UNIVERSITY OF TARTU
FACULTY OF MEDICINE

Ele Prans

Allelic variants of CTLA-4 gene as important markers of immune regulation in type 1 diabetes

Master´s thesis in biomedicine

Supervisors:
Konstantinos Douroudis, Ph.D.
Kalle Kisand, Ph.D.

Tartu
2009

CONTENTS

ABBREVIATIONS

APC	Antigen presenting cell
ARMS	Amplification refractory mutation system
CD	Cluster of differentiation
CI	Confidence interval
Csk	C-terminal Srs tyrosine kinase
CTLA-4	Cytotoxic T lymphocyte antigen-4
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
flCTLA-4	full-length Cytotoxic T lymphocyte antigen-4
GAD65	Glutamic acid decarboxylase 65
HLA	Human leukocyte antigen
H.W.E.	Hardy-Weinberg equilibrium
IA-2	Islet antigen-2
IAA	Insulin autoantibodies
ICA	Islet cell antibodies
ICOS	Inducible T cell co-stimulator
IL-2	Interleukin 2
IL-10	Interleukin 10
INS	Insulin gene
LADA	Latent autoimmune diabetes in adults
LD	Linkage disequilibrium
LYP	Lymphoid specific phosphatase
MHC	Major histocompatibility complex
mRNA	messenger Ribonucleic acid
NFAT	Nuclear factor of activated T cells
OR	Odds ratio
PCR	Polymerase chain reaction
PTPN22	Protein tyrosine phosphatase non-receptor 22
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
ZnT8	Zinc transporter 8

sCTLA-4	soluble Cytotoxic T lymphocyte antigen-4
SNP	Single nucleotide polymorphism
T1D	Type 1 diabetes
TE	Tris-ethylenediaminetetraacetic acid
TGF-β	Transforming growth factor-beeta
TNF-α	Tumor necrosis factor-alpha
UTR	Untranslated region
VNTR	Variable number of tandem repeat

1. INTRODUCTION

Type 1 diabetes (T1D) is a polygenic, multifactorial disorder caused by autoimmune destruction of insulin-producing β cells of the pancreas. The disease occurs worldwide and usually develops at younger age, although it may develop at any age. Multiple genetic factors are believed to be involved in the pathogenesis of T1D, but the factors are largely unknown, except for the role of human leukocyte antigen (HLA) genes on chromosome 6. To understand the mechanisms underlying the development of type 1 diabetes, a search for non-HLA linked genes is crucial. Cytotoxic T lymphocyte antigen-4 (CTLA-4) molecule is an important regulator of T cell activation involved in the down-regulation of immune response. Polymorphisms within the CTLA-4 gene have been suggested to confer susceptibility to autoimmune endocrine disorders, including type 1 diabetes. In order to evaluate the impact of allelic variants of the CTLA-4 gene in type 1 diabetes, MH30C/G, -1147C/T, -318C/T and CT60A/G single nucleotide polymorphisms (SNPs) were studied in a population of Estonian origin, including 154 T1D patients and 230 controls.

2. REVIEW OF THE LITERATURE

2.1. Type 1 diabetes mellitus

Diabetes mellitus represent a heterogeneous group of metabolic disorders with a multifactorial origin involving the interaction of environmental exposures and is characterized by glucose intolerance and hyperglycemia (Robles *et al*. 2001). The American Diabetes Association classification system defines four major forms of diabetes mellitus: type 1, type 2, gestational diabetes and diabetes due to other known causes (genetic defects of β-cell function, genetic defects in insulin action, endocrinopathies, drug- or chemical-induced diabetes). Type 1 diabetes, which accounts approximately 10% of all diabetes forms is often diagnosed at childhood. It is a serious chronic disorder characterized by absolute insulin deficiency caused by selective destruction of the insulin producing β cells in the islets of Langerhans. Type 1 is further subdivided according to whether β cell destruction is caused by an immune (type 1A) or other (type 1B, idiopathic) processes (American Diabetes Association 2006). Immune-mediated diabetes is further divided into a rapid and slowly progressive form. The rapid form appears mainly in infants and children and slowly progressive form in adults, also known as latent autoimmune diabetes in adults (LADA). The most important factor differenting type 1A from type 1B is the presence of islet autoantibodies. The type 1B diabetes etiology is unknown (Al-Mutairi et al. 2007). The term T1D will represent type 1A diabetes through this thesis.

2.1.1. Pathogenesis of human type 1 diabetes

T1D is an organ-specific autoimmune disorder and results from a cellular-mediated autoimmune destruction of the β cells of the pancreas. T1D is considered to be a disease of childhood, with a higher incidence among children 12-13 years of age. However, the disease may develop at any age and large proportion of T1D cases are diagnosed later in life. Before the discovery of insulin in 1922, the diagnosis was associated with 100% mortality in a few years as a result of a diabetic ketoacidosis (Notkins *et al*. 2001). In order to survive, the T1D patients totally rely on exogenous insulin, typically from the early beginning of the onset, since that stage about 60-80% of the insulin producing β-cells are irreversibly destroyed by

the autoimmune attack (Todd 1997). Autoimmune process usually exists for years before the clinical onset of the disease (Atkinson *et al*. 1994). As the access to the biopsies of human pancreas is limited, we do not know much about the events that take place in human, during early phases of T1D. Understanding of the natural progression of the disease is largely based on studies in murine models (the Bio/Breeding [BB] rat and the non-obese diabetic [NOD] mouse) (von Herrath *et al.* 2007).

The development of diabetes can be divided into a series of stages (Figure 1): stage 1, genetic predisposition; stage 2, environmental triggering of autoimmunity; stage 3, appearance of auto-antibodies; stage 4, progressive loss of insulin secretion; stage 5, overt diabetes; stage 6, insulin dependence with loss of almost all islet β-cells (Eisenbarth 2003).

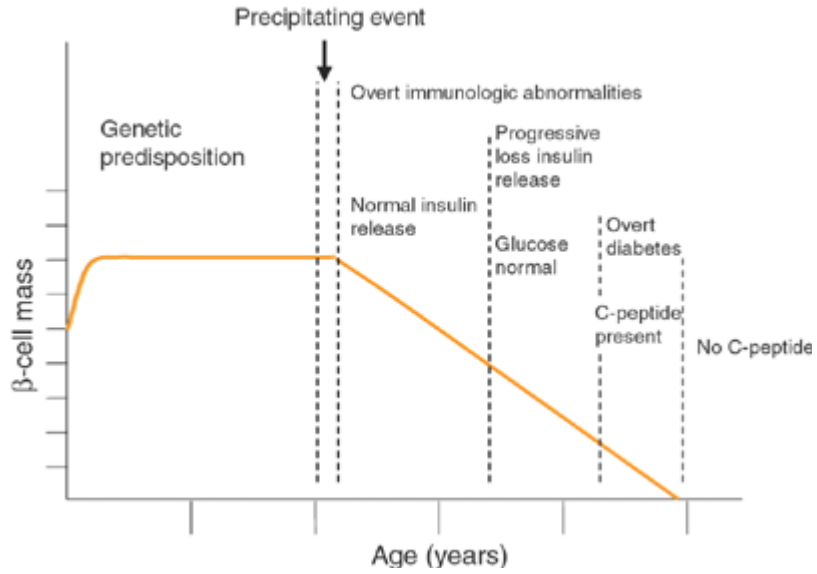

Figure 1. Hypothetical stages in the development of type 1 diabetes (Eisenbarth 1986).

The immune system distinguishes between foreign and self-antigens through the processes of central and peripheral tolerance. Any breakdown in these pathways could initiate an immune response leading to an autoimmune phenotype, including T1D. Central tolerance mechanisms occur in the thymus through positive and negative selection of developing T lymphocytes (Walker and Abbas 2001). Positive selection occurs when immature $CD4^+CD8^+$ T lymphocytes carry unique T-cell receptors interact with HLA class I and II molecules. Upon interaction, the thymocytes receive a protective signal that prevents apoptosis. Thymocytes that have not interacted with MHC molecules undergo cell death. The surviving thymocytes undergo a negative selection, whereby high-affinity interactions between the thymocyte and

the MHC molecules on APC-s result in thymocyte deletion by apoptosis. Autoreactive T cells are present in normal healthy subjects, suggesting that thymic deletion does not completely prevent the escape of such populations, and thus other mechanisms must be controlling these cells. The small fraction of self-reactive T cells that escape negative selection is controlled in the periphery by peripheral tolerance mechanisms such as ignorance, anergy, apoptosis, regulatory T cells (T_{regs}) (Walker and Abbas 2001).

The function of these self-reactive T cells is modulated by a $CD25^{+}highFoxp3^{+}$ subgroup of T cells, referred as T_{regs}. How T_{regs} exert their regulatory function is not completely understood, but such as cell-to-cell contact, secretion of TGF-β (transforming growth factor β) and IL-10 (interleukin 10) or expression of CTLA-4 have been proposed. Thus, polymorphisms affecting these processes, and particular polymorphisms within the CTLA-4 molecule, could have significant effects on susceptibility to autoimmunity, subsequently including T1D (Brand *et al.* 2005; Gough *et al.* 2005).

Markers of the immune destruction of the β cell include islet cell antibodies (ICA), autoantibodies to insulin (IAA), autoantibodies to glutamic acid decarboxylase (GAD_{65}) and autoantibodies to the tyrosine phosphatases (IA-2 and IA-2β) (Knip *et al.* 2005). Recently, ZnT8, a pancreatic β cell specific zinc transporter, has been identified as a candidate autoantigen (Wenzlau *et al.* 2007; Eisenbarth *et al.* 2008; Murgia *et al.* 2009). The autoantibodies can be detected years before clinical diagnosis of disease. Autoantibodies against insulin are often first autoantibodies to appear in the prediabetic state and are usually found during early infancy (Achenbach *et al.* 2004).

Multiple environmental factors have also been proposed in the development of T1D, like toxins (eg N-nitroso derivatives), certain viruses (enteroviruses, cytomegalovirus, rubella virus and rotavirus) and dietary compounds (early exposure to cow´s milk proteins, cereals or gluten) (Atkinson *et al.* 2001; Hirschhorn 2003; Morran *et al.* 2008). The traditional view postulates that an environmental agent triggers the onset of the disease in genetically susceptible individual. It is not known how much of the variation in incidence between countries is due to environmental factors and how much is due to genetic differences between populations. It could also be possible that there is no single environmental T1D trigger, but instead, several factors acting together. The search for environmental factors contributing to the development of diabetes is open and needs further investigation.

2.1.2. Incidence of type 1 diabetes

The incidence of T1D varies among countries. It has been demonstrated that Finland has the highest incidence of T1D in the world followed by Sweden (Karvonen *et al.* 2000). The disease is less common among Asians, Native Americans and Blacks. The incidence ranges from 0.1/100 000 per year in children ≤ 14 years of age in China to 37.4/100 000 per year in children ≤ 14 years of age in Finland and 34.6/100 000 per year in children ≤ 14 years of age in Sweden (Mehra *et al.* 2007). The EURODIAB collaborative study, a registry involving 44 countries in Europe, indicates an annual rate of incidence increase of T1D 3-4% annually (EURODIAB ACE Study Group 2000; Devendra *et al.* 2004) and the largest rate of increase is seen in children under 5 years of age (EURODIAB ACE Study Group 2000).

In 1983-1990, the crude incidence data of T1D in Estonia was reported to be 10.1/100 000 per year and during the years 1991-1998 it was reported to be 12.3/100 000 per year. In both periods, the incidence was highest among children aged 10.0-14.9, but the incidence increased rapidly (10.4% annually) in age group 0-4.9 years. In 1999-2006, the crude incidence rate of T1D was reported to be 16.9/100 000 per year. The average annual increase in incidence over the 24-year period (1983-2006) in Estonia was 3.3%. A shift toward younger patients among children first presenting with T1D is seen: in 1983-1998 the annual incidence was the highest among children aged 10.0-14.9 years, whereas in the last study periood (1999-2006), the 5.0-9.9 year-old children represented a group with the highest incidence (Figure 2). The incidence of childhood-onset T1D in Estonia continues to rise (Podar *et al.* 2001; Teeäär *et al.* 2009).

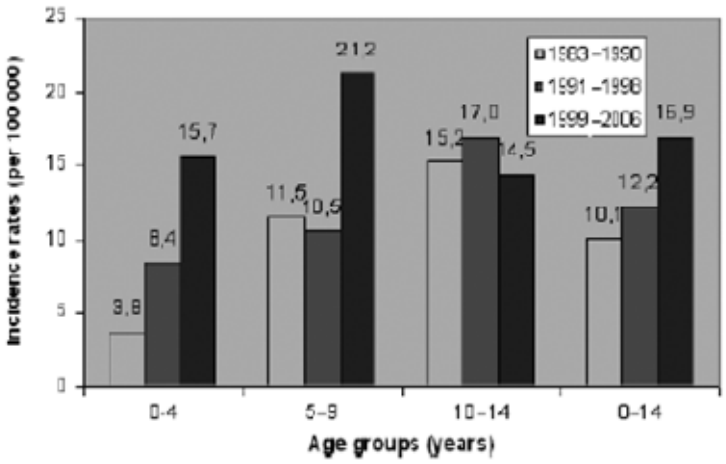

Figure 2. The average crude incidence rates of T1D in Estonian children under 15 years of age in 1983–2006 (Teeäär *et al.* 2009).

2.1.3. Genetic susceptibility to type 1 diabetes

T1D is a polygenic disease, associated with several genes on different chromosomes. The disease clusters within families and based on population-based twin studies, we could notice that the concordance rate for monozygotic twins is approximately 50% and in contrast in dizygotic twins the concordance rate is 5-10% (Concannon *et al.* 2005; Kim *et al.* 2005). The risk to get a disease is approximately 7% for a sibling and 6% for a child of a diabetic (Bach 1994). Differences in risk also depend on which parent has diabetes. The offspring of father with T1D is nearly three times more likely to have T1D than is the offspring of affected mother (Atkinson *et al.* 1994). The background of this difference between mothers and fathers is currently unknown.

Genetic factors in the HLA region are considered to be the most important determinants to T1D susceptibility. The HLA is located on the short arm of chromosome 6 (6p21.3) and occupies a large segment of DNA, extending about 3600 Mbp. It is a region of highly polymorphic genes that form separate gene clusters, class I (telomeric), class II (centromeric) and class III. Class I encode the α-chain of HLA class I molecules, and class II genes encode the loci of both α- and β-chains of heterodimeric HLA class II molecules (HLA-DQ, -DR, and –DP). Class III genes encode a variety of molecules including the components of complement, and tumor necrosis factor-alpha (TNF-α) (Eisenbarth 2003; Simmonds *et al.* 2005; Kantárová *et al.* 2007). Studies have confined major T1D predisposition to the HLA class II genes, DRB1 and DQB1. The risk to develop T1D in Caucasian populations is greater if they are carrying HLA DRB1*0301-DQB1*0201 (DR3) and DRB1*0401-DQB1*0302 (DR4) haplotypes. Among Asian population high risk haplotypes include DRB1*0901-DQB1*0303 (DR9) and DRB1*0405-DQB1*-0401 (DR4) (Ilonen *et al*. 2002; Pociot *et al.* 2002; Aribi 2008). The HLA-region explains approximately 50% of the genetic background of T1D suggesting that additional genetic determinants exist (Davies *et al.* 1994).

Insulin gene (INS, 11p15.5) has also been reported as the second most important genetic susceptibility factor, contributing about 10% of genetic susceptibility to T1D (Al-Mutairi *et al.* 2007). Polymorphism which corresponds to the insulin locus has been identified as allelic variation of a variable number of tandem repeat (VNTR) minisatellite located upstream of the

insulin gene in the promoter region. There are three main VNTR classes defined by VNTR size: class I (26-63 repeats), class II (approximately 80 repeats), and class III (140-200 repeats). Homozygosity for class I VNTR determines high risk for diabetes, while class III VNTRs confers dominant protection (Redondo *et al.* 2001). In Estonian population T1D was also found to be positively associated with INS VNTR class I/I genotype (Haller *et al.* 2007).

The protein tyrosine phosphatase non-receptor 22 (PTPN22, 1p13) and it encodes the lymphoid tyrosine phosphatase (LYP), which is an inhibitor of T cell activation. In 2004, Bottini *et al.* were the first to implicate PTPN22 gene is susceptibility for autoimmune diseases. They showed that an allelic variant 1858T of the PTPN22 gene was significantly more common among patients of T1D compared to controls (Bottini *et al.* 2004). Association of the 1858C-T polymorphism with T1D has been replicated in several studies (Santiago *et al.* 2007; Bottini *et al.* 2006). An association in Estonian population with T1D and PTPN22 1858T allele has also been reported (Douroudis *et al.* 2008).

CTLA-4 molecule is an strong candidate for T cell-mediated autoimmune disease because it mediated T cell apoptosis and is a negative regulator of T cell activation (Nistico *et al.* 1996). Several polymorphisms in the CTLA-4 gene are known to be associated with autoimmune diseases (Kouki *et al.* 2002), including T1D (Kristiansen *et al.* 2000; Ueda *et al*. 2003). Our previous study has reported that a polymorphism at position 49 in exon 1 of the CTLA-4 gene was associated with T1D in Estonian population (Haller *et al.* 2004).

2.2. CTLA-4

Human CTLA-4 (CD152) gene is located on chromosome region 2q33 which contains the T lymphocyte regulatory genes CD28 and inducible co-stimulator (ICOS). It is one of the confirmed T1D susceptibility loci (Ueda *et al.* 2003; Butty *et al.* 2007). CTLA-4 belongs to the immunoglobulin superfamily that is expressed on T cells and at the protein level shares approximately 30% identity with CD28. Both molecules bind to CD80 (B7-1) and CD86 (B7-2) ligands on antigen presenting cells (APCs) (Magistrelli *et al.* 1999) but they carry out different functions. Binding of the costimulatory molecule B7 to CD28 activates T cells by inducing the expression of several cytokines and cytokine receptors, whereas B7 binding to CTLA-4 leads to T cell apoptosis. The affinity of B7 molecules is 20-50 folds higher to CTLA-4 than to CD28, which allows the termination of the immune response by T cell apoptosis in spite of the stimulating CD28 molecules. Therefore, CTLA-4 expression may play a key role in autoimmunity (Carreno *et al.* 2002).

2.2.1. Structure of the gene and the protein

Human CTLA-4 gene exists as a single copy per haploid genome and consists of four exons and three introns encoding different functional domains of the protein (Figure 3). Exons 1 and 2 encode the extracellular portion of the molecule. Exon 1 contains the leader peptide sequence and exon 2 the ligand-binding site. Exon 3 encodes the transmembrane region and exon 4 codes the cytoplasmic tail (Teft *et al.* 2006). The 5' region of the gene includes the sequence encoding the leader sequence, a conserved Kozak consensus sequence with the ATG (Met) initiation of translation signal, an inframe stop codon 26 base pair (bp) upstream this ATG, and a TATA box 75bp upstream the stop codon. Following the coding part of exon 4 is a 3' untranslated region (UTR) of about 1150bp which comprises a stretch of almost 30 AT-repeats (Kristiansen *et al.* 2000).

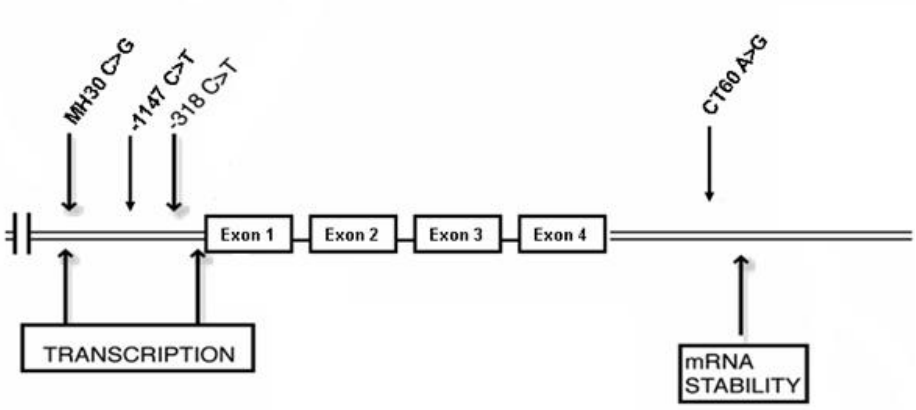

Figure 3. Schematic representation of CTLA-4 gene (modified from Anjos *et al.* 2004; Teft *et al.* 2006).

CTLA-4 is encoded as a 223 amino acid precursor protein. The majority of the protein is localized inside the cell, particularly in the peri-nuclear Golgi. The mature protein contains a 124 amino acid long extracellular region with homology to an Ig-like domain, a 26 amino acid transmembrane region and a 36 amino acid cytoplasmic tale (Kristiansen *et al.* 2000). Within the extracellular domain, the B7 binding motif is centered on the amino acids MYPPPY. The cytoplasmic domain of CTLA-4 encodes the motif YVKM in which the phosphorylation state of tyrosine has been implicated in both signal transduction through SYP/SHP2 phosphatase and the intracellular accumulation of CTLA-4 via AP50 clatharin-mediated endocytosis (Ling *et al.* 1999).

There are two splicing variants of CTLA-4 in humans: the full-length protein (flCTLA-4) encoded by exons 1-4 and a soluble (sCTLA-4), which lacks exon three (transmembrane domain) due to alternative splicing. The flCTLA-4 is a transmembrane glycoprotein. It down-regulates T cell responses by inducing cell cycle arrest and blocking cytokine production. The flCTLA-4 receptor binds the ligand B7 expressed on the surface of APCs. The sCTLA-4 is expressed mainly in inactivated T cells and is also able to bind CD80 and CD86 (Anjos *et al.* 2004; Perez-Garcia *et al.* 2008). Low levels of sCTLA-4 have been detected in normal human serum and increased serum levels have been observed in several autoimmune diseases (e.g. Graves' disease, myasthenia gravis) (Oaks *et al.* 2000; Pawlak *et al.* 2005). A reduction in sCTLA-4 level could lead to reduced blocking of CD80/CD86 molecules, causing T cell activation through CD28 (Ueda *et al.* 2003). On the contrary, higher levels of sCTLA-4 could

compete with membrane bound CTLA-4 for CD80/CD86-binding sites and cause a reduction of inhibitory signaling (Vaidya *et al.* 2004). Mouse T cells can express an additional CTLA-4 transcript that is known as ligand-independent CTLA-4 (liCTLA-4), containing exons 1, 3 and 4 (Teft *et al.* 2006).

2.2.2. Expression

The CTLA-4 gene is mainly expressed in T cells upon activation, although it is constitutively expressed in the CD4+CD25+ T_{reg} subset (Teft *et al.* 2006). T_{reg} cells express the transcription factor Foxp3, which controls the expression of CTLA-4 in T_{regs} (Wing *et al.* 2008). CTLA-4 expression is low in unstimulated cells and increases transiently upon stimulation. Only a minor proportion of total CTLA-4 is expressed at the cell surface under steady state conditions (Gough *et al.* 2005). Expression of the CTLA-4 protein has been shown to peak between 24 and 48 hours post-activation (Jago *et al.* 2004) and returns to background levels by 96 hours (McCoy *et al.* 1999).

The transcriptional regulation of CTLA-4 gene expression is only partially known. It may depend on NFAT (nuclear factor of activated T cells) because modulation of NFAT levels correlates directly with CTLA-4 expression. In addition, inhibition of NFAT activation by cyclosporine A causes a marked decrease in CTLA-4 gene transcription. It has been also shown that NFAT binds to CTLA-4 promoter region (Valk *et al.* 2008).

The regulation of CTLA-4 expression is as pivotal as its concentration on the cell membrane determines the strength of down-regulatory signals for T cells. Thus, maintenance of an optimal surface expression level of CTLA-4 is crucial for the regulation of T cell responses and peripheral tolerance, and for preventing autoimmunity (Karabon *et al.* 2009).

2.2.3. Function

CTLA-4 has a central role as a negative costimulator in T cell activation. T cell activation requires two signals. First signal is provided when the antigen-specific cell surface T cell receptor (TCR; CD3 complex) engages the antigen, which is bound to an MHC class II molecule on the surface of an antigen presenting cell. The second (co-stimulatory) is provided by the interaction of the co-stimulatory molecule, CD28 with its ligands CD80/CD86 on APCs (Figure 4). (Vaidya *et al.* 2004). Following T cell activation T cells begin to induce the expression of CTLA-4, which is very similar to CD28 in sequence. Since CTLA-4 has a higher affinity for B7 molecules it competes with CD28 and eventually out-competes CD28 to bind to C80 and CD86 molecules (Janeway *et al.* 2005).

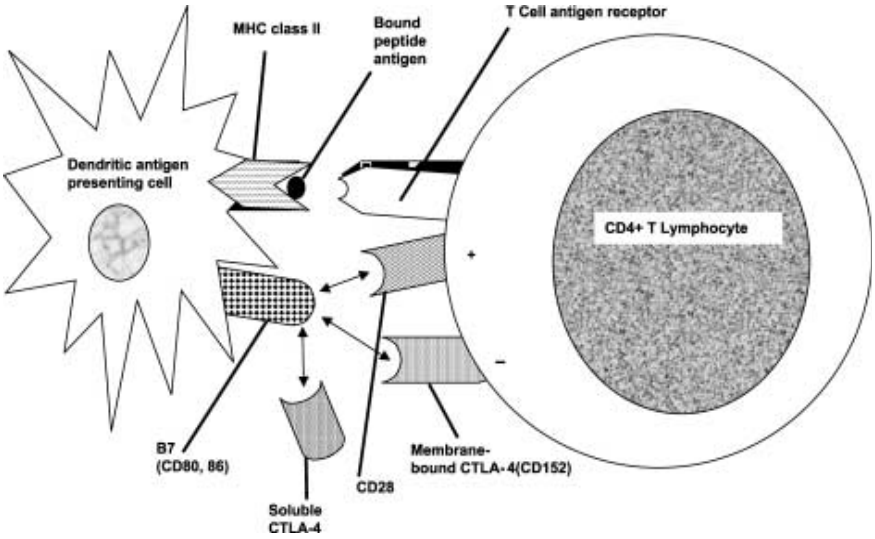

Figure 4. T cell activation (Vaidya *et al.* 2004).

Subsequent to T cell activation, the lymphocyte secretes the cytokine interleukin-2 (IL-2), which drives proliferation and differentiation of the cell. Activated T cells express a high affinity for IL-2 receptors, which binds to the cytokine IL-2. However, when CTLA-4 is induced, it redudes IL-2 production and IL-2 receptor expression. Moreover, mice genetically deficient in CTLA-4 expression develop a lymphoproliferative disease with progressive accumulation of T lymphocytes in peripheral lymphoid organs, as well as in solid organs, such as heart, liver, leading to death by 3-5 weeks of age (Carreno *et al.* 2002; Gough *et al.* 2005).

Since T_{reg} constitutively express CTLA-4, it has been postulated that CTLA-4 is essential for T_{reg} function. In this regard, it has been shown that anti-CTLA-4 treatment blocks T_{reg} function *in vitro* and that T_{reg} from CTLA-4 deficient mice display impaired suppressive activity (Tang *et al.* 2004).

2.2.4. CTLA-4 gene polymorphisms

The CTLA-4 region on chromosome 2q33 has been linked with susceptibility to several autoimmune diseases (Grave's disease, celiac disease) (Kacem *et al.* 2001; Hunt *et al.* 2005). Several CTLA-4 gene variants have been identified. These include polymorphisms in the 5' flanking and promoter region, coding SNP, an A49G variant leading to a threonine to alanine replacement in the signal peptide, polymorphisms in the 3'UTR. Many of these variations have been associated with autoimmune diseases, including T1D, and may be common susceptibility factors in autoimmunity in general (Pociot *et al.* 2002). The most comprehensive SNP and linkage disequilibrium (LD) mapping analysis of this locus (Ueda *et al.* 2003) identified a CT60A/G (renamed as A6230G) SNP in the 3'UTR as the predominant marker for T1D risk. The CT60A/G SNP was reported to correlate with higher mRNA level of sCTLA-4 in unstimulated T cells from individuals heterozygous for the T1D protective haplotype (A49, A6230) compared to the predisposing haplotype (G49, G6230) (Ueda *et al.* 2003). The MH30 SNP represented also one of the highest associated markers with autoimmune diseases across the 2q33 region. Because of its location, the MH30 SNP could affect the CTLA-4 promoter activity and, subsequently, it expression (Ueda *et al.* 2003). The polymorphisms in the promoter region can affect the expression of the CTLA-4 molecule (Wang *et al.* 2002; Howard *et al.* 2002).

3. AIM OF THE STUDY

The aim of the study was to evaluate the impact of CTLA-4 -1147C/T, -318C/T, MH30C/G and CT60A/G polymorphisms in type 1 diabetes patients of Estonian origin.

4. MATERIALS AND METHODS

4.1. Patients and control subjects

Ethnically homogenous population of Estonian origin was enrolled into a case-control study. 154 T1D patients aged between 1.9-78.9 (mean age at diagnosis 21.97±14.26 years, 77 females). 68 T1D patients were selected from local patient registries at the Kuressaare Hospital, Saaremaa County and offices of family doctors. 86 T1D patients were selected from childrens hospital of the Tartu University Hospital (from the southern parts of the country) and from Tallinn Children's Hospital (from the northern parts of the country).

230 individuals were asked to participate in the case-control study as neighborhood controls. To match the age of diabetic patients, the control group was composed of two populations. The younger control population consisted of blood donors (n=139, mean age of 43.3±11.2 years, 85 women) from the blood-donation centre at the Kuressaare Hospital. The older control population consisted of patients hospitalized for various reasons at Kuressaare Hospital (n=91, mean age of 49.8±17.9 years, 54 women). Of note, the hospitalized control patients have not been hospitalized for reasons leading to autoimmune or other metabolic syndromes.

Blood samples were collected January 2001 till June 2004. The study was approved by the Ethics Committee of the University of Tartu, and informed consent was obtained from the adult participants and from the parents of the children involved.

4.2. Genotyping

DNA was extracted from peripheral venous blood samples in 4ml EDTA containing vacuttes by using the salting-out procedure (Miller *et al.* 1988). The isolated DNA was dissolved in Tris-EDTA (TE) buffer. The purity and concentrations of DNA were measured by spectrophotometer (NanoDrop, ND-1000). The DNA samples are stored in -80°C until usage.

4.2.1. CTLA-4 CT60A/G genotyping

The genotyping of the CTLA-4 CT60A/G (rs3087243) SNP was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. The PCR reaction was carried out using a forward primer 5'-CAC CAC TAT TTG GGA TAT ACC-3' and a reverse primer 5'-AGC TCT ATA TTT CAG GAA GGC-3' (Orozco *et al.* 2004) in a volume of 25µl and the components of the reaction are described in Table 1.

Table 1. PCR protocol for determining CTLA-4 CT60A/G SNP.

	x1 (25 µl volume reaction)	Final concentration
DNA	2µl	
10x Buffer (Naxo, Estonia)	2.5 µl	x1
$MgCl_2$ (25mM) (Naxo, Estonia)	2.0 µl	2mM
dNTPs (2.5mM) (Fermentas, Lithuania)	2.0 µl	200 µM
Sense-primer (10µM) (Metabion, Germany)	1.25 µl	0.5 µM
Antisense-primer (10µM) (Metabion, Germany)	1.25 µl	0.5 µM
5U/µl Taq polymerase (Naxo, Estonia)	0.1 µl	0.5U
dH_2O	13.9 µl	

PCR amplification was carried out in a DNA thermal cycler (PTC-100^{TM} Programmable Thermal Controller, MJ Research, Watertown, MA). Following programme was used:

Denaturation	94° C	6'	
Annealing	57° C	2'	1 cycle
Extension	72° C	2'	
Denaturation	94° C	45"	
Annealing	57° C	50"	35 cycles
Extension	72° C	1'	
Extension	72° C	5'	

A 5µl aliquot of the PCR product (216bp) was digested for 3h at 37°C with 5Units of NcoI (Fermentas, Lithuania) enzyme in a reaction volume of 8µl. The components of the reaction are described in Table 2.

Table 2. Restriction protocol for CTLA-4 CT60A/G SNP.

	x1 (10 µl volume reaction)	Final concentration
PCR product	5µl	
10xTango buffer (Fermentas, Lithuania)	0.8µl	x1
NcoI (10U/ µl) (Fermentas, Lithuania)	0.5µl	5U/µl
dH_2O	1.7µl	

Following restriction endonuclease digestion, the products were electrophoresed in 2% w/v agarose gel containing ethidium bromide (0.5µg/ml) and visualized using Fluor-S^{TM} MultiImager (BIO-RAD, USA). In case of the A-allele the restriction analysis produced a fragment of 196bp and in case of the G-allele fragment of 216bp whereas the heterozygosity for the restriction site AG (216bp, 196bp) exhibits both two bands (Figure 5).

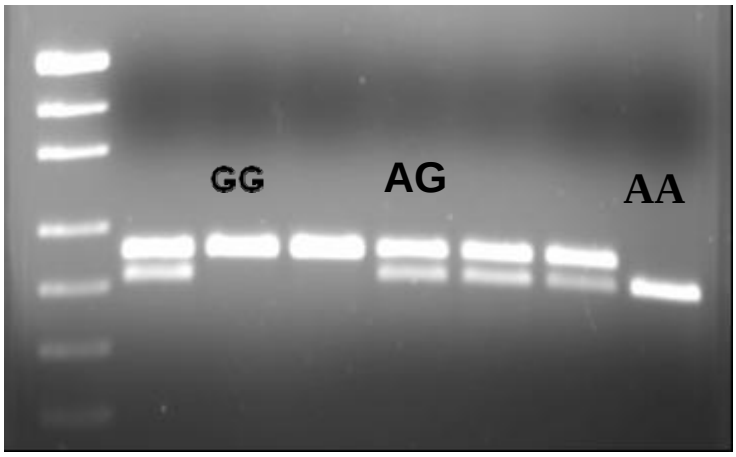

Figure 5. PCR results of CTLA-4 CT60A/G SNP.

4.2.2. CTLA-4 -318C/T genotyping

The CTLA-4 -318C/T (rs5742909) polymorphism was studied using the PCR amplification refractory mutation system (PCR-ARMS) without requiring endonuclease digestion. For each sample two independent reactions were carried out. Both tubes contained common reverse primer 5' AGG-CTC-TTG-AAT-AGA-AAG-C 3' but different forward primers. One with forward 5' CT-TAG-TTA-TCC-AGA-TCC-AC 3' and the other with forward 5' ACT-TAG-TTA-TCC-AGA-TCC-AT 3' (Erfani *et al.* 2006). The different primers are synthesized so that their 3'-ends match either the wild-type or mutant alleles at the point where the sequences

differ. An allele will amplify only if both primers match the template, so whichever reaction generates a product indicates which allele was present in the original DNA. The PCR reaction was carried out in a volume of 25µl and the components of the PCR reaction are described in Table 3.

Table 3. PCR protocol for determining CTLA-4 -318C/T SNP.

	x1 (25 µl volume reaction)	Final concentration
DNA	2µl	
10x Buffer	2.5µl	x1
$MgCl_2$ (25mM) (Naxo, Estonia)	2.5µl	2.0mM
dNTPs (2.5mM) (Fermentas, Lithuania)	2.4µl	200µM
Sense-primer (10µM) (Metabion, Germany)	1.25µl	0.5µM
Antisense-primer (10µM) (Metabion, Germany)	1.25µl	0.5µM
5U/µl Taq polymerase (Naxo, Estonia)	0.4µl	1U
H_2O	12.2µl	

PCR amplification was carried out in a DNA thermal cycler (PTC-100^{TM} Programmable Thermal Controller, MJ Research, Watertown, MA). Following programme was used:

Denaturation	94° C	7'	1 cycle
Denaturation	94° C	20"	
Annealing	53.5° C	20"	30 cycles
Extension	72° C	20"	
Extension	72° C	5'	1 cycle

PCR products were electrophoresed in 2% w/v agarose gel containing ethidium bromide (0.5µg/ml) and visualized using Fluor-S^{TM} MultiImager (BIO-RAD, USA). The homozygous (CC or TT) subjects demonstrating one band and the heterozygous (CT) subjects demonstrating two bands (Figure 6).

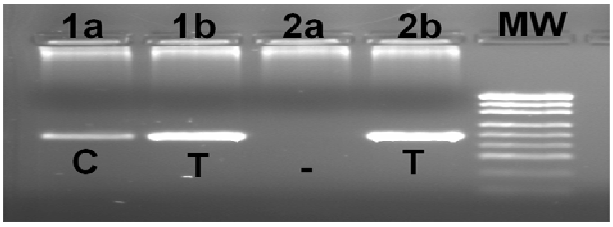

Figure 6. PCR-ARMS results of CTLA-4 -318C/T promoter region.

Lanes 1a, 1b: CT heterozygous. Lanes 2a, 2b: TT homozygous. The a and b represent reactions containing C and T allele specific forward primers, respectively.
Abbreviation: MW-molecular weight marker

To confirm the results obtained by the PCR-ARMS method, 15% of randomly selected samples were also genotyped using the PCR-RFLP analysis. The PCR reaction was carried out using a forward primer 5'-AAA TGA ATT GGA CTG GAT GGT-3' and a reverse primer 5'-TTA CGA GAA AGG AAG CCG TG-3' (Heward *et al.* 1998) in a volume of 25µl and the components of the reaction are described in Table 4.

Table 4. PCR protocol for determining CTLA-4 -318C/T SNP.

	x1 (25 µl volume reaction)	Final concentration
DNA	2µl	
10x Buffer (Naxo, Estonia)	2.5 µl	x1
$MgCl_2$ (25mM) (Naxo, Estonia)	2.0 µl	2mM
dNTPs (2.5mM) (Fermentas, Lithuania)	2.0 µl	200 µM
Sense-primer (10µM) (Metabion, Germany)	1.25 µl	0.5 µM
Antisense-primer (10µM) (Metabion, Germany)	1.25 µl	0.5 µM
5U/µl Taq polymerase (Naxo, Estonia)	0.2 µl	1U
dH_2O	13.8 µl	

PCR amplification was carried out in a DNA thermal cycler (PTC-100^{TM} Programmable Thermal Controller, MJ Research, Watertown, MA). Following programme was used:

Denaturation	94° C	2 min	1 cycle
Denaturation	94° C	30 sec	35 cycles
Annealing	53.5° C	30 sec	
Extension	72° C	30 sec	
Extension	72° C	7 min	1 cycle

A 5µl aliquot of the PCR product (481bp) was digested for 3h at 65°C with 5Units of Tru1/MseI (Fermentas, Lithuania) enzyme in a reaction volume of 10 µl. The components of the reaction are described in Table 5.

Table 5. Restriction protocol for CTLA-4 -318C/T SNP.

	x1 (10 µl volume reaction)	Final concentration
PCR product	5µl	
10xTango buffer (Fermentas, Lithuania)	1.0µl	x1
BseGI/FokI (Fermentas, Lithuania)	0.5µl	5U/µl
dH_2O	3.5µl	

Following restriction endonuclease digestion, the products were electrophoresed in 2% w/v agarose gel containing ethidium bromide (0.5µg/ml) and visualized using Fluor-S^{TM} MultiImager (BIO-RAD, USA). In case of the C-allele the restriction analysis produced fragments of 226bp and 21bp and in case of the T-allele fragments of 130bp, 96bp and 21bp. Whereas the heterozygosity for the restriction site CT (226bp, 130bp, 96bp and 21bp) exhibited all four bands.

4.2.3. CTLA-4 -1147C/T genotyping

The genotyping of the CTLA-4 -1147C/T (rs16840252) SNP was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. The PCR reaction was carried out using a forward primer 5'-GCT GAG GTG TGG ACA ATG G-3' and a reverse primer 5'-TCA GGT GTT CTT AAA AGC CTT AAC-3' (Esteghamati *et al.* 2009) in a volume of 25µl and the components of the reaction are described in Table 6.

Table 6. PCR protocol for determining CTLA-4 -1147C/T SNP.

	x1 (25 µl volume reaction)	Final concentration
DNA	2µl	
10x Buffer (Naxo, Estonia)	2.5 µl	x1
$MgCl_2$ (25mM) (Naxo, Estonia)	2.0 µl	2mM
dNTPs (2.5mM) (Fermentas, Lithuania)	2.0 µl	200 µM
Sense-primer (10µM) (Metabion, Germany)	1.25 µl	0.5 µM
Antisense-primer (10µM) (Metabion, Germany)	1.25 µl	0.5 µM
5U/µl Taq polymerase (Naxo, Estonia)	0.2 µl	1U
dH_2O	13.8 µl	

PCR amplification was carried out in a DNA thermal cycler (PTC-100™ Programmable Thermal Controller, MJ Research, Watertown, MA). Following programme was used:

Denaturation	94° C	4 min	1 cycle
Denaturation	94° C	20 sec	30 cycles
Annealing	53.5° C	30 sec	
Extension	72° C	1 min	
Extension	72° C	10 min	1 cycle

A 5µl aliquot of the PCR product (481bp) was digested for 3h at 55°C with 5Units of BseGI/FokI (Fermentas, Lithuania) enzyme in a reaction volume of 10 µl. The components of the reaction are described in Table 7.

Table 7. Restriction protocol for CTLA-4 -1147C/T SNP.

	x1 (10 µl volume reaction)	Final concentration
PCR product	5µl	
10xTango buffer (Fermentas, Lithuania)	1.0µl	x1
BseGI/FokI (10U/µl) (Fermentas, Lithuania)	0.5µl	5U/µl
dH_2O	3.5µl	

Following restriction endonuclease digestion, the products were electrophoresed in 2% w/v agarose gel containing ethidium bromide (0.5µg/ml) and visualized using Fluor-S™ MultiImager (BIO-RAD, USA). In case of the C-allele the restriction analysis produced

fragment of 481bp and in case of the T-allele fragment of 446bp whereas the heterozygosity for the restriction site CT (481bp, 446bp) exhibits both two bands (Figure 7).

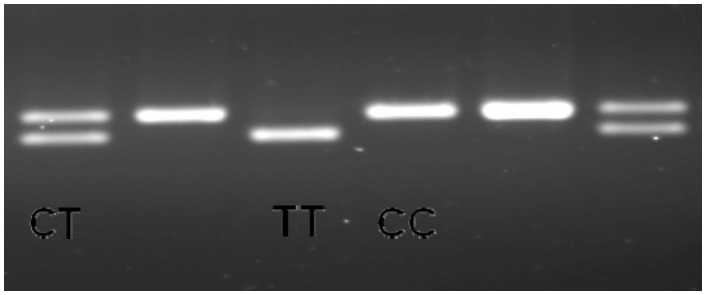

Figure 7. PCR results of CTLA-4 -1147C/T promoter region.

4.2.4. CTLA-4 MH30C/G genotyping

The CTLA-4 MH30 (rs231806) was genotyped using the Taqman SNP 5' allelic discrimination assay (hCV3296021, Applied Biosystems, Foster City, CA, USA). Allelic discrimination assay is a multiplex, end point assay that detects variants of a single nucleic acid sequence. For each sample a unique pair of fluorescent dye detectors (VIC and FAM) was used. The VIC fluorescent dye detector was perfect match for the MH30 C allele and the FAM fluorescent detector was perfect match to the MH30 G allele (Figure 8). PCR reactions were run on an ABI 7500 instrument (Applied Biosystems, Foster City, CA, USA) using the following cycling parameters: after one step at 95˚C 10 min, 45 cycles of denaturation at 92˚C for 15 s and extension at 60˚C for 1 min were performed. The components of the PCR reaction are described in Table 8.

Table 8. PCR protocol for determining CTLA-4 MH30C/G SNP.

	x1 (5 µl volume reaction)	Final concentration
DNA	2 µl	20 ng/µl
2x Buffer (Amplification Master Mix)	2.5 µl	x1
Probe (x40)	0.125 µl	x1
dH_2O	0.375 µl	

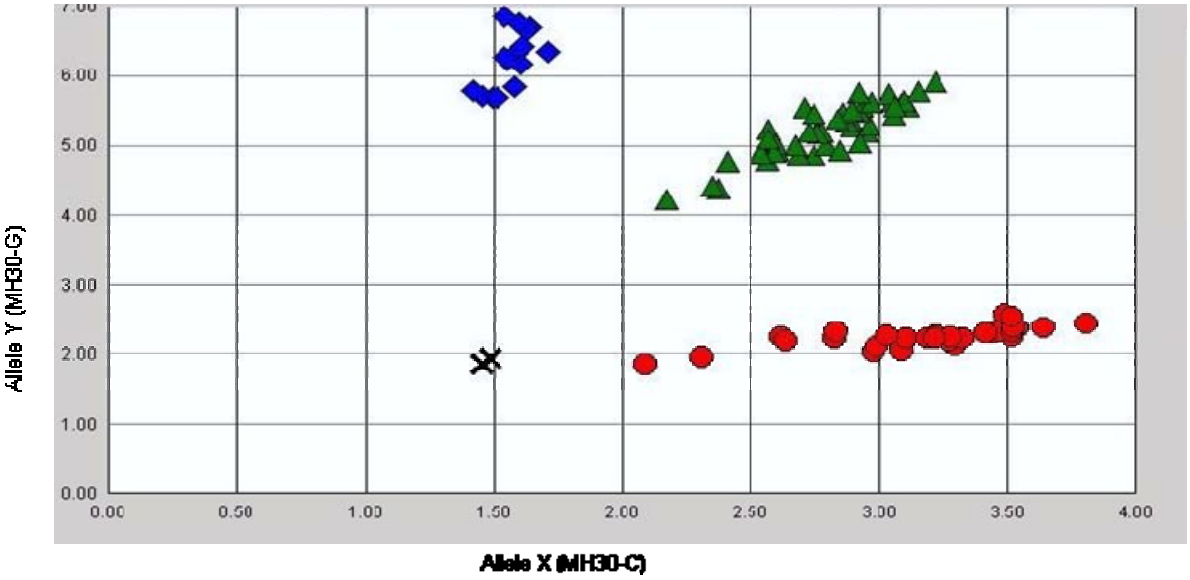

Figure 8. Analysis of SNP MH30.

Blue dots represent homozygous carriers for the G allele, red dots represent homozygous carriers for the C allele, and green dots represent carriers of both alleles, heterozygous carriers. X represent the negative control.

4.3. Statistical analysis

The R 2.3.1 A Language and Environment (Free Software Foundation, USA) was used for statistical analysis. Logistic regression analysis was used to obtain crude odds ratio (OR) values as well as confidence intervals (CI) for genotypes and alleles. H.W.E (Hardy-Weinberg equilibrium) and LD was tested by using the SHEsis software. $P<0.05$ was considered statistically significant for all analysis.

5. RESULTS

5.1. Analysis of alleles and genotypes of CTLA-4 MH30C/G, -1147C/T, -318C/T and CT60A/G SNPs in type 1 diabetes patients and control group.

The MH30, -1147, -318 and CT60 SNPs were genotyped in all studied subjects and the analysis of genotypes distribution showed no significant deviation from H.W.E. in T1D patients (p=0.313, p=0.960, p=0.418, p=0.254, respectively) nor in the control group (p=0.465, p=0.869, p=0.857, p=0.159, respectively). The genotyping success rate was obtained 100% for the SNPs under investigation. In order to verify our findings, 15% of total samples were re-genotyped for each SNP. The re-genotyped analysis revealed 100% identical results with the initial findings.

A significant higher frequency of the CT60 GG (OR=1.76, 95%CI=1.13-2.73, p=0.01) genotype was observed in the group of T1D patients when compared to controls (Table 9).

The frequencies of the CTLA-4 -1147 CC (67.5% vs. 65.2%) and -318 CC (79.2% vs. 77.4%) genotype were higher in T1D patients whereas the frequencies of the CTLA-4 -1147 T (17.9% vs. 19.3%) and -318 T (11.4% vs. 12.0%) allele were lower in T1D patients in comparison to control group, respectively. However, the frequency distribution of alleles and genotypes of -1147 and -318 markers did not differ statistically significantly between T1D cases and controls (Table 9).

Moreover, the analysis of MH30 SNP in the present study did not reveal any significant association between the type 1 diabetes patients and the control group. However, it is of interest that a tendency was observed for the MH30 GG genotype (OR=1.50, 95%CI: 0.97-2.32, p=0.06) in the group of type 1 diabetes when compared to control group (Table 9).

Table 9. Genotype and allele distribution of CTLA-4 MH30C/G, -1147C/T, -318C/T and CT60A/G SNP in T1D patients and controls.

CTLA-4 GENOTYPES and ALLELES	Control, n(%) (N=230)	T1D, n(%) (N=154)	OR (95%CI)	P-value
MH30 (rs231806)				
CC	26 (11.3)	17 (11.0)	1.20 (0.60-2.38)	0.61
CG	110 (47.8)	60 (39.0)	1*	
GG	94 (40.9)	77 (50.0)	1.50 (0.97-2.32)	0.06
C-allele	162 (35.2)	94 (30.5)	1*	
G-allele	298 (64.8)	214 (69.5)	1.24 (0.91-1.69)	0.17
-1147 (rs16840252)				
CC	150 (65.2)	104 (67.5)	1.09 (0.70-1.71)	0.70
CT	71 (30.9)	45 (29.2)	1*	
TT	9 (3.9)	5 (3.3)	0.88 (0.28-2.78)	0.82
C-allele	371 (80.7)	253 (82.1)	1.10 (0.76-1.60)	0.60
T-allele	89 (19.3)	55 (17.9)	1*	
-318 (rs5742909)				
CC	178 (77.4)	122 (79.2)	1.16 (0.69-1.94)	0.58
CT	49 (21.3)	29 (18.8)	1*	
TT	3 (1.3)	3 (2.0)	1.69 (0.32-8.39)	0.54
C-allele	405 (88.0)	273 (88.6)	1.06 (0.67-1.66)	0.80
T-allele	55 (12.0)	35 (11.4)	1*	
CT60 (rs3087243)				
AA	29 (12.6)	19 (12.3)	1.28 (0.66-2.46)	0.46
AG	119 (51.7)	61 (39.6)	1*	
GG	82 (35.7)	74 (48.1)	1.76 (1.13-2.73)	**0.01**
A-allele	177 (38.5)	99 (32.1)	1*	
G-allele	283 (61.5)	209 (67.9)	1.32 (0.97-1.79)	0.07

1*: referent estimate

5.2. Haplotype analysis of CTLA-4 MH30C/G, -1147C/T, -318C/T and CT60A/G SNPs in type 1 diabetes patients and control group.

The LD analysis of the CTLA-4 SNPs revealed a highly significant association (Figure 9) for each locus pair (MH30C/G / -1147C/T, D'=0.999, r^2=0.115), (MH30C/G / -318C/T, D'=0.998, r^2=0.066), (MH30C/G / CT60A/G, D'=0.968, r^2=0.836), (-1147C/T / -318C/T, D'=0.999, r^2=0.575), (-1147C/T / CT60A/G, D'=0.999, r^2=0.129) and (-318C/T / CT60A/G, D'=0.998, r^2=0.074). Five haplotypes (Table 10) at the CTLA-4 loci (MH30, -1147, -318, CT60), were detected both in affected and unaffected subjects. The GCCG haplotype was associated with T1D risk (OR=1.35, 95%CI=1.011-1.81, p=0.042).

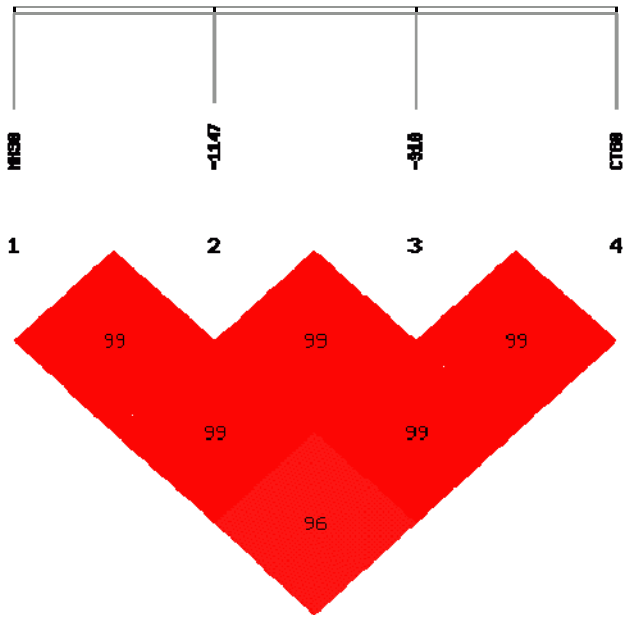

Figure 9. Linkage disequilibrium analysis of the CTLA-4 MH30C/G, -1147C/T, -318C/T and CT60A/G SNPs, based on D´.

Table 10. Haplotype distribution of CTLA-4 polymorphisms in T1D patients and controls.

CTLA-4 HAPLOTYPES* MH30 -1147 -318 CT60	T1D (%) (N=308)	Control (%) (N=460)	OR (95%CI)	p
CCCA	29.5	34.8	0.79 (0.58-1.08)	0.142
GCCA	2.6	3.7	0.69 (0.297-1.63)	0.40
GCCG	49.0	41.9	1.35 (1.01-1.81)	**0.04**
GTCG	6.5	7.4	0.872 (0.49-1.54)	0.64
GTTG	11.4	11.8	0.96 (0.61-1.51)	0.87

*All those frequency <0.03 were ignored in analysis

6. DISCUSSION

The CTLA-4 gene on chromosome 2q33 encodes a molecule that functions as a negative regulator of T-cell activation. It has been reported to confer genetic susceptibility to autoimmune diseases, including type 1 diabetes. The current study is a part of an ongoing project in order to evaluate the impact of CTLA-4 molecule as an important mediator for type 1 diabetes in Estonian population. Previously, an association of CTLA-4 +49A/G SNP with type 1 diabetes patients has been reported by Haller *et al.* 2004. In the present study we tried to evaluate the impact of CTLA-4 MH30C/G, -1147C/T, -318C/T and CT60A/G polymorphisms in T1D patients of Estonian origin.

The CT60 SNP within the CTLA-4 region is one of the strongest candidates and allelic variants of that locus are involved in T cell mediated autoimmune and inflammatory diseases. The CT60 G allele has been reported to be associated with a number of autoimmune diseases, including type 1 diabetes (Mayans *et al.* 2007; Orozco *et al.* 2004; Ueda *et al.* 2003), systemic lupus erythematosus (Torres *et al.* 2004), rheumatoid arthritis (Lei *et al.* 2005). Furthermore, the CT60 G allelic variant also has been reported to correlate with lower mRNA levels of the soluble form of CTLA-4, suggesting that differential expression of alternatively spliced forms of CTLA-4 might have an important role in determining susceptibility to autoimmune diseases (Ueda *et al.* 2003). In contrast the CT60 A allele has been found more frequently among healthy individuals and reported to act as protective allele. The current study is in concordance with previous findings since a statistically significant higher frequency of GG genotype was observed in the T1D patients group when compared to the control group.

Moreover, considering the probable influence of promoter polymorphisms in gene expression, we investigated three promoter polymorphisms. The study performed in 2001 by Ligers *et al.* and in 2004 by Anjos *et al.* indicated the functional significance of -318C/T polymorphism as the expression of the CTLA-4 mRNA was increased in association with T allele. The -318C/T polymorphism might affect the critical region for transcription factor binding with subsequently reduced CTLA-4 transcriptional efficiency or its influence may be the result of the linkage disequilibrium with another polymorphism. The study performed by Wang *et al.* 2002 showed that the -318T allele was associated with a higher promoter activity than the -318C allele.

The -1147C/T promoter polymorphism may affect the CTLA-4 gene expression in a similar way as reported for the SNP -318C/T promoter polymorphism. In a promoter luciferase assay, the -1147 T allele had half the level of luciferase activity of the C allele, suggesting that the -1147 T allele is associated with the reduced expression of CTLA-4 (Howard *et al.* 2002). In favor of it, we would have been expected higher frequency of T allele within the diabetes patients cohort but TT homozygotes were rare in our studied population. The TT genotype frequencies in other populations reveal to be rare as well (Ahmed *et al.* 2001; Kacem *et al.* 2001; Caputo *et al.* 2007; Balic *et al.* 2009).

In the current study we did not observed significant association between CTLA-4 -318 and -1147 SNPs and T1D cases. Our findings in -318 SNP are in agreement with previous study which showed no association for the TT homozygous genotype between T1D patients and control group (Ihara *et al.* 2001) not even when the haplotype (GTCG and GTTG) carried the susceptible associated T allele. The CTLA-4 -318 T allele have been found to be associated with sporadic breast cancer (Wang *et al.* 2007) and systemic sclerosis (Almasi *et al.* 2006). However, the association between -318 CC homozygosity and T1D have been reported in Chinise (OR =1.91, 95%CI 1.28-2.84) (Lee *et al.* 2001) and in non-Hispanic white population (OR=3.52, 95%CI 1.22-10.17) (Steck *et al.* 2005). The association between -318 CC homozygous genotype has also been reported with Graves´ disease (Braun *et al.* 1998).

Our study also supports previous finding which found no association between -1147 TT homozygous genotype and T1D cases (Zhernakova *et al.* 2005). However, Howard *et al*. (2002), reported a positive association of CTLA-4 -1147 T allele with bronchial hyperresponsiveness and asthma. Reasons for the differences in associations of these SNPs and different diseases are not clear. However it is possible that dissimilarities in the genetic background between different populations contribute to the differences in these results. We shoul also consider that the -318 and -1147 variants might not having a direct effect on the T cell activation and the development of autoimmune disease, but rather they have a functional effect unrelated to the disease or that they are in LD with the disease causing alleles.

In a comprehensive study by Ueda *et al.,* a 300kb region of 2q33, including the CD28, CTLA-4 and ICOS genes, was fully analyzed in Graves disease, autoimmune hypothyroidism and T1D patients. 108 SNPs were genotyped and three main peaks of various SNPs related to disease association pattern within the CTLA region were revealed. The MH30 marker

represented one of the peaks and it is of particular interest that due to its location it could influence the promoter activity of the CTLA-4 gene and subsequently its expression, predisposing to autoimmunity. In current study the MH30 marker was not significantly associated with T1D, however we observed a trend association between the MH30 GG disease susceptible genotype and T1D cases (p=0.06). We could not rule out the hypothesis that if we increased the study cohort we might found a significant association with the MH30 SNP and T1D.

In addition, we investigated whether an association could be detected analyzing haplotypes, including all four markers. Haplotype analysis revealed a significant higher frequency of the GCCG haplotype in T1D patients compared to the controls. The strong linkage disequilibrium between the polymorphisms in the 2q33 region makes it difficult to determine which alleles or combination of alleles are directly responsible for the observed association.

Furthermore, taking into account previously reported associations of CTLA-4 molecule with several endocrine disorders as well as the results of the current study, we should consider that the CTLA-4 might confer general risk to develop autoimmunity and the MH30 marker as susceptible candidate. Considering the heterogeneity of studied populations and genetic background of autoimmune disorders, further and larger case-control and family association studies are required in order to evaluate the impact of allelic variants of CTLA-4 gene as disease susceptibility markers. In conclusion the current study confirmed a statistical significant association of CTLA-4 haplotype and CT60 SNP with T1D in Estonian population.

SUMMARY

T1D is a polygenic multi-factorial autoimmune disease, which results in the irreversible destruction of the insulin-producing beta cells in the pancreatic islets of Langerhans. CTLA-4 molecule is an important regulator of T cell activation involved in the down-regulation of immune response. Polymorphisms within the CTLA-4 gene have been suggested to confer susceptibility to autoimmune endocrine disorders, including T1D.

The first part of this master's thesis gives a brief review of the pathogenesis, incidence and genetic susceptibility to T1D. Also an overview of the structure, function and expression of the CTLA-4 gene is presented.

The practical part of this thesis aims to survey the distribution of CTLA-4 gene SNPs in patients with T1D and control subjects of Estonian origin.

Our study demonstrated that:

i) CTLA-4 CT60 SNP GG genotype is related with the risk to T1D in Estonian population;

ii) A trend for association was observed between the CTLA-4 MH30 GG genotype and T1D patients of the studied population;

iii) Haplotype analysis revealed a significant higher frequency of the GCCG haplotype (MH30 -1147 -318 CT60) in type T1D patients compared to the healthy controls;

iv) No significant differences in distribution of allelic or genotypic frequencies of CTLA-4 -1147C/T and CTLA-4 -318C/T polymorphisms were observed between controls and T1D patients in the studied population.

The current study confirms the involvement of the CTLA-4 gene in susceptibility of T1D. The functional role of genetic variants of CTLA4 molecule in pathogenesis of T1D is open and need further investigation.

KOKKUVÕTE

CTLA-4 geeni alleeli variandid kui olulised markerid esimest tüüpi diabeedi immunoregulatsioonis

Ele Prans

Esimest tüüpi diabeet (T1D) on polügeenne multifaktoriaalne autoimmuunhaigus, mis tuleneb insuliini tootvate pankrease saarekeste Langerhansi β-rakkude pöördumatust kahjustusest. CTLA-4 (Cytotoxic T lymphocyte antigen-4) molekul on oluline T raku aktivatsiooni ja immuunvastust pidurdav regulaator. CTLA-4 geeni polümorfismil on leitud seos erinevate autoimmuunsete endokriinhaigustega, kaasa arvatud T1D.

Käesoleva magistritöö kirjanduse osas antakse lühike ülevaade esimest tüüpi diabeedi patogeneesist, esinemusest ning geneetilisest soodumusest. Samuti käsitletakse CTLA-4 geeni struktuurilist ehitust, ekspressiooni ning funktsiooni.

Töö praktilises osas määrati CTLA-4 geeni ühenukleotiidsed polümorfismid: MH30C/G, -1147C/T, -318C/T ja CT60A/G T1D patsientidel ja kontrollisikutel.

Uuringute tulemusel leiti, et

i) CTLA-4 CT60 GG genotüüp oli statistiliselt seotud T1D-kõrgenenud riskiga;
ii) CTLA-4 MH30 GG genotüüp täheldas tendentsi (p=0.06) statistilisele seosele T1D-ga;
iii) Statistiline olulisus ilmnes ka haplotüübi blokis GCCG (MH30 -1147 -318 CT60) võrreldes T1D haigeid kontrollgrupiga;
iv) -1147C/T ja -318C/T alleelide ning nende genotüüpide esinemissagedused ei olnud oluliselt erinevad võrreldes T1D haigeid kontrollgrupi isikutega.

Antud uuring kinnitab CTLA-4 geeni seotust T1D-ga Eesti populatsioonis. CTLA-4 geeni funktsionaalne roll esimest tüüpi diabeedi patogeneesis vajab edasisi uuringuid.

ACKNOWLEDGMENTS

This study was carried out at the University of Tartu, Department of General and Molecular Pathology.

I would like to thank:
My supervisors Dr. Konstantinos Douroudis and Dr. Kalle Kisand who provided guidance and various assistance through the study;
Professor Raivo Uibo for the support and the opportunity to work in his laboratory;
Maire Link for the enthusiasm and interest in my work;
My colleagues at the Department of General and Molecular Pathology for creating a friendly and work-favoring environment.

REFERENCES

Achenbach P, Koczwara K, Kopff A, Naserke H, Ziegler AG, Bonifacio E. Mature high-affinity immune responses to (pro)insulin anticipate the autoimmune cascade that leads to type 1 diabetes. Journal of Clinical Investigation 2004; 114: 589-597

Ahmed S, Ihara K, Kanemitsu S, Nakashima H, Otsuka T, Tsuzaka K, Takeuchi T, Hara T. Association of CTLA-4 but not CD28 gene polymorphisms with systemic lupus erythematosus in the Japanese population. Rheumatology 2001; 40: 662-667

Al-Mutairi HF, Mohsen AM, Al-Mazidi ZM. Genetics of Type 1 Diabetes Mellitus. Kuwait Medical Journal 2007; 6: 107-115

Almasi S, Erfani N, Mojtahedi Z, Rajaee A, Ghaderi A. Association of CTLA-4 gene promoter polymorphisms with systemic sclerosis in Iranian population. Genes and Immunity 2006; 7: 401-406

American Diabetes Association (2006) Diagnosis and classification of diabetes mellitus. Diabetes Care 29; 43-48

Anjos S, Polychronakos C. Mechanisms of genetic susceptibility to type I diabetes: beyond HLA. Molecular Genetics and Metabolism 2004; 81: 187-195

Aribi M. Candidate Genes Implicated in Type 1 Diabetes Susceptibility. Current Diabetes Reviews 2008; 4: 110-121

Atkinson MA, Maclaren NK. The Pathogenesis of Insulin-Dependent Diabetes Mellitus. New England Journal of Medicine 1994; 331: 1428-1436

Atkinson MA, Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. Lancet 2001; 358: 221-29

Bach JF. Insulin-Dependent Diabetes Mellitus as an Autoimmune Disease. Endocrine Review 1994; 15: 516-542

Balic I, Angel B, Codner E, Carrasco E, Perez-Bravo F. Association of CTLA-4 polymorphisms and clinical-immunologic characteristics at onset of type 1 diabetes mellitus in children. Human Immunology 2009; 70(2): 116-120

Bottini N, Musumeci L, Alonso A, Rahmouni S, Nika K *et al.* A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes. Nature Genetics 2004; 36(4): 337-338

Bottini N, Vang T, Cucca F, Mustelin T. Role of PTPN22 in type I diabetes and other autoimmune dioseases. Seminars in Immunology 2006; 18: 207-213

Brand O, Gough S, Heward J. HLA, CTLA-4 and PTPN22: the shared genetic master-key to autoimmunity? Expert Review in Molecular Medicine 2005; 23: 1-15

Braun J, Donner H, Siegmund T, Walfish PG, Usadel KH, Badenhoop K. CTLA-4 promoter variants in patients with Graves´ disease and Hashimoto´s thyroiditis. Tissue Antigens 1998; 51: 563-566

Butty V, Roy M, Sabeti P, Besse W, Benoist C, Mathis D. Signatures of strong population differentiation shape extended haplotypes across the human CD28, CTLA4, and ICOS costimulatory genes. Proceedings of the National Academi of ciences 2007; 104: 570-575

Caputo M, Cerrone GE, Mazza C, Cedola N, Targovnik HM, Gustavo DF. No Evidence of Association of CTLA-4 -318 C/T, 159 C/T, 3 ´STR and SUMO4 163 AG Polymorphisms with Autoimmune Diabetes. Immunological Investigations 2007; 36: 259-270

Carreno BM, Collins M. The B7 Family of Ligands and Its Receptors: New Pathways for Costimulation and Inhibition of Immune Responses. Annual Review of Immunology 2002; 20: 29-53

Concannon P, Erlich HA, Julier C, Morahan G, nerup J, Pociot F, Todd JA, Rich SS, and the Type 1 Diabetes Genetic Consortium. Type 1 Diabtees. Estimation for Susceptibility Loci from Four Genome-Wide Linkage Scans in 1,435 Multiplex Families. Diabetes 2005; 54: 2995-3001

Davies JL, Kawaguchi Y, Bennett ST, *et al.* A genome-wide search for human type 1 diabetes susceptibility genes. Nature 1994; 371: 130-136

Devendra D, Liu E, Eisenbarth GS. Type 1 diabetes: recent developments. British Medical Journal 2004; 328: 750-754

Douroudis K, Prans E, Haller K, Nemvalts V, Rajasalu T, Tillmann V, Kisand K, Uibo R. Protein tyrosine phosphatase non-receptor type 22 gene variants at position 1858 are associated with type 1 and type 2 diabetes in Estonian population. Tissue Antigens; 2008; 72: 425-430

Eisenbarth GS. Type 1 diabetes mellitus: a chronic autoimmune disease. New England Journal of Medicine 1986; 314: 1360-1368

Eisenbarth GS. Insulin autoimmunity: Immunogenetics/ Immunopathogenesis of Type 1A diabetes. Annals of the New York Academi of Sciences 2003; 1005: 109-118

Eisenbarth GS, Jeffrey J. The Natural Historyof Type 1A Diabetes. Arquivos Brasileiros de Endocrinologia & Metabiologia 2008; 52(2): 146-155

Erfani N, Razmkhah M, Talei AR, Pezeshki AM, Doroudchi M, Monabati A, Ghaderi A. Cytotoxic T lymphocyte antigen-4 promoter variants in breast cancer. Cancer Genetics and Cytogenetics 2006; 165: 114-120

Esteghamati A, Khalilzadeh O, Anvari M, Tahvildari M, Amiri HM, Rashidi A, Solgi G, Parivar K, Nikbin B, Amirzargar A. Association of CTLA-4 gene polymorphism with Graves' disease and opthalmopathy in Iranian patients. European Journal of Internal Medicine 2009; 20: 424-428

EURODIAB ACE Study Group. Variation and trends in incidence of childhood diabetes in Europe. Lancet 2000; 355: 873-876

Gough S.C.L, Walker LS.K, Sansom DM. CTLA-4 gene polymorphism and autoimmunity. Immunological Reviews 2005; 204: 102-115

Haller K, Kisand K, Nemvalts V, Laine A-P, Ilonen J, Uibo R. Type I diabetes in insulin-2221 MspI and CTLA-4 +49A/G polymorphism dependent. European Journal of Clinical Investigation 2004; 34: 543-548

Haller K, Kisand K, Pisarev H, Salur L, Laisk T, Nemvalts V, Uibo R. Insulin gene VNTR, CTLA-4+49A/G and HLA-DQB1 alleles distinguish patent autoimmune diabetes in adults from type 1 diabetes and from type 2 diabetes groups. Tissue Antigens 2007; 69: 121-127

Heward JM, Allahabadia A, Carr-Smith J, Daykin J, Cockramt CS, Gordon C, Barnett AH, Franklyn JA, Gough SCL. No evidence for allelic association of a human CTLA-4 promoter polymorphism with autoimmune thyroid disease in either population-based case-control or family-based studies. Clinical Endocrinology 1998; 49: 331-334

Hirschhorn JN. Genetic epidemiology of type 1 diabetes. Pediatric Diabetes 2003; 4: 87-100

Howard TD, Postma DS, Hawkins GA, Koppelman GH, Zheng SL, Wysong AK, Xu J, Meyers DA, Bleeker ER. Fine mapping of an IgE-controlling gene on chromosome 2q: Analysis of CTLA4 and CD28. Journal of Allergy and Clinical Immunology 2002; 110(5): 743-751

Hunt KA, McGovern D, Kumar PJ, Ghosh S, Travis S, *et al.* A common CTLA-4 haplotype associated with coeliac disease. European Journal of Human Genetics 2005; 13: 440-444

Ihara K, Ahmed S, Nakao F, Kinukawa N, Kuromaru R *et al.* Association studies of CTLA-4, CD28, and ICOS geme polymorphisms with type 1 diabetes in the Japanese population. Immunogenetics 2001; 53: 447-454

Ilonen J, Sjöroos M, Knip M, Veijola R, Simell O, Akerblom H, Paschou P, Bozas E, Havarani O, Malamitsi-Puchner A, Thymelli J, Vazeou A, Bartsochas C.S. Estimation of Genetic Risk for Type 1 Diabetes. American Journal of Medical Genetics 2002; 115(1): 30-36

Janeway CA, Travers P, Walport M, Shlomchik M. Immunobiology: the immune system in healt and disease. 2005, Garland Publishing, New York

Jago CB, Yates J, Câmara NO, Lechler RI, Lombardi G. Differential expression of CTLA-4 among T cell subsets. Clinical and Experimental Immunology 2004; 136: 463-471

Kacem HJ, Bellassoued M, Bougacha-Elleuch N, Abid M, Ayadi H. CTLA-4 Gene Polymorphisms in Tunisian Patients with Graves' Disease. Clinical Immunology 2001; 110: 361-365

Kantárová D, Buc M. Genetic susceptibility to type 1 diabetes mellitus in humans. Physiological Research 2007; 56(3): 255-266

Karabon L, Kosmaczewska A, Bilinska M, Pawlak E et al. The CTLA-4 gene polymorphisms are associated with CTLA-4 protein expression levels in multiple sclerosis patients and with susceptibility to disease. Immunology 2009; 128: 787-796

Karvonen M, Viik-Kajander m, Moltchanova E, Libman I, LaPorte R, Tuomilehto J. Incidence of Childhood Type 1 Diabetes Worldwide. Diabetes Care 2000; 10: 1516-1526

Kim MS, Polychronakos C. Immunogenetics of Type 1 Diabetes. Hormone Research 2005; 64: 180-188

Knip M, Veijola R, Virtanen SM, Hyöty H, Vaarala O, Åkerblom HK. Environmental Triggers and Determinants of Type 1 Diabetes. Diabetes 2005; 54: 125-136

Kouki T, Gardine CA, Yanagawa T, Degroot LJ. Relation of the three polymorphisms of the CTLA-4 gene in patients with Graves' disease. Journal of Endocrinological Investigation 2002; 25: 208-213

Kristiansen OP, Larsen ZM, Pociot F. CTLA-4 in autoimmune diseases–a general susceptibility gene to autoimmunity? Genes and Immunity 2000; 1: 170-184

Lei C, Dongqing Z, Yeqing S, et al. Association of the CTLA-4 gene with rheumatoid arthritis in Chinese Han population. European Journal of Human Genetetics 2005; 13: 823-828

Lee YJ, Lo FS, Shu SG, Wang CH, Huang CY, Liu HF *et al.* The promoter region of the CTLA-4 gene is associated with type 1 diabetes mellitus. Journal of Pediatric Endocrinology 2001; 14(4): 383-388

Ligers A, Teleshova N, Huang WX, Hillert J. CTLA-4 expression is influenced by promoter and exon 1 polymorphisms. Genes and Immunity, 2001; 2: 145-152

Ling V, Wu PW, Finnerty HF, Sharpe AH, Gray GS, Collins M. Complete sequence Determination of the Mouse and Human CTLA4 Gene Loci: Cross-Species DNA Sequence Similarity beyond Exon Borders. Genomics 1999; 60: 341-355

Magistrelli GM, Jeannin P, Herbault N, Benoit de Coignac A, Gauchat J-F, Bonnefoy J.Y, Delneste Y. A soluble form of CTLA-4 generated by alternative splicing is expressed by nonstimulated human T cells. European Journal of Immunology 1999; 29: 3596-3602

Mayans S, Lackovic K, Nyholm K, Lindgren P, Ruikka K, Eliasson M, Cilio CM, Holmberg D. CT60 genotype does not affect CTLA-4 isoform expression despite association to T1D and AITD in northern Sweden. BMC Medical Genetics 2007; 8: 1-8

McCoy K, Le Gros G. The role of CTLA-4 in the regulation of T cell immune responses. Immunology and Cell Biology 1999; 77: 1-10

Mehra NK, Kumar N, Kaur G, Kanga U, Tandon N. Biomarkers of susceptibility to type 1 diabetes with speciaö reference to the Indian population. Indian Journal of Medical Research 2007; 125: 321-344

Miller S.A, Dykes D.D, Polesky H.F. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Research 1988; 16: 1215

Morran MP, Omenn GS, Pietropaolo M. Immunology and Genetics of Type 1 Diabetes. Mount Sinai Journal of Medicine 2008; 75: 314-327

Murgia C, Devirgiliis C, Moncini E, Donadel G, Zalewski P, Perozzi G. Diabetes-linked zink transporte ZnT8 is a homodimeric proteiin expressed by distinkt rodent endocrine cell types in the pancreas and ohter glands. Nutrition, Metabolism & Cardiovascular Diseases 2009; 19: 431-439

Nistico L, Buzzetti R, Pritchard LE, Van der Auwera B, Giovannini C *et al.* The CTLA-4 region on chromosome2q33 is linked to, and associated with, type 1 diabetes. Human Molecular Genetics 1996; 5: 1075-1080

Notkins AL, Lernmark Å. Autoimmune type 1 diabetes: resolved and unresolved issues. Journal of Clinical Investigations 2001; 108: 1247-1252

Oaks MK, Hallett KM. Cutting Edge: A Soluble Form of CTLA-4 in Patients with Autoimmune Thyroid Disease. The Journal of Immunology 2000; 164: 5015-5018

Orozco G, Torres B, Nunez-Roldan A, Gonzalez-Escribano MF, Martin J. Cytotoxic T-lymphocyte antigen-4-CT60 polymorphism in rheumatoid arthritis. Tissue Antigens 2004; 64: 667-670

Pawlak E, Kochanowska IE, Frydecka I, Kielbinski M, Potoczek S, Bilinska M. The soluble CTLA-4 receptor: a new marker in autoimmune diseases. Archivum Immunologiae et Therapie Experimentalis 2005; 53: 336-341

Perez-Garcia A, Camara R, Roman-Gomez J, jimenez-Velasco A, Encuentra M *et al*. CTLA-4 polymorphisms and clinical outcome after allogeneic stem cell transplantation from HLA-identical sibling donors. Blood 2008; 1: 461-467

Pociot F, McDermott MF. Genetics of type 1 diabetes mellitus. Genes and Immunity 2002; 3: 235-249

Podar T, Solntsev A, Karvonen M, Podaiga Z, Brigis G, Urbonaite B, Viik-Kajander M, Reunanen A, Tuomilehto J. Increasing incidence of childhood-onset Type I diabetes in 3 Baltic countries and Finland 1983-1998. Diabetologia 2001; 44: 17-20

Redondo MJ, Fain PR, Eisenbarth GS. Genetics of Type 1A Diabetes. Recent Progress in Hormone Research 2001; 56: 69-89

Robles D.T., Eisenbarth G.S. Type 1A Diabetes Induced by Infection and Immunization. Journal of Autoimmunity 2001; 16: 355-362

Santiago JL, Martinez A, Calle H, fernandez-Arquero M, Figueredo MA, Concha eG, Urcelay E. Susceptibility to type I diabetes conferred by the PTPN22 C1858T polymorphisms in the Spanish population. BMC Medical Genetics 2007; 8(54): 1-5

Simmonds MJ, Gough SCL. Genetic insights into disease mechanisms of autoimmunity. British Medical Bulletin 2005; 71: 93-113

Steck AK, Bugawan TL, Valdes AM, Emery LM, Blair A, Norris JM, Redono MJ, Babu SR, Erlich HA, Eisenbarth GS, Rewers MJ. Association of Non-HLA Genes With Type 1 Diabetes Autoimmunity. Diabetes 2005; 54: 2482-2486

Zhernakova A, Eerligh P, Barrera P, Weseloy JZ, Huizinga T.J.W. CTLA-4 is differentially associated with autoimmune diseases in the Dutch population. Human Genetics 2005; 118: 58-66

Tang Q, Boden EK, Henriksen KJ, Bour-Jordan H, Bi M, Bluestone JA. Distinct roles of CTLA-4 and TGF-β in $CD4^{+}CD25^{+}$ regulatory T cell function. European Journal of Immunology 2004; 34: 2996-3005

Teeäär T, Liivak N, Heilman K, Kool P, Šor R, Paal M, Einberg Ü, Tillmann V. Increasing incidence of childhood-onset type 1 diabetes mellitus among Estonian children in 1999-2006. Time trend analysis 1983-2006. Pediatric Diabetes 2009; x-x

Teft WA, Kirchhof MG, Madrenas J.A Molecular Perspective of CTLA-4 Function. Annual Review of Immunology 2006; 24: 65-97

Todd, J.A. 1997. Genetics of type 1 diabetes. Pathologie Biologie 1997; 45(3): 219-227

Torres B, Aguilar F, Franco E, et al. Association of the CT60 marker of the CTLA4 gene with systemic lupus erythematosus. Arthritis and Rheumatism 2004; 50: 2211–2215

Ueda H, Howson JMM, Esposito L, et al. Association of the T-cell regulatory gene CTLA-4 with susceptibility to autoimmune disease. Nature 2003; 423: 506-511

Vaidya B, Pearce S. The emerging role of CTLA-4 in autoimmune endocrinopathies. European Journal of Endocrinology 2004; 150: 619-626

Valk E, Rudd CE, Schneider H. CTLA-4 trafficking and surfake expression. Trends in Immunology 2008; 29: 272-279

Von Herrath M, Sanda S, Herold K. Type 1 diabetes as a relapsing- remitting disease? Nature Immunology 2007; 7: 988-994

Walker LSK, Abbas AK. The enemy within: Keeping self-reactive T cells at the bay in the periphery. Nature reviews 2001; 2: 11-19

Wang L, Li D, Fu Z, Li H, Li D. Association of CTLA-4 gene polymorphisms with sporadic breast cancer in Chinise Han population. BMC Cancer 2007; 7: 173

Wang XB, Zhao R, Giscombe R, Lefvert AK. A CTLA-4 gene polymorphism at position -318 in the promoter region affects the expression of protein. Genes and Immunity 2002; 3: 233-234

Wenzlau JM, Juhl K, Yu L, Moua O, Sarkar SA, Gottlieb P, Rewers M, Eisenbarth GS, Jensen J, Davidson HW, Hutton JC. The cation efflux transporte ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes. The Proceedings of the National Academy of Sciences 2007; 104(43): 17040-17045

Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, Nomura T, Sakaguchi S. CTLA-4 contol over Foxp3^{+} Regulatory T Clee Function. Science 2008; 322: 271-275