isolated from blood using Wizard Genomic DNA Purification Kit (Promega, USA) following the manufacturer instructions.

The isolated DNA was stored at -20C◦ until analysis of *CTLA-4* gene polymorphisms.

3.3.2. PCR primers reconstitution

Primers were received in a lyophilized state. Primer containers were first centrifuged at 13,000 rpm for 3 minutes, and then reconstituted with ultrapure water (nuclease free water) to create a stock solution of each primer with a final concentration of 100pmol/µl. The stock primer solution was then mixed using vortex. 10 µl aliquot was taken from stock primer and diluted with 90 µl nuclease free water to make 10pmol/µl working solution.

3.3.3. Analysis of CTLA-4 gene polymorphisms by PCR-RFLP

The four SNPs were genotyped using PCR-RFLP technique. The specific PCR primers were designed using online Primer3 software based on the genomic sequence deposited in gene bank and the sequence of each SNP was retrieved from NCBI-SNP database as presented in **Appendix** 1.

Then restriction enzymes required for the PCR-RFLP identification of each SNP were selected from new England Biolabs database, as shown in **Table** 3.3.

PCR primers and conditions, restriction enzyme digestion and results interpretation were done as indicated in **Tables** 3.3 and 3.4.

Table (3.3): The PCR mix and reaction conditions used for genotyping *CTLA-4* SNPs.

SNP	SNP primer	PCR product size	PCR program	PCR mix condition
+49A/G	**F:** TCCTGAAGACCTGAACACCG	222 bp	94◦C, 5 min, 94◦C, 30 sec; 56◦C, 45 sec; 72◦C, 45 sec; 35 cycle 72◦C, 5min	Total volume of 20 μL, with 10 μL Taq PCR Master mix (Promega, USA), 2μL (10pmol) of primers, 4μL Nuclease-free water, 2μL (50ng) of genomic DNA
	R: TGCCTTTGACTGCTGAAACA			
-318C/T	**F:** GGCTCAGAAAGTTAGCAGCC	247 bp	94◦C, 5 min 94◦C, 30 sec; 57◦C, 45 sec; 72◦C, 45 sec; 35 cycle 72◦C, 5min	
	R: ACAACCTCAAGCACTCAACTG			
-1661A/G	**F:** CTAAGAGCATCCGCTTGCACCT	486 bp	94◦C, 5 min 94◦C, 30 sec; 60◦C, 45 sec; 72◦C, 45 sec; 35 cycle 72◦C, 5min	Total volume of 30 μL, with 15 μL Taq PCR Master mix (Promega, USA), 2μL(10pmol) of primers, 9μL Nuclease-free water, 2μL (50ng) of genomic DNA.
-1722 T/C	**R:** TTGGTGTGATGCACAGAAGCCTTT			

Table (3.4): Restriction enzymes, digestion conditions and length of digested fragments

	+49A/G	**-318C/T**	**-1661A/G**	**-1722 T/C**
Enzyme	APeK1	Mn1I	DRA1	APeK1
The enzymatic digestion condition	A 10μL aliquot was digested with 0.12 μL of APek 1 restriction enzyme (NEB, UK) at 75◦C for 30 minute	A 10μL aliquot was digested with 0.2 μL of Mn1I restriction enzyme (NEB, UK) at 37◦C for 1 hour	A 10μL aliquot was digested with 0.2 μL of DRA1 restriction enzyme (NEB, UK) at 37 C for overnight incubation.	A 10μL aliquot was digested with 0.12 μL of APek 1 restriction enzyme (NEB, UK) at 75◦C for 30 minute
Length of digested fragments (bp)	**A allele:** 222 bp	**C allele:** 96+70+67+14 bp	**A allele :** 334 + 152 bp	**T allele :** 486 bp
	G allele : 79 bp + 143 bp	**T allele:** 137 +96 + 14 bp	**G allele :** 486 bp	**C allele :** 270 + 216 bp
- Digested PCR products was then electrophoresed on 3.0% agarose gel and was visualized by ethidium bromide staining.				

3.4. Agarose gel electrophoresis (3.0%)

1. Dried agarose gel (2.4 gm) was dissolved in 80 ml 1x Tirs-Acetate-EDTA buffer (2M Tris base 1M Glacial Acetic Acid, 0.05 M EDTA) by heating.

2. Then 4.0µl Ethidium Bromide(10mg/ml) was added and mixed, the gel was casted into a mold which was fitted with wells-forming comb.

3. The agarose gel was submerged in electrophoresis buffer within a horizontal electrophoresis apparatus.

4. After amplification, the PCR products and a DNA ladder size marker (Promega, Madison, WI, USA) were loaded into the sample wells to aid in fragment size determination.

5. PCR fragments were detected by size in the agarose gel.

6. Electrophoresis was performed by using Electrophoresis power supply (BioRad, USA) at 70 volts for 40 min at room temperature, and the DNA bands were visualized and documented using a UV trans-illuminator documentation system

3.5. Statistical analysis

The Hardy-Weinberg equilibrium (HWE) equation was used to calculate the expected genotype frequencies. Difference between expected and observed genotypes was assessed by X^2 test. *P-value* ≤ 0.05 was considered statistically significant.

The frequencies of the alleles and genotypes were compared between patient and control groups by the Chi square test when appropriate. The odds ratio (OR) and 95% confidence interval (CI) were also estimated in order to test the relation between RPL and the investigated polymorphisms.

Chapter 4

Results

Chapter 4

Results

4.1. PCR-RFLP Genotyping Results

The following figures **(4.1** through **4.4)** represent in a respective manner, genotyping examples of the *CTLA-4* "+49A/G, -318C/T, -1722 T/C and -1661 A\G" gene polymorphisms investigated in this study.

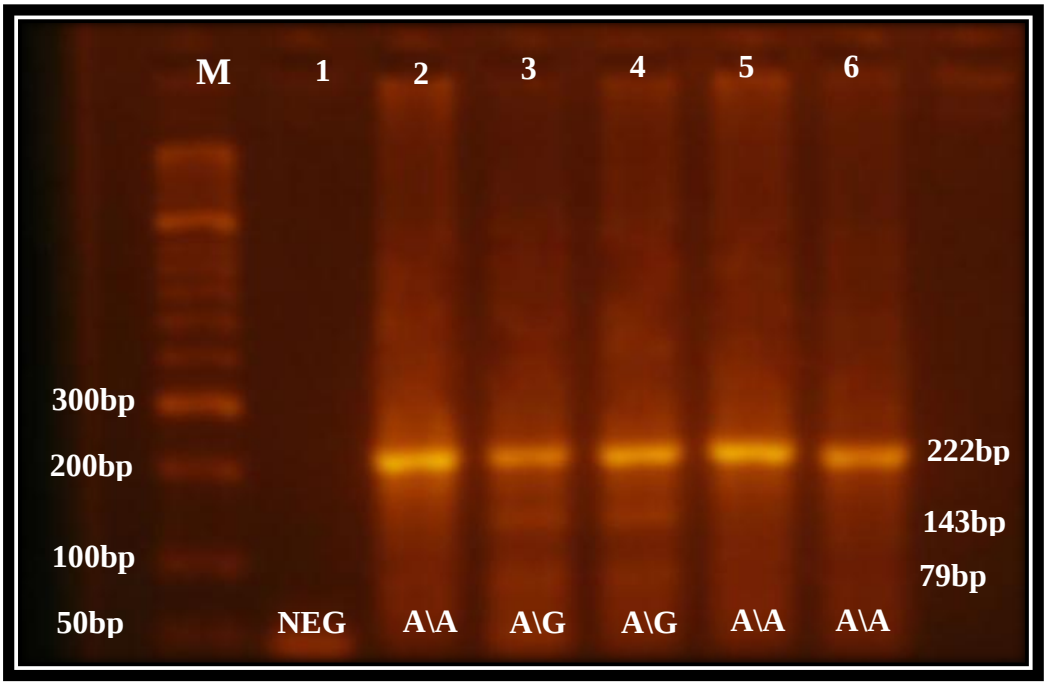

Figure (4.1): A photograph of ethidium bromide stained 3% agarose gel showing the PCR-RFLP products of *CTLA-4* "+49A/G polymorphism.

Lane M: 50 bp DNA ladder. Lane 1 indicates the negative control, lanes 2,5,6 indicate homozygous samples for A\A (222 bp) and lanes 3,4 indicate heterozygous A\G genotype (222 +143+ 79 bp).

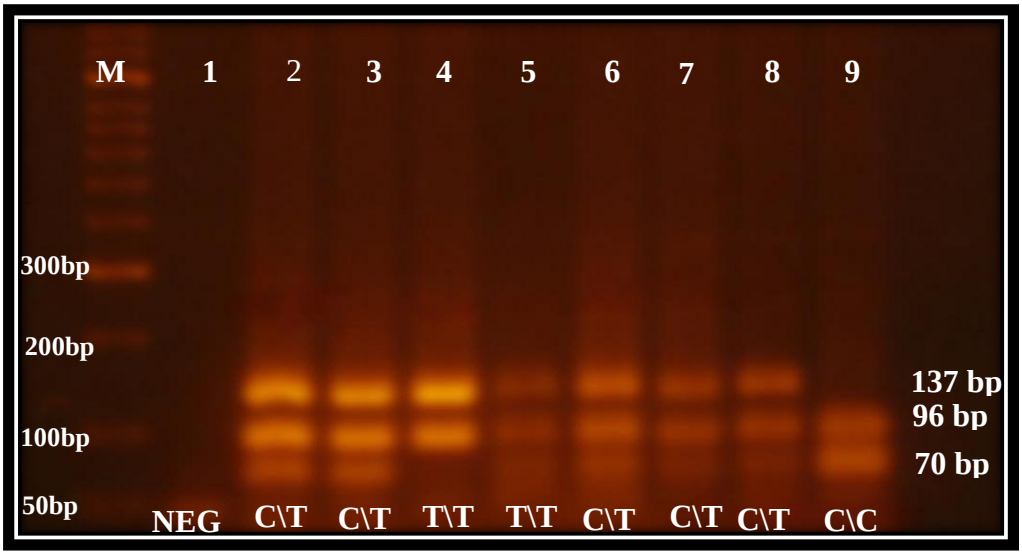

Figure (4.2): A photograph of ethidium bromide stained 3% agarose gel showing the PCR-RFLP products of *CTLA-4* "-318 C/T polymorphism.

Lane M: 50 bp DNA ladder, lane 1 indicates the negative control, lanes 2,3, indicate heterozygous C\T samples (137+ 96 + 70 bp), lanes 4,5 indicate homozygous T\T (137 + 96 bp) and lane 9 illustrates a homozygous C\C (96 + 70 bp).

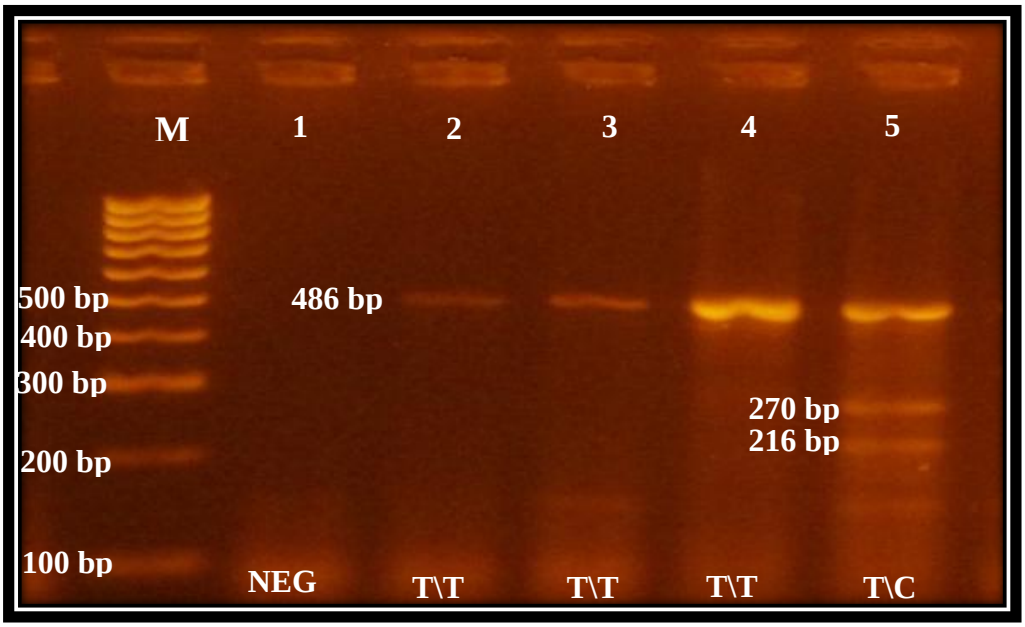

Figure (4.3): A photograph of ethidium bromide stained 3% agarose gel showing the PCR-RFLP results of *CTLA-4* "-1722 T/C polymorphism. Lane M: 100 bp DNA ladder, lane 1 indicate the negative control, lanes 2,3,4 indicate homozygous T\T (486 bp) samples and lane 5 indicates a heterozygous T\C (486, 270+ 216 bp).

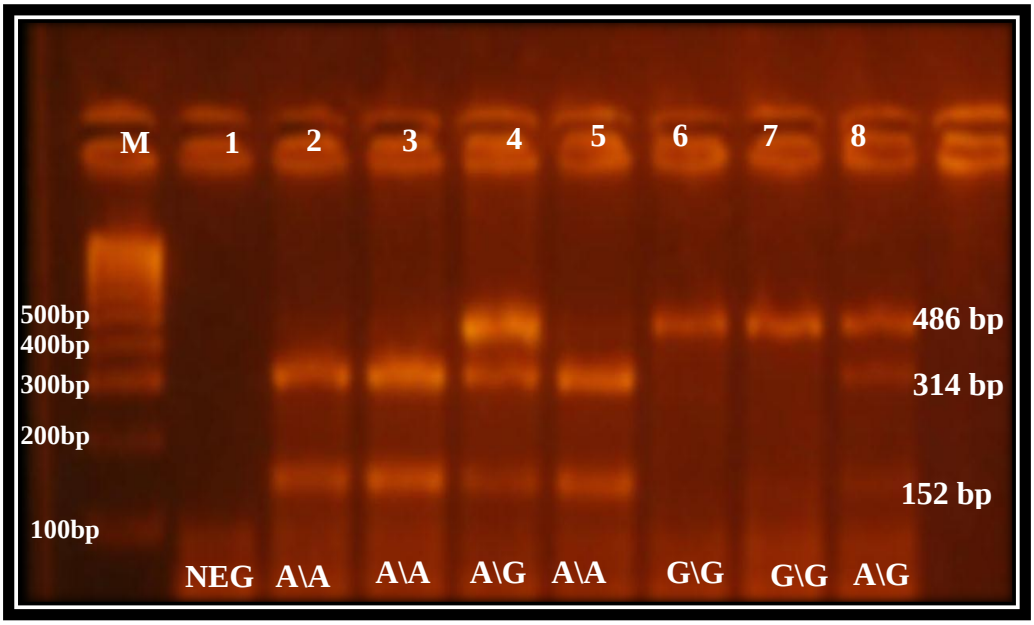

Figure (4.4): A photograph of ethidium bromide stained 3% agarose gel showing the PCR-RFLP products of *CTLA-4* "-1661 A/G polymorphism.

Lane M: 100bp DNA ladder, lane 1 indicates the negative control, lanes 2,3,5 indicate homozygous A\A genotype (314 +152 bp), lanes 4,8 indicate heterozygous A\G (486 +314 + 152 bp) and lanes 6,7 indicate G\G homozygous genotype (486 bp).

4.2. Genotype and allele frequencies of *CTLA-4* gene polymorphisms in patients and controls

Table 4.1 illustrates genotypes and alleles frequencies, odds ratio, 95% confidence intervals and *P* values for the four *CTLA-4* gene polymorphisms among RPL patients and controls.

Statistical analyses of genotypic and allelic frequencies for the tested SNPs revealed **no significant** (all P values are > 0.05) difference between RPL patients and controls.

Table (4.1): Genotype and allele frequencies of *CTLA-4* gene polymorphisms in RPL patients and controls.

SNP	Allele	Patients n=200	Controls n=200	OR (95%CI)	P-Value
CTLA-4 +49A/G	A\A	111 (55.5%)	112 (56%)	0.98 (0.66 to 1.45)	0.92
	A\G	89 (44.5%)	88 (44%)	1.02 (0.68 to 1.51)	0.92
	G\G	0	0	-	-
	Normal A	311(77.75%)	312 (78%)	0.98 (0.70 to 1.37)	0.93
	Mutant G	89 (22.25%)	88 (22%)		
CTLA-4 -318C/T	C\C	145 (72.5%)	159 (79.5%)	0.68 (0.43 -1.08)	0.1
	C\T	51 (25.5%)	37 (18.5%)	1.04 (0.64 -1.7)	0.86
	T\T	4 (2%)	4 (2%)	1 (0.24 - 4.05)	1
	Normal C	341(85.25%)	355 (88.75%)	0.73 (0.48 to 1.10)	0.14
	Mutant T	59 (14.75%)	45 (11.25%)		
CTLA-4 -1661A/G	A\A	95 (47.5%)	108 (54%)	0.77 (0.52 to 1.14)	0.19
	A\G	90 (45%)	79 (39.5%)	1.25 (0.84 to 1.86)	0.26
	G\G	15 (7.5%)	13 (6.5%)	1.16 (0.54 to 2.52)	0.7
	Normal A	280 (70%)	295 (73.75%)	0.83 (0.61 to 1.13)	0.23
	Mutant G	120 (30%)	105 (26.25%)		
CTLA-4 -1722 T/C	T\T	170 (85%)	167 (83.5%)	1.11 (0.65 to 1.92)	0.68
	T\C	30 (15%)	33 (16.5%)	0.9 (0.52 to 1.53)	0.68
	C\C	0	0	-	-
	Normal T	370 (92.5%)	367 (91.75%)	1.10 (0.66 to 1.85)	0.73
	Mutant C	30 (7.5%)	33 (8.25%)		

4.3. Hardy-Weinberg equilibrium in the four *CTLA-4* polymorphisms

Deviation from Hardy-Weinberg equilibrium (HWE) was assessed as given in the following representative example.

HWE for *CTLA-4* +49A\G:

Frequency of major allele A (p) = 112X2 +88X1\200X2 = 0.78

Frequency of minor allele G (q) = 0 X2+ 88X1\200X2 = 0.22

Expected genotype frequencies:

Genotype AA: p2 x200 = $(0.78)^2$ X 200 = 121.68 individuals.

Genotype AG: 2pq x200 = 2X0.78X0.22 X 200 = 68.64 individuals.

Genotype GG: q^2 x200 = $(0.22)^2$X 200 = 9.68 individuals.

The difference between observed and expected genotype frequencies in the control group was determined by using Chi (X^2) square test.

Table 4.2 illustrates the observed and expected genotype frequencies of *CTLA-4* polymorphisms in the control group. Chi square testing showed that there is no significant deviation from Hardy-Weinberg equilibrium for -318C\T, -1661 A\G and -1722 T\C SNP genotypes. *CTLA-4* +49A\G genotypes, however, showed a significant deviation from Hardy-Weinberg equilibrium with a ***P*-value =0.0001.**

Table (4.2): Observed and expected genotype frequencies of the four *CTLA-4* gene polymorphisms in the control group.

	Observed Genotype	**Expected Genotype**	**P-value**	**Chi square (X^2)**
+49A\G				
A\A	112	121.7	0.0001*	15.9
A\G	88	68.64		
G\G	00	9.68		
-318 C\T				
C\C	159	157.5	0.3	1.08
C\T	37	39.9		
T\T	4	2.5		
-1661 A\G				
A\A	108	108.78	0.77	0.081
A\G	79	77.4		
G\G	13	13.78		
– 1722 T\C				
T\T	167	168.36	0.2	1.6
T\C	33	30.28		
C\C	00	1.36		

***df* =1.**

*** Significant difference**

4.4. The frequencies, odds ratio and P-values of the four *CTLA-4* gene polymorphisms among RPL and control subjects under recessive model

Table 4.3 illustrates the frequencies, odds ratio and *P*-values of the *CTLA-4* gene SNPs among RPL and control subjects under recessive model. The statistical analyses showed that there is no significant difference between the two groups.

Table (4.3): The frequencies, odds ratio and *P*-values of the *CTLA-4* gene polymorphisms among RPL and control subjects under recessive model.

SNP	Allele	Patients n=200	Controls n=200	OR (95%CI)	*P*-Value
CTLA-4 +49A/G	A\A+A\G	200 (100%)	200 (100%)	1 (0.02 to 50.64)	1.00
	G\G	0	0	-	-
CTLA-4 -318C/T	C\C +CT	196 (98%)	196 (98%)	1 (0.24 to 4.05)	1.00
	T\T	4 (2%)	4 (2%)	1 (0.24 to 4.05)	1.00
CTLA-4 -1661A/G	A\A+A\G	185 (92.5%)	187 (93.5%)	0.86 (0.4 to 1.85)	0.69
	G\G	15 (7.5%)	13 (6.5%)	1.16 (0.54 to 2.52)	0.69
CTLA-4 -1722T/C	T\T+T\C	200 (100%)	200 (100%)	0.68 (0.43 -1.08)	1.00
	C\C	0	0	-	-

4.5. Distribution of *CTLA-4* gene polymorphisms in the study population under dominant model

Table 4.4 illustrates the frequencies, odds ratio and *P*-values of the *CTLA-4* gene SNPs among RPL and control subjects under dominant model. The statistical analyses indicates no significant difference between the two groups under this model.

Table (4.4): The frequencies, odds ratio and P-values of the *CTLA-4* gene polymorphism among RPL and control subjects under dominant model.

SNP	Allele	Patients n=200	Controls n=200	OR (95%CI)	*P*-Value
CTLA-4 +49A/G	A\A	111 (55.5%)	112 (56%)	0.98(0.66 to 1.45)	0.92
	A\G +G\G	89 (44.5%)	88 (44%)	1.02(0.68 to 1.51)	0.92
CTLA-4 -318C/T	C\C	145 (72.5%)	159 (79.5%)	0.68 (0.43 -1.07)	0.10
	C\T+T\T	55 (27.5%)	41 (20.5%)	1.47(0.92 to 2.34)	0.10
CTLA-4 -1661A/G	A\A	95 (47.5%)	108 (54%)	0.77(0.52 to 1.14)	0.19
	A\G+G\G	105 (52.5%)	92 (46%)	1.3 (0.87 to 1.92)	0.19
CTLA-4 -1722T/C	T\T	170 (85%)	167 (83.5%)	0.68 (0.43 -1.08)	0.10
	T\C+C\C	30 (15%)	33 (16.5%)	1.04 (0.64 -1.7)	0.86

4.6. Distribution of *CTLA-4* gene polymorphisms in the study population under co-dominant model

Table 4.5 illustrates the frequencies, odds ratio and *P*-value of the *CTLA-4* gene SNPs among RPL and control subjects under co-dominant model. The statistical analyses revealed no significant difference between the two groups under this model.

Table (4.5): The frequencies, odds ratio and P-values of the *CTLA-4*" gene polymorphisms among RPL and control subjects under co-dominant model.

SNP	Allele	Patients n=200	Controls n=200	OR (95%CI)	P-Value
CTLA-4 +49A/G	A\G(***wt/mt***)	89 (44.5%)	88 (44%)	1.02 (0.68 to 1.51)	0.92
	A\A+G\G ***wt/wt+mt/mt***	111 (55.5%)	112 (56%)	0.98 (0.66 to 1.45)	0.92
CTLA-4 -318C/T	C\T(***wt/mt***)	51 (25.5%)	37 (18.5%)	0.68 (0.43 -1.08)	0.10
	C\C+T\T ***wt/wt+mt/mt***	149 (74.5%)	163 (81.5%)	1.04 (0.64 -1.7)	0.86
CTLA-4 -1661A/G	A\G(***wt/mt***)	90 (45%)	79 (39.5%)	1.25 (0.84 to 1.86)	0.26
	A\A+G\G ***wt/wt+mt/mt***	110 (55%)	121 (60.5%)	0.78 (0.53 to 1.18)	0.26
CTLA-4 -1722T/C	T\C(***wt/mt***)	30 (15%)	33 (16.5%)	0.68 (0.43 -1.08)	0.10
	T\T+C\C ***wt/wt+mt/mt***	170 (85%)	167 (83.5%)	1.04 (0.64 -1.7)	0.86

Chapter 5

Discussion

Chapter 5
Discussion

Pregnancy loss is one of the serious complications of pregnancy, which affects 1-2% of women attempting to conceive. Despite years of effort to determine the factors involved in miscarriage, the cause remains elusive in around 50% of the cases hence, this reflects the heterogeneous nature of this malady (Rasiti and Nasiri, 2016).

Several mechanisms have been proposed to function actively in the protection of the semi-allogeneic fetus from maternal immune system. The presence of regulatory T cells (Tregs) and the expression of immunomodulatory molecules at the fetal maternal interface have been identified as crucial factors for fetomaternal tolerance (Riella et al., 2013).

The regulation of Tregs in turn, is mediated by antigen independent co-stimulatory signals, which act on the surface of Treg cells (Gupta et al., 2012). Co-stimulation signal of T cells plays a key role in maintaining the immune tolerance to fetal tissue, and also provides a new strategy of therapeutic intervention for RPL (Gupta et al., 2012).

CTLA-4 is a member of the co-stimulatory molecules. CTLA-4 acts as a negative regulator of activated T cells and may have a critical role in Th1/Th2 equilibrium (Dehaghani et al., 2005). Its expression could induce nuclear localization of Foxo3 transcription factor, leading to inhibition of IL-6 production and other inflammatory cytokines (Tait and Hunter, 2009). CTLA-4 expression in placental fibroblasts and deciduas supports its role in the maintenance of pregnancy and fetomaternal tolerance (Rasti and Nasiri, 2016). Therefore, it is suggested that abnormal expression of *CTLA-4*, effected by genetic polymorphism(s), may be associated with RPL. Indeed, some *CTLA-4* single nucleotide polymorphisms (SNPs) have been implicated as potential risk factors for RPL in certain populations.

The present study was carried out in order to investigate whether the *CTLA-4* "-318 C/T, -1661 A\G, -1722 T\C and +49A\G" polymorphisms contribute to RPL

susceptibility. The four polymorphisms were investigated in 200 Palestinian women suffering from RPL and 200 healthy controls.

- **Association between *CTLA-4* gene exon-1 "+49 A/G" "dbSNP ID rs231775" Polymorphism and RPL:**

The study results revealed that this SNP does not predispose to RPL since, the genotype and allele frequencies did not show any significant difference between the RPL and the control groups. This relation remained true under dominant, co-dominant and recessive models.

Interestingly, the homozygote "GG" genotype was not observed in either the case or the control group. Based on our data the minor allele (G) frequency (MAF) for this polymorphism in the control group is 0.22 meaning that this genotype is expected to occur in about 10 individuals of the 200 control samples, as illusterated in **Table** 4.1 and 4.2. Therefore, the absence of this genotype in our population may be a consequence of selection against this "deleterious?" genotype. This also explains the observed deviation of this SNP genotypes from Hardy-Weinberg equilibrium.

Literature search on association of this polymorphism and RPL revealed contradictory results. Whereas (Wang et al., 2005; Gupta et al., 2012; Messaudi et al., 2014 and Rasiti and Nasiri, 2016) showed that there is a significant association between *CTLA-4* +49 A\G polymorphism and RPL. (Chaili, 2010; Pendeloski et al., 2011; Bonyadi et al., 2014; Naderi , 2014 and Hayashi et al., 2015) showed that there is no association between *CTLA-4* +49 A\G polymorphism and RPL.

- **Association between *CTLA-4* Gene "-318 C/T" "dbSNP ID rs5742909" Promoter Polymorphism and RPL:**

Regarding *CTLA-4* "-318 C/T" polymorphism the C/T genotype was more frequent in the RPL patients as compared to control women (25.5%, 18.5%, respectively) but this difference did not reach statistical significance (***P* value= 0.86).** On the other hand, the C/C genotype frequency was higher in the control group relative to the patients (79.5%, 72.5%, respectively) but the difference also was not significant (***P* value= 0.1).**

In analogy to *CTLA-4* +49 A\G polymorphism and RPL, studies from different population showed divergent results. (Chaili, 2010 and Naderi, 2014) have reported that there is a significant association between *CTLA-4* "-318 C/T" polymorphism and RPL. (Messaoudi et al., 2014) however, did not replicate such an association.

- **Association between *CTLA-4* Gene "-1661 A/G" "dbSNP ID rs4553808" Promoter Polymorphism and RPL:**

The A/A genotype of this polymorphism was observed more frequently in the control women as compared to the RPL patients (54%, 47.5% respectively) but this difference was not significant (**P value= 0.19).** The A/G genotype was higher in the RPL patients relative to the control group (45%, 39.5%, respectively) but again the difference was not statistically significant (**P value= 0.26).**

In harmony with our results, (Chaili, 2010) showed that *CTLA-4* "-1661 A/G" polymorphism is not associated with RPL.

- **Association between *CTLA-4* Gene "-1722 T/C" "dbSNP ID rs733618" Promoter Polymorphism and RPL:**

Concerning this polymorphism, the comparison of genotype and allele frequencies in the case and control groups did not show significant differences. Similar results were reported by (Chaili, 2010) who also showed a lack of association between *CTLA-4* "-1722 T\C " polymorphism and RPL.

Moreover, the CC homozygote genotype was not encountered in any individual of the investigated RPL and control subjects. The explanation for the absence of the CC genotype is mainly due to the uncommon occurrence of the C-allele (MAF ~ 0.08) in our population. Given this C-allele frequency and the sample size of the study it is expected to find only one individual harboring the CC genotype in the study sample, as shown in **Table** 4.2.

Contradictory results are a common place in genetic association studies performed on different populations. Possible explanations for discrepant results include one or more of the following, differences in the ethnicity (genetic background), the sample size, the inclusion and exclusion criteria of the study, presence of nucleotide polymorphism(s) somewhere else in the examined gene, epigenetic alterations,

linkage disequilibrium to other sequence variants in the vicinity of the studied locus, and prevailing environmental conditions.

Ethnic differences may interfere with allele/genotype frequencies and influence the results. Therefore, one SNP may be associated with RPL in a specific population, but not in a different one. On the other hand, the heterogeneity of RPL may affect the involvement and nature of genetic susceptibility factors.

A major problem of most published studies on *CTLA-4* polymorphism and RPL up to the present time is their modest sample size. As RPL occurs in approximately 2% of all women, it is difficult for a single center to recruit a representative number of RPL patients. The limited number of cases included in most studies reduces the statistical power often leading to non-significant results.

Moreover, RPL seems to be a multifactorial disorder rather than a single disease. This implies that it is probably caused by multiple (genetic, epigenetic and non-genetic) factors, and therefore, RPL might be related to polymorphisms in a more than expected number of genes.

Chapter 6

Conclusion and Recommendations

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Conclusion:

The present study focused on the of *CTLA-4* gene polymorphisms: (+49A\G, -318 C\T, -1661 A\G and -1722T\C) in 200 Palestinian women suffering from RPL and 200 matching controls. The results can be summarized as follows:

1- The study revealed that there is no statistically significant association between the examined *CTLA-4* gene polymorphisms and RPL in our population.

2- The minor allele frequencies (MAFs) of the *CTLA-4* gene polymorphisms in the control group are as follow: +49A\G: 0.22, 318 C\T: 0.11, -1661 A\G: 0.26 and -1722T\C: 0.08.

3- The A/G genotype of -1661 A\G SNP was higher in the RPL patients relative to the control group (45%, 39.5%, respectively) but the difference was not statistically significant (P value= 0.26).

4- The control group *CTLA-4* "+49A\G" polymorphism genotypes deviated significantly from Hardy-Weinberg equilibrium probably due to a deleterious effect of the GG genotype.

5- The control group genotypes of CTLA-4 "– 1661 A\G", "-318 C\T "and "-1722 T\C" polymorphisms genotypes were in Hardy-Weinberg equilibrium.

6- The lack of significant difference in the alleles/genotypes frequency between RPL and control groups implies that the investigated polymorphisms do not represent a risk factor for RPL in the study sample.

Recommendations:

1. Performing studies on a larger sample size to confirm the lack of association between *CTLA-4* "- 1661 A\G" gene polymorphism and RPL.
2. Testing additional *CTLA-4* gene polymorphisms in the RPL patients.
3. Examining other genes polymorphisms involved in immune-tolerance in RPL cases.
4. Performing further studies to correlate the level of CTLA-4 with RPL.

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Appendix 1

(Primer Design)

A- Primers designed

SNP	Primers positions*
49 A/G	CAAACACATTTCAAAGCTTCAGGATCCTGAAAGGTTTTGCTCTACT**TCCTGAAG** **ACCTGAACACCG**CTCCCATAAAGCCATGGCTTGCCTTGGATTTCAGCGGCACAA GGCTCAGCTGAACCTGGCT**A**CCAGGACCTGGCCCTGCACTCTCCTGTTTTTTCT TCTCTTCATCCCTGTCTTCTGCAAAGGTGAGTGAGACTTTTGGAGCATGAAGAT GGAGGAGGTGTTTCTCCTACCTGGGTTTCAT**TTGTTTCAGCAGTCAAGGGCA**GT GATTTA
318C/T	CTGCCATTAGCCCAAGGGCTCAGAAAGTTAGCAGCCTAGTAGTTTTGGAGATGT CAATG**AAATGAATTGGACTGGATGG**TTAAGGATGCCCAGAAGATTGAATAAAAT TGGGATTTAGGAGGACCCTTGTACTCCAGGAAATTCTCCAAGTCTCCACTTAGT TATCCAGATCCT**C**AAAGTGAACATGAAGCTTCAGTTTCAAATTGAATACATTTT CCATCCATGGATTGGCTTGTTTTGTTCAGTTGAGTGCTTGAGGTTGTCTTTTCG ACGTAACAGCTAAACC**CACGGCTTCCTTTCTCGTAA**AACCAAAACAAAAAGGCT TTCTATTCAAGTGCCTTCTGT
1661A/G & **1722 T/C**	TCATTGGCCCTTGCTG**CTAAGAGCATCCGCTTGCACCT**TCTGCTCATCCCCAGA CAAGCTTTGTCCTGTGACCATAATGAACTCTTCATGCCGTTTCCAACTTTAGCC CATGTTATTCTTCTTGTCTGAATATCCACCCTTTTCTCTGTTCTCAATAATAAG TTCAGGCTTTTCGTCTTCTGAGAAGCCCTTTCTGACTTCCACAGGCTGAACCAC TGGCTTCTGCTCCTCTACATAATACTTCAACTCCAGCATTGATCTCACTCTATC ATGATCATGGGTTTAGCTG**T**CTGTCCCTGCCACTGCTGTGTGTTCCTCTTGAGG GCAGGAACATTTGTTTTTCACTTTTT**A**AAAAACCTCTGTTGCCCAGTCTGGCAT TAGGAAGTGCCCATTAGGTTGTTATTGCTTGTTGGCGCTTGAGCTGGGGCTTGA AGATTTCTATAATGTGTAGCAGTGTATAGAAAACAGGCAGGTCAGA**AAAGGCTT** **CTGTGCATCACACCAA**CATGGCACATGTATACATATGTAACAAATCTGCATGTT GTGCACATGTAC

* Forward and reverse primers as well as the SNPs are highlighted

B- Single nucleotide polymorphisms (SNP) sequence:

SNP	(SNP) sequence
rs231775	GCACAAGGCTCAGCTGAACCTGGCT[A/G]CCAGGACCTGGCCCTGCACTCTCCT
rs5742909	GTCTCCACTTAGTTATCCAGATCCT[C/T]AAAGTGAACATGAAGCTTCAGTTTC
rs4553808	CAGGAACATTTGTTTTTCACTTTTT[A/G]AAAAACCTCTGTTGCCCAGTCTGGC
rs733618	GAACACACAGCAGTGGCAGGGACAG[A/G]CAGCTAAACCCATGATCATGATAGA