T.R.N.C

NEAR EAST UNIVERSITY

GRADUATE SCHOOL OF HEALTH SCIENCES

HLA CLASS II POLYMORPHISMS AS A RISK FACTOR IN JORDANIAN PATIENTS WITH TYPE I DIABETES MELLITUS

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MEDICAL BIOCHEMISTRY PROGRAM

MASTER OF SCIENCE THESIS

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SUPERVISOR
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The Directorate of Graduate School of Health Sciences,

This study has been accepted by the Thesis Committee in Medical Biochemistry Program as a Master of Science Thesis.

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According to the relevant articles of the Near East University Postgraduate Study – Education and Examination Regulations, this thesis has been approved by the above mentioned members of the thesis committee and the decision of the Board of Directors of the Institute.

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ABSTRACT

Obaied M.H. HLA Class II Polymorphisms as a Risk Factor in Jordanian Patients with Type I Diabetes Mellitus. Near East University, Graduate School of Health Sciences, M.Sc. Thesis in Medical Biochemistry Program, Nicosia, 2018.

Type 1 diabetes mellitus (T1D) is a chronic autoimmune disease characterized by the absence of insulin as a result of the immuno-selective destruction of beta cells of the langerhans islets in pancreas. Environmental and genetic risk factors play a role in the occurence of T1D. Human leukocyte antigen (HLA) class II molecules particulary DQA1 and DQB1 haplotypes have been discovered to be related with the T1D. In our study, it was aimed to detect haplotypes which make individuals susceptible to or protect against T1D in Jordanian population. DNA was purified from blood taken from healthy participants and T1D patients. HLA-DQA1, HLA-DQB1 and cytotoxic T-lymphocyte associated protein 4 (CTLA4) gene regions were amplified by PCR followed by restriction digestion. Finally digestion products were applied to the agarose gel electrophoresis to evaluate different haplotypes. Only DQA1*0301 has been found to increase susceptibility for T1D. On the other hand, DQA1*0201 and DQB1*0501 have been detected as protective against T1D. Other haplotypes did not reveal significant differences in control and patient groups. Also, no significant correlation has been observed in terms of CTLA4 polymorphisms.

Keywords: Type one diabetes mellitus, human leukocyte antigen class II, cytotoxic T-lymphocyte associated protein 4

ÖZET

Obaied M.H. Ürdün Popülasyonundaki Tip 1 Diabetes Mellitus'lu Hastalarda Risk Faktörü Olarak HLA Class II Polimorfizmleri. Yakın Doğu Üniversitesi, Sağlık Bilimleri Enstitüsü, Tıbbi Biyokimya Programı, Yüksek Lisans Tezi, Lefkoşa 2018.

Kronik otoimmün bir hastalık olan tip 1 diabetes mellitus (T1D) pankreasta bulunan langerhans adacıklarının beta hücrelerinin immüno-seçici olarak harabiyete uğramasının sonucunda insülinin üretilememesi ile karaterize edilmektedir. T1D'nin oluşumunda çevresel ve genetik faktörlerin rolü bulunmaktadır. İnsan lökosit antijeni (HLA) sınıf II moleküllerinin, özellikle DQA1 ve DQB1 haplotiplerinin T1D ile ilişkili olduğu gösterilmiştir. Çalışmada Ürdün populasyonunda T1D'ye neden olabilecek ya da hastalıktan koruyucu etki gösterebilecek haplotiplerin araştırılması amaçlanmıştır. Sağlıklı bireylerden ve T1D hastalarından alınan kan örneklerinden saflaştırılan DNA'nın HLA-DQA1, HLA-DQB1 ve sitotoksik T-lenfosit ilişkili protein 4 (CTLA4) gen bölgeleri PCR ile çoğaltılmış ve sonrasında restriksiyon enzimleri ile kesim işlemi uygulanmıştır. Kesim ürünleri haplotiplerin değerlendirilmesi için agaroz jel elektroforezinde ayrılarak görüntülenmiştir. T1D ile DQA1*0301 haplotipi arasında korelasyon gözlenirken, DQA1*0201 ve DQB1*0501 haplotiplerinin T1D'ye karşı koruyucu olduğu bulunmuştur. Kontrol ve hasta grubu arasında diğer haplotipler bakımından anlamlı bir farklılık gözlenmemiştir. Ayrıca CTLA4 polimorfizmleri bakımından gruplar arasında bir farklılık bulunmamıştır.

Anahtar Kelimeler: Tip 1 diabetes mellitus, insan lökosit antijeni sınıf II, sitotoksik T-lenfosit ilişkili protein 4

TABLE OF CONTENTS

ABBREVIATIONS

ADA	American Diabetes Association
Anti-GAD	Anti-Glutamic Acid Decarboxylase
Anti- IA2	Anti-Tyrosine Phosphatase-Like Insulinoma Antigen 2
Anti-ICA	Anti- Islet Cell Autoantigen
Anti- ZNT8A	Anti-Zinc Transporter Protein 8
APC	Antigen-Presenting Cell
CD	Cluster of Differentiation
CLEC16A	C-Type Lectin Domain Containing 16A
CTLA-4	Cytotoxic T-lymphocyte Associated Protein-4
DC	Dendritic Cell
GAD	Glutamic Acid Decarboxylase
HSP	Heat Shock Protein
HLA	Human Leukocyte Antigen
IAA	Insulin Autoantibody
IDDM	Insulin Dependent Diabetes Mellitus
IFIH-1	Interferon Induced with Helicase C Domain-1
IFN-γ	Interferon Gamma
IGRP:	Islet-Specific Glucose-6-Phosphatase Catalytic Subunit-Related Protein
IL	Interleukin
IL2RA	Interleukin 2 Receptor- Alpha
INS	Insulin
LADA	Latent Autoimmune Diabetes of Adults
MHC	Major Histocompatibility Complex
NIDDM	Non-Insulin-Dependent Diabetes Mellitus
NK	Natural Killer
NOD mice	Non Obese Diabetic mice
PCR	Polymerase Chain Reaction
PI	Proinsulin
PTPN2	Protein Tyrosine Phosphatase Non-Receptor Type 2

PTPN22	Protein Tyrosine Phosphatase Non-Receptor Type 22
TBE	Tris/Borate/EDTA
TCR	T-cell Receptor
T1D	Type 1 Diabetes Mellitus
T1DA	Type 1 Diabetes Mellitus A
T1DB	Type 1 Diabetes Mellitus B
T2D	Type 2 Diabetes Mellitus
TNF-α	Tumor Necrosis Factor-α
VDR	Vitamin D Receptor
WHO	World Health Organization

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

Diabetes mellitus is a disease that affects the ability of the body to produce or use insulin, that causes high levels of glucose in the blood or so called hyperglycemia. Chronic diabetes includes type 1 diabetes (T1D) and type 2 diabetes (T2D). Long term complications of diabetes include neuropathy, retinopathy, kidney damage (nephropathy), diabetic foot and heart disease (van Belle et al., 2011).

T1D is characterized by the insufficiency of beta cells to make insulin hormone due to an attack of immune system to pancreas. Therefore T1D is accepted as an autoimmune disease (Wong and Wen, 2003). Diabetes is more common in childhood, but some cases it is diagnosed in adulthood which is called late autoimmune diabetes. The presence of a damage in beta cells can be detected by the presence of insulin (IAA), glutamic acid decarboxylase (GAD65), protein tyrosine phosphatase (ICA-512 or IA-2) autoantibodies (Katahira, 2011).

T1D is caused by several factors. Although some have not been discovered yet, it has been confirmed that its appearance depends on genetic and environmental factors. In most of the cases genetic factors and genetic predisposition play a major role (Mehers and Gillespie, 2008). Studies have revealed a link between T1D and human leukocyte antigen (HLA) gene which is divided into three groups; HLA class I, class II and class III genes (Noble et al., 2010). The HLA class II gene is made up of multiple forms like DQ, DR and DP each with its own functions (Wong and Wen, 2003). Since HLA gene is multi haplotype gene, initially researchers gave their attention to identify the haplotype which is linked with T1D. DR haplotype was found to be responsible for the genetic aspect of the disease (Katahira, 2011). Then recently DQA and DQB haplotypes have been found to cause susceptibility for more than 50% of the diabetic cases (Zayed, 2016). It was a multiple haplotypes of the HLA DQB and DQA genes make it a double-edged sword where the genetic cause of the disease can be diagnosed and identified more precisely, but the multiplicity of these genes makes it necessary for further studies by taking geographical distribution of these haplotypes into consideration. In the Caucasian communities there is close association between T1D and DRB1*0301-DQA1*0501- DQB1*0201 and the DRB1*0405- DQA1*0301-DQB1*0302, DRB1*0401-DQA1*0301-DQB*0302, and DRB1*0402-DQA1*0301-DQB1*0302 haplotypes, followed by the DRB1*0404-

DQA1*0301-DQB1*0302 and the DRB1*0801-DQB1*0401-DQB1*0402 haplotypes. DRB1*1501-DQA1*0102-DQB1*0602, DRB1*1401-DQA1*0101-DQB1*0503 are considered as protective (Ehrlich et al., 2008). In contrast, DQA1*0103, DQA1*0201, DQA1*0401, DQB1*0301, DQB1*0402, DQB1*0501, DQB1*0503, DQB1*0601 and DQB1*0602 alleles are considered protective in Chinese population (Morran et al., 2015). Therefore, ethnicity and the place of residence should be considered when evaluating HLA DQA and DQB haplotypes.

Some studies have suggested that there are non-immune genes like insulin (INS) gene, cytotoxic T-lymphocyte associated protein 4 (CTLA-4), lymphoid protein tyrosine phosphatase, non-receptor type 22 (PTPN22), interleukin 2 receptor alpha (IL2RA), interferon induced with helicase C domain 1 (IFIH1), vitamin D receptor (VDR), KIAA0350 and phosphotyrosine protein phosphatase, non-receptor 2 (PTPN2) that may be correlated with T1D (Mehers and Gillespie, 2008).

It must also be noted that environmental factors are suggested to promote T1D. Although evidence is not sufficient, enteroviral infections, nutritional factors, exposure to harmful environmental pollutants are thought to be effective on the predisposition of T1D in early childhood (Bodin et al., 2015).

Having various genetic haplotypes brings complexity to disease (Mehers and Gillespie, 2008). Depending on the ethnical origin, some countries show the existence of genetic forms that contribute to the prevention of T1D while other forms contribute significantly to the formation of the disease (Jerram and Leslie, 2017). Thus, it was reasonable to conduct intensive studies in different parts of the world to detect the genetic causes of T1D and whether there are other factors that interfere with the occurrence of disease (Katahira, 2011). Considering the high prevalence of T1D in Arab countries where endogamous and consanguineous marriages (10–70%) and also first-cousin marriages are also common, Jordanian population is one of the best populations in this area to study HLA class II haplotypes (Zayed, 2016). Thus, two hundred patients were included in this study to reveal the relationship between HLA class II DQA1, DQB1 and CTLA-4 haplotypes and T1D.

2. GENERAL INFORMATION

2.1. Diabetes Mellitus

Diabetes mellitus is one of the most common diseases worldwide. Diabetes mellitus is related with a wide range of clinical manifestations from being asymptomatic to ketoacidosis or coma, depending on the degree of metabolic disorder, but most common is hyperglycemia due to insufficient insulin action. Both genetic and environmental factors play a role in the occurence of diabetes (Seino et al., 2010). Diabetes mellitus can be categorized into four groups:

1- Diabetes mellitus type 1 (T1D) is described as a chronic autoimmune disease caused by an attack to the insulin secreting pancreatic beta cells. T1D is genetically predisposed with the presence of environmental stimuli and also called insulin dependent diabetes mellitus (IDDM) (Mehers and Gillespie, 2008) with different onset mode during the age (Katahira, 2011). The World Health Organization (WHO) and the American Diabetes Association (ADA) categorized T1D into two categories; immune-mediated (autoimmune) and idiopathic (Katahira, 2011).

2- Diabetes mellitus type 2 (T2D) is characterized with diminished β-cell function and insulin resistance. Obesity is the major risk factor for the development of T2D. T2D is non-insulin dependent diabetes mellitus (NIDDM) which usually occurs after the age of 40. Environmental factors, life style habits like lack of exercise, high fat-carbohydrate diet and as well as genetic factors influence the formation of the disease. Both low insulin secretion and insulin resistance involve in the onset of T2D but the ratio of participation differs from one patient to other (Butler et al., 2003).

3- Gestational diabetes is the most common complication of pregnancy. It might be associated with maternal and neonatal adverse outcomes. In order to maintain stable glucose concentration in blood, diet and exercise are the first treatment strategies. If these fail to achieve glycemic goals, insulin injection is suggested. In paralel with the increase in the prevalence of T2D and obesity, its prevalence tends to increase worldwide (varying from 1-20%). Various risk factors are associated with the gestational diabetes. Most commons are obesity, history for

gestational diabetes, family history for diabetes, older maternal age, ethnicity and polycystic ovary syndrome (Alfadhli, 2015).

4- Diabetes due to other specific mechanisms and diseases can be divided into two groups; 1. Those in which specific mutations have been identified which cause abnormalities in pancreatic β-cell function and abnormalities in insulin action. 2. Those associated with other diseases or conditions like diseases of exocrine pancreas, endocrine disease, liver disease, drug- or chemical-induced, infections, rare forms of immune-mediated diabetes, various genetic syndromes often associated with diabetes (Seino et al., 2010).

2.2. Type 1 Diabetes Mellitus

Although protecting the body against foreign subjects is a specialized function of the immune system, in some cases immune system starts to destroy the normal cells which causes autoimmune diseases like T1D. In T1D, body is deprived of insulin due to a destruction of β-cells of the langerhans islets in pancreas. What triggers this autoimmune attack is not clear but there is a general acceptance that environmental and genetic factors have some roles (Noble and Valdes, 2011). Type 1 diabetes can be diagnosed at early stages of life (in the age group of 5-7 years) or it might be slightly delayed until puberty (van Belle et al., 2011).

The risk of T1D varies from one geographical location to another also from one ethnical origin to another. As an example; three Scandinavian countries, Norway, Sweden and Finland have the highest incidence rate among European countries. United States of America reported 23.7 incidence rate in 100,000 in population under the age 15. (Patterson et al., 2014). In Jordan, as the target research region, the frequency is 32/100,000 a year (Ajlouni et al., 1999).

The way patients develop T1D is not completely understood, but there are some hypotheses: For example, the disease is triggered by an infection of inflammatory cells driven by dendritic cells (DCs) and macrophages as a top level, accompanied by B and T lymphocytes in non obese diabetic (NOD) mice. Furthermore, in people same process takes place with a less grade of infiltration (Willcox et al., 2009). In the study of the T1D, 26 gene loci were identified and at least 6 of them are shared between NOD mice model and human in terms of the risk

for the disease (Bluestone et al., 2010). It has been shown that the deceptive initial occurance in T1D is a rise in the course of insulitis (Eizirik et al., 2009) through apoptotic β-cells when DCs are stimulated. In reality however, this is led by the autoreactive T cells in the pancreatic lymph nodes (Turley et al., 2003). The existence of one or a complex of intra- and extra-thymic can reduce tolerance ratio and manage diabetogenic T cell reproduction, which might then arrive to the islets. A hypothetical function has been transferred to specific pathogens in human T1D ability, while pathogens may additionally award contrasting susceptibility for T1D onset through the inducement of inflammatory or immunoregulatory mediators (Tanaka et al., 2013).

Environmental factors also trigger T1D in individuals which have genetic risks for the disease. They lead to a stimulation of dendritic cells (DCs) and β cellular antigen-specific T cells in pancreatic lymph nodes. T cell activation is mediated by proinflammatory cytokines along with interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), and interleukins (IL-1β, IL-6, IL-17, IL-12). Numerous infiltrating immune cells make a contribution to the inflammatory microenvironment: DC, $CD4^{+}$-T_h (helper) 1 cells, monocytes, macrophages, and $CD8^{+}$-T_c (cytotoxic) cells. This inflammatory microenvironment inside the islets might also up-regulate primary Fas molecules and the major histocompatibility complex-I (MHC-I) that recognised by infiltrated diabetogenic T cells. Crucial evidence of the function of regulatory T and B cells and natural killer (NK) cells stil remains unclear. This autoimmune event is mostly gradual, and development may change in diabetic individuals. Subsequently, just a little insulin-generating cells are present in the islets during the first months of T1D (Tian et al., 2006; Barcala Tabarrozzi et al., 2013).

2.2.1. Classes of Type 1 Diabetes Mellitus

The American Diabetes Association divided T1D into two categories: Type 1 Diabetes Mellitus A (T1DA) and Type 1 Diabetes Mellitus B (T1DB).

-Type 1 Diabetes Mellitus A (T1DA)

The immune-mediated elimination of pancreatic beta cells is called T1DA. Diabetes is often diagnosed with fast-onset in children, but it does not prohibit the occuring of

the disease in adults, but in very few cases which is usually common in adults, delayed symptoms and lack of insulin suggest latent autoimmune diabetes (LADA). This expression has been usually used to indicate the autoimmune forms of diabetes that started without insulin requirement (Katahira, 2011). This type is correlated with autoantibodies targeting insulin-producing cells: anti-tyrosine phosphatase (anti-IA2), anti-glutamic acid decarboxylase (anti-GAD), anti-islet cell antigen (anti-ICA) and anti-zinc transporter 8 protein (anti-ZnT8) (Alves et al., 2016). Beta cell destruction in autoimmune T1DA may occur quickly in some patients or it might be delayed in others, also it has genetic susceptibility with environmental risk (Atkinson and Eisenbarth, 2001; Seino et al., 2010).

–Type 1 Diabetes Mellitus B (T1DB)

This type of diabetes is idiopathic and uncommon. No signs of autoimmune response against pancreatic beta cells and genetic predisposition have been detected (American Diabetes Association, 2015)

2.2.2-Stages of Type 1 Diabetes Mellitus

T1D occurs at all ages but usually common in children. Clinical manifestations of T1D are polyuria, polydipsia and wight loss. Patients have diabetic ketoacidosis which may cause life threatening complications if it is not treated (Fajans et al., 1978). There are three stages of T1D that help us to know and distinguish T1D from other types.

-Existence of islet autoantibodies

The existence of auto antibodies like anti-IA2, anti-GAD and anti-ICA automatically gives a clear signal for an evidence of autoimmune diabetes (van Belle et al., 2010). It has been shown that main autoantibodies like proinsulin (PI), GAD65, I-A2, and ZnT8 have a role in the destruction of beta cells in the islets of the Langerhans (Taplin and Barker, 2008). The main focus of the process is $CD4^+$ cells are originated from GAD65, IA-2, and PI with a little contribution by heat shock protein 60 (HSP-60), islet spesific glucose 6-phosphatase catalytic subunit-related protein (IGRP) and HSP-70 (van Belle et al., 2010). Beta cell destruction requires

coexistence of $CD4^+$ and $CD8^+$ T cells, that either of these T lymphocyte subsets are required eventually (Hammond et al, 1998). T1D prevention has been proposed to be achieved by isolating CD4 and CD8 T cells from the beta islets (Wong et al., 1996). Also eradication of major histocompatibility complex (MHC) class I is one of the suggestions for T1D prevention. The deficiency in beta 2-microglobulin gene causes a decrease in MHC class I gene expression which also results in the supression of insulitis (Hamilton-Williams et al., 2003). Recent studies revealed that the primary responsible factor for the destruction of beta cells in the islets of langerhans is CD4 and then CD8 T cells. This has been clearly demonstrated in non obese diabetic (NOD) mice which is similar to humans (Baxter et al., 1997) In practice, beta-cell breakdown can be initiated by CD8 T lymphocytes which make granzyme and perforine containing cytolytic granules and followed by Fas and Fas ligand-dependent interactions. By releasing cytokines like interleukin 1b, 6, 17 and 12, CD4 contributes to the action of CD8 T and B cells. Also, the interaction of CD40 and CD40L with antigen-presenting cells makes a positive feedback (Hammond et al, 1998). Both T and B lymphocytes are important in the formation of the disease. However, little importance has been given to B lymphocytes, they are vital antigen presenting cells (APC) during the response against autoantigens. APC inclusive of macrophages and DCs might also spark off and provoke responses against B- cell autoantigens. However, B- cells seem to play a completely single position with the aid of enlargement and the variety of T cell clones. B lymphocytes play a large spectrum of capabilities and it is observed in each pathogenic and regulatory process in T1D (Noorchashm et al., 1997).

-Disease Onset Mode

T1D can be classified according to the onset mode as: fulminant, rapid and slow onset. They also differ from each other in terms of their insulin requirements (Imagawa et al., 2000). Fulminant T1D appears within 1–2 weeks from the beginning of the disease. Slow onset does not need insulin or requirement might arise after 6 months from the begining of the disease, while rapid onset needs insulin between 2 weeks to 6 months from the beginning of disease (Atkinson, and Eisenbarth, 2001).

-Insulin deficiency

In some cases, pancreatic cells continue to produce insulin for few months but in low levels, this period is called honeymoon for T1D patient (Atkinson et al., 2014). In fact, the T1D is characterized with insulin deficiency that results from the breakdown of beta cells in the langerhans islets of pancreas (Abdul-Rasoul et al., 2006). Clinically, insulin deficiency is described by decreased serum or urine C peptide levels. In the diabetic conditions, C-peptide in serum is < 0.5 ng per ml (0.17 nmol/L), or fasting C-peptide in serum is < 0.3 ng/ml (0.1 nmol/L), and the C-peptide level in urine is < 10 μg per day (3.3 nmol/day), after a meal load or (glucagon taken) (Imagawa et al., 2003; Imagawa and Hanafusa, 2006). In rapid-onset diabetes fasting C-peptide in serum is < 0.4 ng/ml (0.13 nmol/L), or C-peptide serum is < 1.0 ng/ml (0.33 nmol/L) and C-peptide level in urine is < 20 μg/day (6.6 nmol/day), after taking glucagon or meal (Stenström et al., 2005). Although slow-onset diabetes has two phases in terms of insulin requirement, due to hyperglycemia in both types of diabetes, there is a need for insulin (Imagawa et al., 2000).

2.2.3. Risk Factors for Type 1 Diabetes Mellitus

2.2.3.1. Genetic Risk Factors

A disease is difficult to study since there are many forms of genes that interfere with the development of disease. Genetic factors play important roles in many diseases that may affect various organs in human body. More than 50 candidate genes have been studied to reveal the association with T1D and about 80% has been found to be related with the disease which makes T1D as multigenetic disease (Wong and Wen, 2003). The HLA class II DQ and DR allels play roles and give progress in T1D. Particularly, DQA1 and DQB1 polymorphisms are the main risk factors for T1D (Polychronakos and Li, 2011). In addition, there are other genes that function in the development of T1D like protein tyrosine phosphatase, non-receptor type 22 (PTPN22), insulin (INS), KIAA0350 and cytotoxic T lymphocyte-associated protein 4 (CTLA-4). On other side, some genes have been identified as protective against T1D like DQB1*0602 (Qiu et al., 2014).

2.2.3.2. Environmental risk factors

Studies showed that genes may not be sufficient to cause T1D, also there are environmental risk factors assuming in T1D diseases (Akerblom et al., 2002). Environmental factors trigger T1D in patients with genetic susceptibility (van Belle et al., 2011). Environmental factors include some viruses, childhood infection, diet and gestational events (Bodin et al., 2015). There is a broad scientific evidence that gives a clear indication about the contribution of non-genetic factors in the promotion of the disease. It is now accepted that the stimulation of T1D occurs due to some environmental factors that in turn interact with genes that are genetically prepared and initiate together an autoimmune attack towards beta cells in the pancreas (Akerblom et al., 2002).

-Enterovirus

The presence of the enterovirus in the blood is an environmental factor that causes the development of T1D due to the selective infection of beta cells. Additionally, scientists have discovered that there is a similarity between Cox P2-C protein in Coxsackievirus and the GAD autoantibody, that is a major autoantibody in the T1D (Tian et al., 1994). One of the events that confirmed the link was the same response of peripheral blood mononuclear cells to both Cox P2-C and GAD peptides (Atkinson et al., 1994). The most common T1D diabetes, which is very typical in Japan, has been found to have a rapid onset and clinical symptoms without the development of autoimmune antibodies and this disease usually starts with a sudden hyperglycemia and ketone body elevation among infected individuals which may be similar to about 70% with symptoms of acute mumps disease, intestinal viruses, human virulence virus (Imagawa and Hanafusa, 2011). Response to the presence of viruses in individuals with type 1 diabetes points to a strong relationship between viruses and genes that stimulate the destruction of beta cells in the pancreas (Tanaka et al., 2013).

-Bacteria

It is known that the intestinal bacteria may affect the development of T1D and this has been shown in tests conducted on rodents. These experiments explain the

development of the disease in people living in places devoid of most of the other environmental factors causing the disease, or when giving antibiotics. Although antibiotics can lower the risk of growing T1D, utilization of some antibiotics may alter gut microbiota balance which causes increased permeability of the mucosal membranes and following autoimmune activation (Vaarala et al., 2008).

2.3. Overview of Type 1 Diabetes Mellitus Genetics

T1D is considered as the most complex genetic disorder because there are environmental and genetic factors. All these factors play important roles in the development of the disease. Several studies have been done to elucidate the relationship between genetic risk factors and T1D in different areas of the world (Polychronakos and Li, 2011). The study of T1D helps to discover the complexity of genetic inheritance compared to some diseases with Mendelian inheritance pattern which might be easier to study with. Still, there are many studies being done to identify the gene pool behind the T1D (Jerram and Leslie, 2017). The strong evidence in genetic participation to T1D can be explained by the fact that 65% of the identical twins of T1D proband will develop T1D before the age of 60. Also the children born with an affected family have 5% of T1D risk by the age 20 in contrast with 0.3% risk of growing T1D with children who have not affected family members (Noble and Valdes, 2011).

Recently discovered that major histocompatibility complex (MHC) region located on chromosome 6 in vertebrate genome is related with the T1D (Atkinson et al., 2014). MHC, also known as human leukocyte antigen (HLA) in humans, is a surface protein of white blood cells which is responsible for antigen binding in the acquired immune system (Campbell and Trowsdale, 1993). HLA is mapped at 6p21.31 and have classical loci which encodes 4 MB long gene. The main function of HLA antigens is to provide protection against pathogens (Horton et al., 2004). There are three classes of HLA; class I, class II and class III. Class I HLA gene presents endogenous antigens to $CD8^+$ T cells, class II works on exogenous antigens for the elimination by $CD4^+$ T cells and class III (HLA_peptide_TCR) generates the immune response (Horton et al., 2004). Studies have indicated that there was a correlation between the HLA region and the susceptibility to T1D (Hamilton-

Williams et al., 2003). A detailed view of HLA region in human genome reveals the localization of class I and II HLA's which are telomeric and centromeric ends, respectively. Three HLA regions make up more than 200 genes inherited from the two parents and some genes are included in immune response process (Campbell and Trowsdale, 1993). HLA class I has A, B and C genes while HLA class II includes DR, DQ and DP genes. HLA class III is located in between class I and class II HLA regions (Figure 2.1). The product of this area is structurally homologous cell surface protein, it binds antigenic peptides and presents them to T cells (Horton et al., 2004). More recently, studies reported that the HLA class II genotype (DQA1, DQB1, DR) plays an essential role in T1D than the class I genes, especially DQ genes are more essential than DR genes (Mehers and Gillespie, 2008).

Nomenclature system in literature describes the locus. For example; DQB1*04:05 haplotype in which allele name is DQB1, serologic group is 04 and individual allele is 05 (Noble and Valdes, 2011).

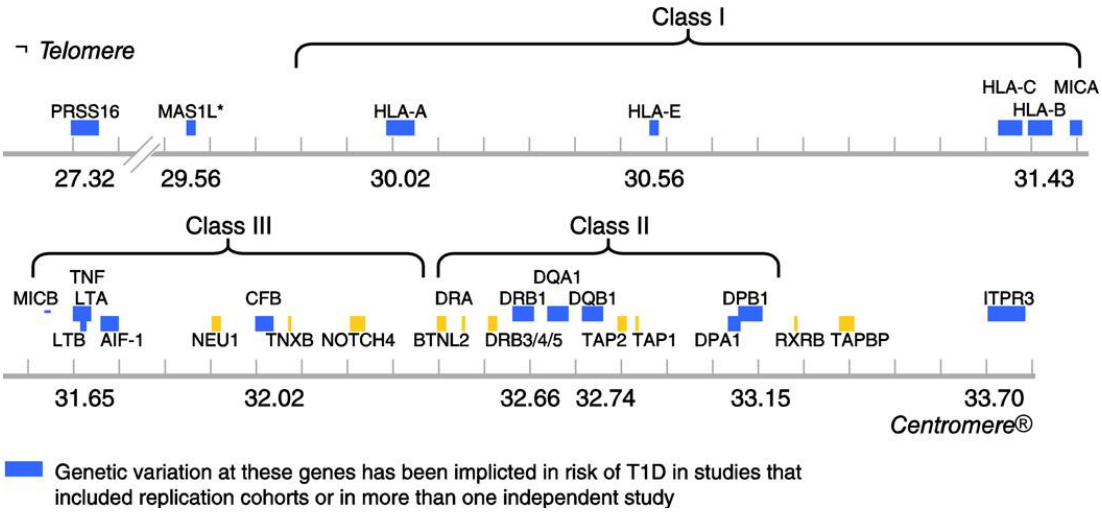

Figure 2.1. Map of HLA region showing genes important for T1D (Noble and Valdes, 2011)

Worldwide reports and research has stated that HLA class II loci hold extreme importance in susceptibility to or protection against T1D. It has been observed that HLA class II genotypes do vary with ethnic differences. DQB1*0201, DRB1*0301, DQA1*0501 and/or DQB1*0302, DRB1*0401, DQA1*0301, DQB1*0302 and many other haplotypes have shown to be associated with T1D

(Wong and Wen, 2003). These genotypes are very common within a Caucasian population with T1D, and about 10% of Caucasian patients with T1D have both of those genotypes (Patterson et al., 2014). HLA class II genotypes display highest relevance with T1D in which DQB1*0302 and DRB1*0401 and DQB1*0201 and DRB1*0301 represent the highest susceptibility and DQB1*0602, DRB1*0501 and DQA1*0102 genotypes prevent T1D (Polychronakos and Li, 2011). While the DR4 polymorphism is uncommon and DR3 polymorphism is absent in Japanese population, that may explain the lowest incidence of T1D in the Japanese population (Katahira, 2011).

Genetically speaking, the HLA alleles are observed in every human population which are inherited from the parents. Major role can be attributed to the HLA class II DR and DQ and to a lesser extent to HLA class II DP in the development of T1D (Noble and Valdes, 2011). Polymorphisms in these alleles may result in various variations (Jerram and Leslie, 2017).

2.4. Other Genes Contributing Type 1 Diabetes Mellitus

Although, HLA particularly class II appears to be highly related with T1D, there are other gene regions correlated with odds ratio higher than 1.1 (Atkinson et al, 2014). These loci are related to the risk of T1D. They stimulate immune response, consisting of the expansion and renewal of tolerance. This pathway may help to give an explanation for the progression of differing quotes for T1D in adults compared to children, where it is reflected through minor differences in genetic susceptibility as have been stated. Fourty eight genes have been found susceptible to the impact during environmental stimuli or physiological pathways (Barrett et al., 2009).

2.4.1. Insulin gene

According to the studies of insulin (INS) gene expression, it has been observed that class I alleles are correlated with elevated INS expression inside the pancreas while as contrasted with class III alleles. Furthermore, INS gene is genuine within the thymus in which class I alleles are expressed at 2 to 3 fold minimal stages. This was probably to modify the choice of T cells in the thymus (Barrett et al., 2009)

2.4.2. Protein Tyrosine phosphatase, Non-Receptor Type 22

According to studies in 2000s, protein tyrosine phosphatase, non-receptor type 22 (PTPN22) has been found to localized on chromosome 1p13. Gene product is related with the increased susceptibility to T1D due to poor T cell activation. As the T1D-related allele is noticeably not unusual inside the well known populace, identification of PTPN22 as a T1D susceptibility gene prominence the significance of T cell activation (Bjornvold et al., 2008).

2.4.3. Cytotoxic T-Lymphocyte Associated Protein 4

The cytotoxic T-lymphocyte associated protein 4 (CTLA-4) gene is located on chromosome 2q33. It produces T cell receptor which is important in T cell apoptosis. It is also negative modulator of the activation of T cells. Transmitted changes during CTLA-4 gene expression can increase T cells' self reactivity and consequently autoimmune diseases such as T1D (Nistico et al., 1996).

2.4.4. KIAA0350

KIAA0350, or C-Type Lectin Domain Containing 16A (CLEC 16A), is a gene with unknown feature located on chromosome 16p13.2. It is recognized with the aid of cDNA library sequencing. Its shape, expected from the coding sequence, submits that it has a C-type lectin-binding area, showing that it might act as cellular surface receptor. It is recognized by immune cells, especially by B lymphocytes (Jerram and Leslie, 2017).

However, all these four genes contribute to the occurence of T1D, still the HLA class II and the CTLA-4 play an essential role in the T1D susceptibility. Recent sudies found that more than 50 percent of the "diagnosed novel genes" (one hundred HLA genes/171 novel genes) are positioned on the HLA region. However, within the 53 T1D-related genes tested by using replication research and various expression studies, 28 genes have been found as non-HLA genes. The function of these non-HLA genes at once is predominantly unknown, but some of these genes play vital role in immune response (Qiu et al., 2014).

2.5. Type 1 Diabetes Mellitus and HLA Class II Polymorphisms

Genes encoding HLA molecules are placed on chromosome 6. The HLA is divided into three classes: Class I (HLA-A, B and C), class II (HLA-DR, HLA-DQ, HLA-DP) and class III. The class I and class II proteins coded by using the pertinent genes are transmembrane cell surface glycoproteins, which are crucially take much more attention due to their ability to serve foreign antigens to T cells (Horton et al., 2004). Although there are some complexities in the understanding of the HLA class II gene polymorphisms, studies have summarized four major forms (DQA, DRA, DQB and DRB) which are difficult to dissect the genetic importance of pure genetic means, but some studies have revealed that there are forms (DR, DQA, DQB) related to nearly 80% of T1D cases (Erlich et al., 2008; Zayed, 2016). A large number of evidence in the human body shows the importance of genetic factors of T1D for susceptibility and protection. Developments in the sequencing technology and genotyping methods make the studies in this field much more easier. Also the different haplotypes in the HLA region direct many studies to test and find out which genes are responsible for T1D. Many researchers tested and found 23–25 gene correlations between T1D and HLA class II (Tandon, 2015). The way by which the class II genes can induce susceptibility to T1D, or the protection from T1D, is still not certain (Brown et al., 1993). One theory is that efficient antigen-binding depends on the modification of the antigen-binding site on the DQ dimer. The two conclusive remnants, DQα 52 and DQβ 57 are put at the obverse ends of the α helices that form the antigen-binding site of the DQ molecule. Another theory is that a exchange of an amino acid of the DQ molecule drives considerable changes of the antigen-binding site and, in order to a adjustment of the ability of the class II molecule for the diabetogenic peptides (Nishimoto et al., 1987). As an extension to this study it is recognized that in the DR molecule, Asp-57 is involved in hydrogen and salt bonding with the antigenic peptide and the Arg-76 situated in the α chain. Modulation in the DRα Arg-76 remnant could also retardate the antigen binding site. This is difficult to observe physiologically even with the DRα chain existence because of the lack of polymorphisms in this chain (Peakman et al., 1999). Other theory was provided by the studies in a transgenic non-obese diabetic (NOD) mouse pattern. Replacement of β chain (which is duplicate to the human class II DQB1 position) Asp 57 with Ser 57

(I-ANOD) aids to conserve these mice from diabetes (Miyazaki et al., 1990). In comparison, the expression of Pro56 and the normal His56 in the I-A β chain shows the same impact. Moreover, expression of certain I-E (which is the duplicate of the human HLA-DR locus) shows resistance to diabetes (Nishimoto et al, 1987; Lund et al., 1990). However, the therapy of NOD mice with an anti-I-A-monoclonal antibody appears to inhibit the diabetes (Boitard et al., 1988). These judgments, conducted in an animal pattern of T1D, clarify the role of both HLA-DQ and HLA-DR in human T1D. The interplay in the members of the trimolecular complex ($CD4^+$ T cell receptors, self-peptide, and MHC class II molecules) has a vital function in the pathogenesis of autoimmune diseases. The expansion of therapies targeting different components of the trimolecular complex is being followed intensively for the prevention of T1D (Michels, 2013).

A structural variation may lead to a huge functional alterations in the antigen presenting ability of the class II molecules. One might imagine that the cells of a body that are heterozygous for both DQα and DQβ would consist all four chain groups on their surface. Competition in antigen binding could occurred with efficient antigen binding is dictated by the modulation of the antigen-binding sites on either DQ dimer (Gough and Simmonds, 2007). Changes at DQα-52 or DQβ-57, that are situated at complementary ends of the alpha helices affect the antigen-binding property. These changes would probably inflict a huge conformational trail on the molecule's antigen-presenting strength. Such conformational variance may be slightly responsible for the susceptibility within the non-Asp-57 allele population, and for the diversity in the degree of preservation accorded by either allele during the group of Asp-57 alleles. Therefore, the susceptive allele non-Asp-57 DQB1*0302 governs over the preservative impact of Asp-57 DQB1*0301 (Tandon, 2015; Jerram and Leslie, 2017). In addition, presence of Asp-57 can also detect genetic resistance against T1D. In different populations, the DQB1*0602 allele frequency is uncommon among T1D patients (Pugliese et al., 1999). Using this allele would help in the preventive process of diabetes in thymic development stage. An unknown diabetogenic peptide can bind to the DQB1*0602 molecule preferentially because of the comparatively higher affinity than to other DQ molecules. This may result in a depletion of self-peptide reactive T cells. Patients with DQB1*0602 allele have high

levels of T cells in thymic maturation stage and that will protect them from diabetes. Lately, protective DQB1*0602 allele carriers and their first-degree relatives are under inverstigation in large scale studies like Diabetes Prevention Trial, which is being acted in the USA. This trial has been designed to diagnose T1D earlier in people who have the high risk for the disease (Davey et al., 1994; Mahon et al., 2009). In another study, there are different suggestions about the pre-diabetics carrying different HLA polymorphisms like DQA1*0102 and DQB1*0602 that have the probability to be antibody negative for all islet autoantigens (Pietropaolo et al., 2002). Seven percent of pre-diabetics in the same study carried the HLA DQB1*0602 polymorphisms and that opposed the observations of the protective effect associated with DQB1*0602 (Pugliese et al., 1999; Greenbaum et al., 2000).

HLA genotypes showed different patterns in various ethnic societies. In the Caucasian population there is a strong association between T1D and DQA1*0501, and DQB1*0201 and/or DQA1*0301 and DQB1*0304. About 10% of Caucasian T1D patients have neither of this polymorphisms, and there are small number of T1D patients do not carry this haplotypes (Gerasimou et al., 2018). In contrast the DR4 haplotype is uncommon and the DR3 is missing in the Japanese population. While studying the relevance of HLA class II haplotypes with T1D disease susceptibility, both ethnic and geographical differences must be taken into account (Mehers and Gillespie, 2008). Ethnic differences might be exemplified as:

1. DQB1*0401 and DQA1*0303 polymorphisms are present in East Asia and Japan but they have not been observed in other ethnic populations like Blacks and Caucasians. They are also found in Philippines, Japanese and Taiwanese population but they are absent in Korean people (Katahira, 2011).
2. DQB1*0302 and DQA1*0301 haplotypes give a susceptibility of T1D in Caucasian, Blacks and East Asian populations in contrast they are very rare in Japanese population (Katahira, 2011).
3. DQB1*0303 and DQA1*0302 haplotypes are highly seen in East Asian populations like Japanese population and Caucasians, but it is very rare in Black and Mexican-Americans (Katahira, 2011).

4. DQB1*0604 and DQA1*0102 haplotypes are shown in all ethnicities but some reseachers conclude that these haplotypes give high susceptibility in Japanese, Caucasian and Latin American populations (Katahira, 2011).
5. DQB1*0302 polymorphisms are suggested to have a vital function in Caucasians with T1D. It was also observed that combined with other alleles, DQB1*0302 has a neutral or protective effect in some populations.
6. The DQB1*0201 polymorphism has been mentioned to be related with the risk for T1D. However in some Arab countries, individuals that carry DQB1*0201 polymorphism have been shown to be prone for the development of T1D, but in other Arab countries the same polymorphism has been found as protective (Zayed, 2016).
7. In the study investigating the occurence of HLA class II polymorphisms in African Americans revealed that HLA-DRB1*03 haplotype had two functions. HLA-DRB1*0301 haplotype bears susceptibility of the disase while HLA-DRB1*0302 allele showed protection (Howson et al., 2013).

Among Arab countries, Jordan has been selected as geographical target area in our study. It has been stated that like Caucasians DQB1*0201-DRB1*0301 haplotype is linked with the highest susceptibility to T1D in individuals from Tunisia, Bahrain and Lebanon. Oppositely, DQB1*0402-DQA1*0401 showed high T1D susceptibility in Moroccan and Mediterranean populations. DQB1*0204-DQB1*02 and DQA1*0201 haplotypes proved a resistance to T1D (Zayed, 2016). The intense correlation between HLA and T1D appears in Egyptian population (DRB1*0301-DRB3*0201-DQA1*0501-DQB1*0201), while the DRB1*0403-DQA1*03-DQB1*0302 haplotype showed protection against T1D (Zayed, 2016).

2.6. CTLA-4 and Type 1 Diabetes Mellitus

The cytotoxic T-lymphocyte associated protein 4 (CTLA-4) gene is located in 2q33 chromosome (Ueda et al., 2003). CTLA-4 gene encodes T cell receptor which regulates apoptosis and proliferation of T cells. T lymphocyte specific receptor protein appears upon antigen presentation. Therefore, CTLA-4 is strongly associated with immune and autoimmune responses and may have role in T-cell mediated autoimmune events. A polymorphism has been identified in the first exon of CTLA-4

gene at position 49 which is an exchange of A/G (Ongagna et al., 2002). CTLA-4 took much of an interest due to an inhibitory impact on T-cell proliferation in patients bearing the A/A genotype than the patients bearing the G/G genotype. Therefore, the existence of G allele has been observed as a stimulatory factor in the formation of messenger RNA and proteins required for the proliferation of T cells (Al-Khaldi et al., 2013).

2.7. Development of Type 1 Diabetes Mellitus

Formation of the disease is still not clear and has high complexity. Based on previous studies, it can be speculated that T1D occurs when there is a genetic predisposition to HLA class II polymorphisms. Structure of HLA results in the selection of autoreactive T cell repertoire. It causes a reduction in thymic regulatory cells. Alteration in the expression of MHC class II molecules, activation of antigen presenting cells, increased expression of autoantigenic peptides are accompany with environmental factors. T cell activation together with the presence of B lymphocytes and development of autoantibodies cause $CD4^+$ and $CD8^+$ T cells to destroy pancreatic tissue (Figure 2.2) (Wong and Wen, 2003).

2.8. Type 1 Diabetes Mellitus in Jordan

The Hashemite Kingdom of Jordan is an Arab Kingdom in Western Asia. The total population is 9.46 million people. Some reports indicate that 37% of the population is under the age of 15. Jordan has the highest refugee (Syria, Iraq, Palestine, Libya, etc.) to citizen ratio among all Arab countries. About 2 million refugees do create a fertile environment for the formation and spread of new diseases and environments conducive to diseases such as T1D.

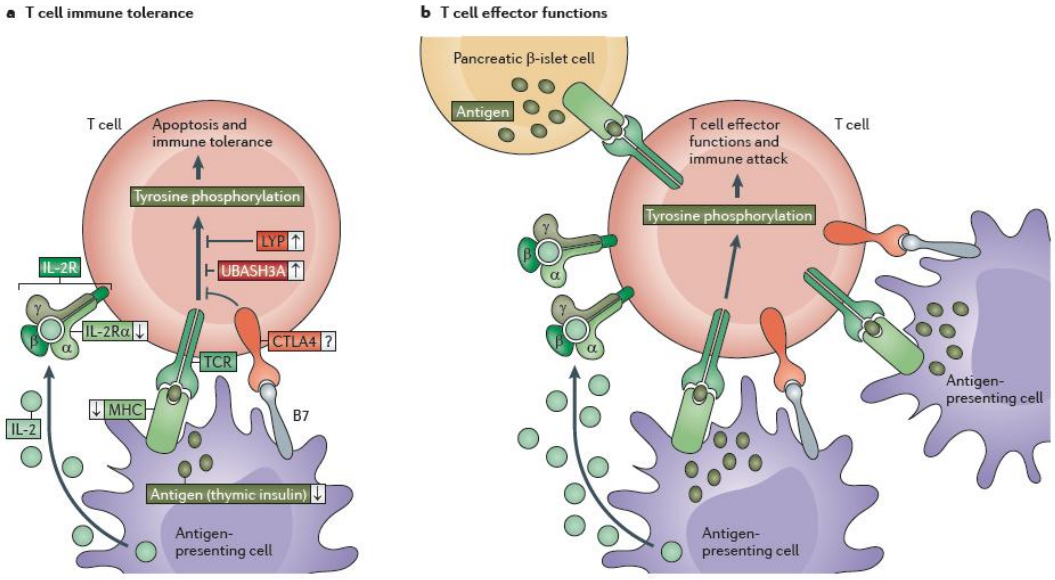

Figure 2.2. Scheme showing the process of correlation between HLA class II molecules with T1D (Polychronakos and Li, 2011).

In 2050 around 3.4 million Jordanians are prospective to be growing diabetes mellitus (Jundi and Ghammaz, 2014). A study conducted by Ajlouni et al. showed an increase in the incidence of T1D over years in Jordanian population. In 1992 it was recorded as 2.8/100,000, in 1994 about 3.2/100,000 and in 1996 it was 3.6/100,000 meaning that the risk of T1D is continually increasing among the Jordanian population. The highest incidence has been detected in 3.2/100,000 between the age of 10-14 and the lowest incidence has been observed in 1.3/100,000 in patients below the age of 4 (Ajlouni et al., 1999). The incidence of T1D in Jordan increased about 40% in 2010 compared to 1998 (Ajlouni et al., 1999). High incidence rate makes Jordan as a suitable population to study T1D and its genetic grounds. Another important factor is to give a decision to work in this region is the existence of tribal traditions like marriage of relatives, especially with cousins which contributes greatly to the existence of many inherited diseases in society (Jundi and Ghammaz, 2014).

3. MATERIALS AND METHODS

3.1. Chemicals

DNA extraction kit was procured from Qiagen (Germany). PCR master mix, nuclease free water, restriction digestion enzymes and 50 bp DNA ladder for agarose gel electrophoresis were obtained from New England Biolabs (United Kingdom). Primers were ordered from Burjuan (Jordan). Ethidium bromide and 10X TBE (Tris/Borate/EDTA) buffer were purchased from Bio Basic (Canada). Agarose was procured from Cleaver Scientific (United Kingdom). All the disposible tools were obtained from Tarson (India).

3.2. Patient and Control Samples

Two hundred (whole blood) control samples (96 male, 104 female) were collected from the healthy individuals choosen from Jordanian population between the age of 1-15. Only five of them had a family history for T1D.

Two hundred samples were collected from patients (81 male, 119 female) between the age of 1-15 who have been diagnosed with T1D. All patients were originally from Jordan. Thirty nine of them had a family history for T1D.

All samples were collected by the cooperation of the Royal Medical Service Hospitals in Jordan after getting written consent. This study was approved by the Ethical Committee of the Near East University (Project no: YDU/2017/48-427).

3.3. Methods

DNA extraction, polymerase chain reaction (PCR) and cleavage of PCR products by restriction enzymes were performed according to the previously published protocol (Ongagna et al., 2002) with some modifications.

3.3.1. DNA Extraction

DNA was purified according to the manufacturer's protocol (Qiagen QIAamp DNA Mini Kit). Twenty µL of proteinase K was added to 200 µL of blood sample. Then 200 µL Buffer AL was added to the mixture. Sample was mixed for 15 s by vortexing and incubated at 56^{o}C for 10 min. Sample was briefly centrifuged to

remove drops from the inside of the lid. Two hundred µL of ethanol (96-100%) was added and sample was mixed by vortexing for 15 s. After mixing, sample was again briefly centrifuged to remove drops from the inside of the lid. The mixture was applied to the QIAamp Mini Spin Column and centrifuged at 6000xg for 1 min. Filtrate was discarded and column was placed into a clean collection tube. Five hundred µL of Buffer AW1 was added to the column and sample was centrifuged at 6000xg for 1 min. Filtrate was discarded and column was placed into a clean collection tube. Five hundred µL of Buffer AW2 was added to the column and sample was centrifuged at 20,000xg for 3 min. Filtrate was discarded and column was placed into a clean collection tube. One hundred µL of Buffer AE was added, the column was incubated at room temperature for 10 min and sample was centrifuged at 6000xg for 5 min for the elution of DNA from the column.

3.3.2. Polymerase chain reaction

PCR mixture contained 25 µL of Master Mix (Taq DNA polymerase, dNTPs, $MgCl_2$, KCl and stabilizers), 1 µL reverse primer (50 nmol stock concentration), 1 µL forward primer (50 nmol stock concentration), 18 µL nuclease free water, 5 µL DNA sample. Primers designed to amplify DQA1, DQB1 and CTLA-4 genes are shown in Table 3.1. PCR cycles were as follow:

Initiation at 95^{o}C for 70 s

Denaturation at 94^{o}C for 30 s
Annealing at 57^{o}C for 30 s } 35 cycles
Extention at 72^{o}C for 45 s

Final extention at 72^{o}C for 5 min.

Agarose gel electrophoresis was performed in order to confirm the amplification of the genes. For this purpose 2.5% agarose gel was prepared in 1xTBE buffer. Samples were run at 125 V for 50 min.

3.3.3. Restriction Digestion

In order to digest PCR products Dde I, Fok I and Rsa I restriction enzymes were used to detect DQA1 polymorphisms. Similarly, DQB1 polymorphisms were detected by cutting the PCR product with Acy I, Hae III, Hha I and Hpa II restriction enzymes. Only one restriction enzyme, Bbv I, was used for CTLA-4 gene. All

enzymes, their target genes and size of the restriction products were shown in Table 3.1. Mixture was prepared for 12 samples. Briefly, 156 µL nuclease free water, 12 µL restriction enzyme, 12 µL buffer were added first, then this mixture was divided into 12 tubes. 10 µL of PCR product was added and samples were incubated at 36^{o}C for 1 h and at 65^{o}C for 20 min.

Table 3.1. Primers used for PCR and restriction enzymes

Primer	Restriction Enzyme	Location	Target Band (bp)	Expected band (Bp)
5'-GGTGTAAACTTGTACCAG-3' 5'-GGTAGCAGCGGTAGAGTTG-3'	Dde I	HLA-DQA1	225	225, 127, 118, 113
	Fok I			225, 187
	Rsa I			225, 186, 183
5'-GATTTCGTGTACCAGTTTAAG-3' 5'-CCACCTCGTAGTTGTGTCTGC-3'	Acy I	HLA-DQB1	241	241, 137, 104, 70
	Hae III			241, 150, 127, 95
	Hha I			189, 141, 112, 90
	Hpa II			241, 198, 126, 115
5'-GCTCTACTTCCTGAAGACCT-3 5'-AGTCTCACTCACCTTTGCAG-3'	Bbv-I	CTLA-4	162	162, 88, 74

3.3.4. Agarose Gel Electrophoresis

Restriction digestion products were visualized by 3% agarose gel electrophoresis. Agarose (3 g) was dissolved in 100 mL of 1xTBE buffer in the microwave. Gel was poured into the electrophoresis tray after the addition of 2 µL of ethidium bromide (final concentration: 0.5 µg/mL) and let it solidify at room temperature for 20 min.

Samples (10 µL-from restriction digestion step) were mixed with 2 µL of gel loding dye. Five µL DNA ladder and 10 µL sample were loaded to the gel. Samples were run at 125 V for 50 min.

3.3.5. Statistical Analysis

Statistical analyses conducted in this research were done using IBM SPSS Software package, Version 19.0. Subjects' demographics, i.e., gender, age, onset of the disease, family history with diabetes as well as specific types of genes were analyzed using frequencies and percentages. Correlations among diabetes type I and demographic data along with genes under study were analyzed on the basis of Pearson correlation coefficients at a significance level of 0.01

4. RESULTS

4.1. Type 1 Diabetes Mellitus and Gender

As seen in Table 4.1, 59.5% (n = 119) of the patients were female and 40.5% (n = 81) were male. No correlation was found between gender and T1D (*R*= 0.76 and sig=.132).

Table 4.1. Description of study groups in terms of gender

	Frequency	Percent	Valid Percent	Cumulative Percent
Patient group				
Male	81	40.5	40.5	40.5
Female	119	59.5	59.5	100.0
Total	200	100.0	100.0	
Control group				
Male	96	48.0	48.0	48.0
Female	104	52.0	52.0	100.0
Total	200	100	100	

R= significant with T1D. When the sig<=0.01

4.2. Type 1 Diabetes Mellitus and Age

Out of 200 diabetic patients, the percentage of patients between the age of 1-5, 6-10 and 11-15 was 17% (n = 34), 43.5% (n = 87) and 39.5% (n = 79), respectively (Table 4.2). There is a significant correlation between the T1D and age (*R*= 0.076, sig = 0.132).

4.3. Type 1 Diabetes Mellitus and the Age of Onset

Table 4.3 indicates that 29.5% (n = 59) of the subjects were diagnosed with T1D between the age of 1-3 years, while 28.5% (n = 57) of them were diagnosed when they were between 4 to 6 years old. On the other hand, 26% (n = 52) of the subjects experienced the disease at the age of 7-9 years and 14% (n = 28) at the age of 10-12 years. Individuals who were diagnosed with the disease after the age of 12

years were only 2% (n = 4). There is no correlation found between the T1D and age of onset (*R*=0.064, sig=0.132).

Table 4.2. Description of study groups in terms of age

	Frequency	Percent	Valid Percent	Cumulative Percent
Patient group				
1-5 years	34	17.0	17.0	17.0
6-10	87	43.5	43.5	60.5
11-15	79	39.5	39.5	100.0
Total	200	100.0	100.0	
Control group				
1-5	58	29.0	29.0	29.0
6-10	100	50.0	50.0	79.0
11-15	42	21.0	21.0	100
Total	200	100	100	

R = significant with T1D. When the sig<=0.01

Table 4.3. Description of patient group in terms age of onset

Age of onset	Frequency	Percent	Valid Percent	Cumulative Percent
1-3 years	59	29.5	29.5	29.5
4-6	57	28.5	28.5	58.0
7-9	52	26.0	26.0	84.0
10-12	28	14.0	14.0	98.0
Older than 12	4	2.0	2.0	100.0
Total	200	100.0	100.0	

R = significant with T1D. When the sig<=0.01

4.4. Type 1 Diabetes Mellitus and Family History

The results in Table 4.4 show the number of individuals with a family history in terms of T1D. It was revealed that 94.5% (n = 189) of the subjects had no family

history with diabetes. According to the previous data analyzed there is no correlation between the family history and T1D (*R*=0.079, sig= 0.266).

Table 4.4. Description of study groups in terms of family history with T1D

	Frequency	Percent	Valid Percent	Cumulative Percent
Patient group				
YES	11	5.5	5.5	5.5
NO	189	94.5	94.5	100.0
Total	200	100.0	100.0	
Control group				
Yes	5	2.5	2.5	2.5
No	195	97.5	97.5	100.0
Total	200	100	100.0	

R= significant with T1D. When the sig<=0.01

4.5. Evaluation of the Restriction Digestion Products on Agarose Gel Electrophoresis

By using the image of agarose gel electrophoresis (Figure 4.1), restriction digestion products were evaluated in terms of HLA class II DQA1, DQB1 and CTLA-4 haplotypes. All evaluations have been made according to the code given in Tables 4.4, 4.5 and 4.6.

4.6. DQA1, DQB1 and CTLA-4 Haplotype Analysis

Samples from 200 control and 200 T1D patients were analyzed and only DQA1*0201, DQA1*0301 and DQB1*0501 were found to be associated with T1D. Only DQA1*0301 haplotype has been found as a risk factor for T1D. Percentage of DQA1*0201 and DQB1*0501 haplotypes was significantly higher in control group than patient group. Therefore, these haplotypes are thought to provide protection against T1D. No significant association was found between CTLA-4 and T1D.

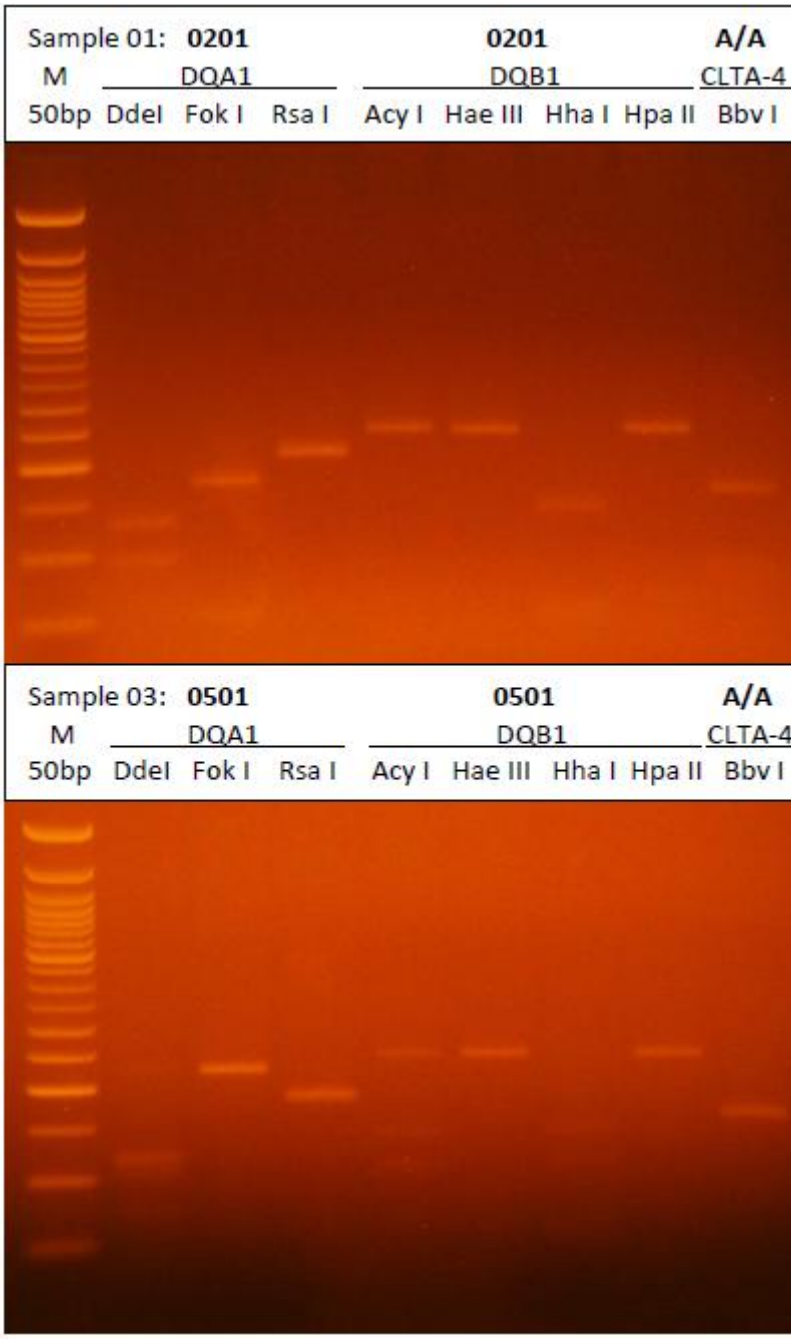

Figure 4.1. Agarose gel electrophoresis

Table 4.5. DQA1 genotype code (Maeda et al., 1989; Teutsch et al., 1996; Du et al., 2000; Ongagna et al., 2002)

DQA1 Genotype									
Enzyme	**Band Size**	**0101**	**0102**	**0103**	**0201**	**0301**	**0401**	**0501**	**0601**
Dde I	225		+			+			
	127				+				
	118						+	+	+
	113	+	+	+					
	107						+	+	+
	88				+				
	70	+	+	+				+	
Fok I	225	+	+	+		+		+	
	187				+		+		+
Rsa I	225			+	+				+
	186/183	+	+			+	+	+	

Table 4.6. DQB1 genotype code (Uryu et al., 1990; Nomura et al., 1991; Teutsch et al., 1996; Ongagna et al., 2002; Zhao et al., 2012)

DQB1 Genotype																
Enzyme	**Band Size**	**0501**	**0502**	**0503**	**0504**	**0601**	**0602**	**0603**	**0604**	**0605**	**0201**	**0301**	**0302**	**0303**	**0401**	**0402**
Acy I/ BsaH I	241		+	+		+		+		+	+	+				
	137	+			+		+		+				+	+	+	+
	104	+			+		+		+				+		+	
	84													+		+
	70															
Hae III	241	+	+								+		+	+		
	150			+		+		+		+					+	
	127				+		+		+			+				+
	95			+	+	+	+	+	+	+		+			+	+
Hha I	189											+	+	+		
	141	+		+		+			+	+	+				+	+
	112				+		+	+							+	
	90		+													
	65	+	+	+	+	+	+	+	+	+	+					+
	52/48	+	+	+	+	+	+	+	+	+	+	+	+	+		+
Hpa II	241	+									+				+	+
	198				+	+				+		+				
	150						+	+	+				+	+		
	126		+	+	+	+				+		+				
	100-115				+	+	+	+	+	+		+	+	+		
	40-50		+	+	??+	??+				??+		??+				

Table 4.7. CLTA-4 genotype code (Kinjo et al., 2002; Ongagna et al., 2002)

CLTA Genotype				
Enzyme	**Band Size**	**G/G**	**G/A**	**A/A**
Bbv I	162		+	+
	88	+	+	
	74	+	+	

Table 4.8. Correlation between DQA1, DQB1, CTLA-4 haplotypes and T1D

	Patient Group (n=200) (%)	**Control Group (n=200) (%)**	**R**	**Sig-**
DQA1				
DQA1*0101	27.0	30.5	. 039	.——
DQA1*0102	9.5	8.5	.017	.——
DQA1*0401	11.0	9.5	.033	.——
DQA1*0201	10.5	37.5	.316**	0.00
DQA1*0501	10.5	8.5	.043	.——
DQA1*0301	31.5	5.5	.343**	0.00
DQB1				
DQB1*0201	73.0	64.5	.092	.——
DQB1*0501	4.0	13.5	.168**	.001
DQB1*0303	6.0	10.5	.082	.——
DQB1*0302	17.0	11.5	.079	.——
CTLA4				
CTLA-4 A/A	62.5	59.0	.036	.——
CTLA-4 G/G	9.5	12.5	.048	.——
CTLA-4 A/G	28.0	28.5	.006	.——

R= significant with T1D. When the sig< =0.01*

5. Discussion

In this study, it was aimed to study the relationship between HLA class II polymorphisms and T1D in Jordanian population. For this purpose blood samples were taken from 200 control and 200 diabetic individuals. Each participant was informed about the study and asked some questions to obtain demographic status of the study groups. Questionnaire filled by each participant can be found at the end of the thesis. After DNA isolation, DNA was amplified by using primers designed for HLA DQA1, HLA DQB1 and CTLA-4 gene regions. Gene fragments were treated with different restriction enzymes to identify possible polymorphic areas and finally samples were run in agarose gel electrophoresis to visualize digestion products.

No significant relationship has been observed between gender and T1D. This is in accordance with the previous study (Ajlouni et al., 1999). In our study most of the patients fell into age between 6-10 years which was accounted for 43.5% (n = 87) and the second largest group constituted patients with age between 11-15 years (39.5%; n = 79). Finally, the percentage of patient group members ranged from 1-5 years was 17% (n = 34). There was a significant association between the age and T1D which was also in line with the literature (Ajlouni et al., 1999). No correlation has been found between age of onset and family history and T1D.

DQA1*0101 (frequencies were 54, 61 in patient and control groups, respectively) showed no significant correlation with T1D with higher frequency in control group than the patient group which does not show any statistical evidence to be accepted as a protective genotype among the Jordanian population. Same genotype has been shown as protective against T1D in Algerian (Djoulah et al., 1992) and Moroccan (Izaabel et al., 1996) populations. Also in parallel with the results obtained here other researchers found no significant correlation between protective effect of this haplotype and T1D in Sudanese population (Magzoub et al., 1991), Italian population (Lazio region) (Petrone et al., 2001) and Greek population (Khalil et al., 1993).

DQA1*0201 (frequencies were 21, 76 in patient and control groups, respectively) showed a significant positive correlation (R=.316, sig=.001) with T1D in terms of protection aganist the disease. Similarly this genotype also showed a significant correlation in Moroccan (Izaabel et al., 1996), French and Spanish

(Zayed, 2016) and Italian (Petrone et al., 2001) populations in terms of protection against T1D. However, in other countries like Sudan (Magzoub et al., 1991) and Greeks as Europeans (Khalil et al., 1993) no significant relationship has been found between protective function of DQA1*0201 and T1D.

DQA1*0501 (frequencies were 21, 16 in patient and control groups, respectively) showed no significant correlation in Jordanian population. The higher frequency in patient group does not mean an increase in the susceptibility for T1D. Other studies reported an increase in the frequency of this genotype in the patient groups from different countries (Djoulah et al., 1992; Khalil et al., 1993 Petrone et al., 2001; Zayed, 2016). However, same genotype showed a significant correlation with susceptibility to T1D in Egyptian (Zayed, 2016), Moroccan (Izaabel et al., 1996) populations.

DQA1*0102 (frequencies were 19, 17 in patient and control groups, respectively) showed no significant correlation with T1D in Jordanian population. Same genotype has been found to be correlated with the protection against T1D in Moroccan (Izaabel et al., 1996) and Algerian (Djoulah et al., 1992) populations.

DQA1*0401 (frequencies were 22, 19 in patient and control groups, respectively) showed no significant correlation with T1D in Jordanian population. On the other hand same haplotype was found to be associated with T1D in Moroccan population (Izaabel et al., 1996).

DQA1*0301 (frequencies were 63, 11 in patient and control groups, respectively) showed significant correlation with the increasing susceptibility to T1D (R=.343, sig=0.00) similar to other populations like Moroccan (Izaabel et al., 1996, Algeria (Djoulah et al., 1992) and Italy (Petrone et al., 2001).

DQB1*0201 (frequencies were 146, 129 in patient and control groups, respectively) showed no significant correlation with T1D development in the Jordanian population. While same genotype was detected to be correlated with the T1D in Moroccans (Izaabel et al., 1996), Algerians (Djoulah et al., 1992), Bahrainis (Al-Harbi et al., 2004), Greeks (Khalil et al., 1993) and Italians (Petrone et al., 2001).

DQB1*0501 (frequencies were 8, 21 in patient and control groups, respectively) was found to be significantly correlated with the protection against T1D (R= .168, sig= .001). Similar results have been obtained from Moroccan (Izaabel et

al., 1996) and Bahrain (Al-Harbi et al., 2004) populations. However, in Algerian (Djoulah et al., 1992) and Italian (Petrone et al., 2001) populations, no significant correlation has been reported in terms of the protective role of the same genotype

DQB1*0303 (frequencies were 12, 27 in patient and control groups, respectively) has not shown any significant correlation with T1D which is similar to Italian (Petrone et al., 2001), Moroccan (Izaabel et al., 1996), Algerian (Djoulah et al., 1992) and Greek (Khalil et al., 1993) populations.

DQB1*0302 (frequencies were 34, 23 in patient and control groups, respectively) showed no significant correlation with development T1D. In contrast, it was significantly correlated with T1D susceptibility among Algerians (Djoulah et al., 1992), Moroccans (Izaabel et al., 1996), Bahrainis (Al-Harbi et al., 2004), Italians (Petrone et al., 2001) and Greeks (Khalil et al., 1993).

CTLA-4 A/A (frequencies were 126, 118 in patient and control groups, respectively), A/G (frequencies were 55, 57 in patient and control samples respectively) and G/G (frequencies were 19, 25 in patient and control groups, respectively) showed no significant correlation with T1D, same results were also observed in Jordanian population (Al-Khaldi et al., 2011).

6. Conclusion

Among all HLA-DQA1, DQB1 and CTLA-4 genotypes studied only DQA1*0301 genotype showed correlation in terms of the susceptibility to T1D. On the other hand, two of them DQA1*0201 and DQB1*0501 have been found to be protective against T1D. CTLA-4 genotypes (A/A, A/G, and G/G) did not show any difference between study groups. These results can help others to focus on the significant genes which are associated with T1D to find prevention and treatment methods to these disease.

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Hello,

I am Master of Science student at Near East University/North Cyprus, Faculty of Medicine, Department of Medical Biochemistry. We are doing study about “HLA Class II polymorphisms as a Risk Factor in Jordanian Patients with Type 1 Diabetes Mellitus”

My study comes to examine the genetic factors that cause and develop the type 1 diabetes that will help the patient for early detection of the disease and avoid the factors that cause the increase in the incidence of the disease.

Please fill in the form to help us in the field of our research while keeping the confidentiality of the information and take all in consideration of your credibility in the answer

With all respect

The reasearcher

1-Do you have type one diabetes?

☐ Yes ☐ No

2- Can we use your sample to conduct our research?

☐ Yes ☐ No

3- Gender ?

☐ Male ☐ Female

4- How old are you?

☐ 1-5 ☐ 6-10 ☐ 11-15

5-At what age the disease appeared?

…… Years Old.

6- Do you have a history of type one diabetes in the family?

☐ Yes ☐ No

7- Do you have member with Type I Diabetes in the same family, if the answer is yes who is he/she ?

☐ Father ☐ Mother ☐ Brother ☐ Sister

8- originally are you from the Hashemite kingdom of Jordan?

☐ No ☐ Yes

9-Do you take a regular treatment for diabetes?

☐ Yes ☐ No

10- Do you regularly check for diabetes?

☐ Yes ☐ No ☐ Mostly

11- Is diabetes an economic burden?

☐ Yes ☐ No

12- How much does your treatment cost you monthly?

☐ Less than 500 $ ☐ 600- 1000 $ ☐ More than 1000 $

Thank you