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The Genetic Landscape of Type 1 Diabetes in a Genetically Heterogeneous Population: the Role of HLA-G

by

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ABSTRACT

Type 1 Diabetes Mellitus (T1DM), also known as insulin-depended diabetes mellitus (IDDM), is an autoimmune disorder, characterised by the destruction of insulin-producing β-cell in the pancreas via the combined action of infiltrating T cells and macrophages. Self-antigens are presented to other immune cells via a group of peptide presenting molecules, the Human Leukocyte Antigens (HLA). Genotypic combinations that phenotypically portray greater risk include DRB1*03:01-DQA1*05:01-DQB1*02:01, DRB1*04:xx-DQA1*03:01-DQB1*03:02 (DR3-DQ2.5/DR4-DQ8). However disease related HLA haplotypes vary throughout different populations. The Cypriot population HLA is highly polymorphic and the combination of HLA alleles is very heterogeneous, so that the already proposed T1DM susceptibility alleles may either be confirmed or novel HLA susceptibility loci may be identified. It has been suggested that risk for T1DM lies in polymorphisms of the telomeric MHC locus, a region closest to classical and non-classical HLA class I alleles. Therefore, this study investigates the involvement of a 14bp insertion/deletion polymorphism (*rs371194629*) at the 3' untranslated region (UTR) of HLA-G in the context of T1DM and age of onset, as well as the overall involvement of HLA-G in the disease pathogenesis. Findings that emerged from this study show a strong association between the HLA-G 14bp-polymorphism and T1DM with respect to the age of onset. Specifically, the deletion/deletion (*DEL/DEL*) genotype was found to be associated with an early age of onset, while the presence of the insertion allele was associated to a later age of onset, thus indicating a possible dominant effect over the deletion allele, a role in delaying disease onset and an overall involvement of HLA-G in the pathogenesis of Type I diabetes. HLA-G gene expression was also found to be 2-fold lower in the early age of onset group when compared to the late one. In addition, this 14bp polymorphism is part of the most common HLA-G extended haplotype (Promo-G*0104a/G*01:04/UTR-3) that has not only been associated with T1DM but has also been found to be involved in disease progression since it is commonly found in the early onset group. Moreover, new HLA-G promoter haplotypes, genotypes and 3'UTR haplotypes have been recognised in the Cypriot population due to new SNP combinations and the high diversity of this population.

The significant differences between T1DM patients and controls concerning genetic susceptibility in the CTLA-4 and the TGF-beta genes alludes to the possible involvement of regulatory T cells (Treg), since both factors are heavily required for their augmentation.

Therefore, this is the first study that proposes that HLA-G localised at the pancreatic level but also HLA-G positive Tregs may fail to render immune modulation during autoimmune response due to HLA-G genotypic differences and thus identify these cells as potential therapeutic targets.

Lastly, evaluation of the HLA alleles at the protein level suggests a strong genetic contribution of the HLA molecules DR/DQ. Amino acids DQβ L^{26} and A^{57} which contribute to the stability of the heterodimer and are part of the binding pocket , are exclusively found in the risk DQB1 alleles *02:01 and *03:02. Similarly, all the amino acids on the risk DQA1 *03:01 and *05:01 alleles are entirely different from all the other alleles. This set of ten DQα amino acids (**$Y^{11}R^{52}R^{55}F^{61}T^{64}I^{66}L^{69}V/L^{76}H^{129}E/K^{175}$**), DRβ **$Q^{70}$** and DQβ **$L^{26}A^{57}$** have significant function, either as pocket amino acids, T-cell receptor (TCR) contact sites or part of the dimer of heterodimer formation, suggesting that these alleles, due to their conserved protein composition, are the main contributors in the disease pathogenesis while other genetic factors may act as disease modifiers.

ΠΕΡΙΛΗΨΗ

Ο Σακχαρώδης διαβήτης τύπου 1 (ΣΔΤ1), γνωστός επίσης και ως ινσουλίνο-εξαρτώμενος διαβήτης, είναι μια αυτοάνοση διαταραχή, που χαρακτηρίζεται από την καταστροφή των β-κυττάρων στο πάγκρεας μέσω της συνδυασμένης δράσης των διηθητικών κυττάρων Τ και των μακροφάγων. Τα αντιγόνα εαυτού παρουσιάζονται στα κύτταρα του ανοσοποιητικού συστήματος, μέσω μιας ομάδας παρουσίαση μορίων/ πεπτιδίων, τα αντιγόνα ιστό-συμβατότητας (HLA). Οι γονοτυπική συνδυασμοί που απεικονίζουν φαινοτυπικά μεγαλύτερο κίνδυνο περιλαμβάνουν τους DRB1 *03:01-DQA1 *05: 01-DQB1 *02:01, DRB1 *04: xx-DQA1 *03:01-DQB1 * 03:02 (DR3-DQ2.5 / DR4-DQ8). Ωστόσο, οι HLA απλότυποι ρίσκου για ΣΔΤ1 ποικίλλουν στους διαφορετικούς πληθυσμούς. Οι HLA απλότυποι του κυπριακού πληθυσμού είναι εξαιρετικά πολυμορφικοί και ο συνδυασμός των αλληλόμορφων HLA είναι πολύ ετερογενής. Επομένως, μέσο αυτού του πληθυσμού μπορεί είτε να επιβεβαιωθούν οι ήδη προτεινόμενοι απλότυποι ρίσκου ΣΔΤ1 ή να βοηθήσει στην αναγνώριση νέων HLA ρίσκου.

Περαιτέρω, έχει προταθεί ότι ο κίνδυνος για ΣΔΤ1 έγκειται σε πολυμορφισμούς στην τελομερική θέση του μείζονος συμπλέγματος ιστοσυμβατότητας (MHC), μια περιοχή που βρίσκεται πλησιέστερα προς αλληλόμορφα κλασικής και μη κλασικής HLA τάξης Ι. Ως εκ τούτου, αυτή η μελέτη διερευνά την εμπλοκή ενός πολυμορφισμός 14 βάσεων (rs371194629) στην 3 'αμετάφραστη περιοχή (UTR) του HLA-G, στην ηλικία έναρξης της νόσου, καθώς επίσης και τη συνολική συμμετοχή του HLA-G στην παθογένεια της νόσου. Ευρήματα που προέκυψαν από αυτή τη μελέτη δείχνουν μια ισχυρή συσχέτιση μεταξύ του HLA-G 14bp-πολυμορφισμού και της ηλικίας έναρξης του ΣΔΤ1. Συγκεκριμένα, ο γονότυπος διαγραφή / διαγραφής (DEL / DEL) βρέθηκε να σχετίζεται με την πρώιμη ηλικία έναρξης, ενώ η παρουσία του αλληλόμορφου εισαγωγής συσχετίστηκε με την μεγαλύτερη ηλικία έναρξης, υποδεικνύοντας έτσι μια πιθανή δεσπόζουσα επίδραση επί του αλληλόμορφου διαγραφής, ένα ρόλο στην καθυστέρηση της έναρξης της νόσου και συνολικά την συμμετοχή του HLA-G στην παθογένεια του διαβήτη τύπου Ι. Η γονιδιακή έκφραση HLA-G βρέθηκε επίσης να είναι 2 φορές χαμηλότερη στην πρώιμη ηλικία εμφάνισης σε σύγκριση με την μεγαλύτερη ηλικία έναρξης. Επιπλέον, ο 14bp πολυμορφισμός είναι μέρος του πιο κοινού HLA-G απλότυπου (Promo-G * 0104a / G * 01:04 / UTR-3) που δεν έχει μόνο συσχετιστεί με τον ΣΔΤ1 αλλά έχει επίσης βρεθεί να εμπλέκετε στην εξέλιξη της ασθένειας.

Οι σημαντικές διαφορές μεταξύ των ασθενών με ΣΔΤ1 και την ομάδα ελέγχου όσον αφορά τη γενετική προδιάθεση στα γονίδια CTLA-4 και TGF-β παραπέμπει στην πιθανή συμμετοχή των ρυθμιστικών Τ κυττάρων (Treg), καθώς και οι δύο παράγοντες είναι αναγκαίοι για την αύξηση τους. Ως εκ τούτου, αυτή είναι η πρώτη μελέτη που προτείνει ότι το HLA-G σε παγκρεατικό επίπεδο, αλλά και τα HLA-G θετικά Tregs αποτυγχάνουν να αντισταθμίσουν την αυτοάνοσης αντίδραση λόγο γονοτυπικών διαφορών του HLA-G. Έτσι προτείνουμε αυτά τα HLA-G θετικά Tregs κύτταρα ως πιθανούς θεραπευτικούς στόχους.

Τέλος, η αξιολόγηση των HLA αλληλόμορφων σε επίπεδο πρωτεΐνης υποδηλώνει κύρια γενετική συμβολή των HLA μορίων DR / DQ στον ΣΔΤ1. Τα αμινοξέα DQβ L^{26} και A^{57} που συμβάλλουν στη σταθερότητα του ετεροδιμερούς και αποτελούν μέρος του θύλακα δέσμευσης, βρίσκονται πρώτιστα στα αλληλόμορφα ρίσκου DQB1 * 02:01 και *03:02. Ομοίως, όλα τα αμινοξέα στα DQA1 αλληλόμορφα κινδύνου *03:01 και *05:01 είναι εντελώς διαφορετικά από όλα τα άλλα αλληλόμορφα. Αυτό το σύνολο των δέκα DQα αμινοξέων (**$Y^{11}R^{52}R^{55}F^{61}T^{64}I^{66}L^{69}V/L^{76}H^{129}E/K^{175}$**), το DRβ **$Q^{70}$** και το DQβ **$L^{26}A^{57}$**, έχουν σημαντική λειτουργία, είτε σαν αμινοξέα πρόσδεσης, θέσεις επαφής υποδοχέα Τ-κυττάρων (TCR) ή μέρος του διμερούς σχηματισμού ετεροδιμερούς, γεγονός που υποδηλώνει ότι αυτά τα αλληλόμορφα, λόγω της συντηρημένη πρωτεϊνική σύνθεση τους, είναι οι κύριοι συνεισφέροντες στην παθογένεια της νόσου, ενώ άλλοι γενετικοί παράγοντες μπορούν να ενεργούν ως τροποποίητες της νόσου.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLE

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION TO T1DM AND AUTOIMMUNITY

Type 1 Diabetes Mellitus (T1DM), also known as insulin-depended diabetes mellitus (IDDM), is an autoimmune disorder, characterised by the destruction of insulin-producing β-cell in the pancreas via the actions of infiltrating T cells and macrophages. This metabolic disorder of multiple aetiology results in defects in insulin secretion and/or insulin action leading to disturbances of fat, protein and carbohydrate metabolism.

The main symptoms of T1DM include polyuria, blurring of vision, thirst and weight loss. Diabetes Mellitus is characterised by chronic hyperglycaemia and left untreated can develop retinopathy with potential blindness, nephropathy, neuropathy and ketoacidosis. Affected individuals have increased risk of cardiovascular, peripheral vascular and cerebrovascular disease. (Cooke and Plotnick, 2008)

The incidence rate of T1DM varies regionally but it appears to be escalading in all publications. In Northern Europe and the U.S.A, the rate ranges from 8 to 17 per 100,000. The highest rates appear in Scandinavia, with Finland as high as 40/100,000, while Japan and China are as low as 1/100,000 (Kasper, 2005). In Cyprus, the incidence rate also portrays a rising trend, from 10.80 per 100,000 during the 1990-1999 period to 14.44 per 100,000 person/years between 2000 and 2009 (Skordis et al., 2012). This study further revealed that significantly more children were diagnosed during the cold months as opposed to warmer months of the year ($p<0.001$). The same pattern is observed in other population studies on both hemispheres. Therefore, these results present an interaction between environmental factors and genetic background that initiate the immune-mediated process that leads in β-cell autoimmunity.

Autoimmunity is a process in which the immune system malfunctions and begins to produce auto-antibodies and T cells directed to attack self-component leading in loss of self-antigen tolerance. Immunological tolerance to self-antigens is maintained through clonal deletion, clonal anergy, and active suppression mechanisms in order to preserve the biologic integrity of self. Disruptions in these mechanisms lead to loss of self-tolerance and autoimmune disease.

Self-tolerance occurs in three forms: central, peripheral, and acquired tolerance. Central tolerance is the process by which immature T and B cells are rendered non-reactive to self-antigens, during lymphocyte development, in the thymus and the bone marrow respectively. In autoimmune disease, autoreactive T cells escape thymic selection and can, therefore, trigger a

cascade of immune responses. In the thymus, T cells undergo positive and negative selection in order to give rise to T cells that recognise self-major histocompatibility complex (MHC) molecules. Positive selection within the thymic cortex is facilitated by thymic epithelial cells, rich in surface MHC molecules. Immature T cells that have binding affinity and adhere to these MHC molecules are saved from programmed cell death. In contrast, cells that fail to bind die via apoptosis. Therefore, a positive selection mechanism ensures that only T cells that are capable of recognising MHC related antigens may survive. The importance of this mechanism lay in the primary functions of T cells to identify and respond to infected host cells through an MHC facilitated manner. During this process, the immature thymocytes are transformed to either CD4+ T helper cells or CD8+ T cytotoxic cells depending on whether they recognise MHC class II or MCH class I respectively (Charles A. Janeway, 2001).

On the contrary, negative selection takes place in the cortex, the cortico-medullary junction and the medulla, and is predominately mediated by medullary thymic epithelial cells (mTECs) (Derbinski and Kyewski, 2010). This type of cells are responsible for displaying self-antigens to developing T cells and signal auto-reactive T cells, that bind to antigen to undergo apoptosis. This process is regulated by a transcription factor, the Autoimmune Regulator (AIRE). AIRE regulates the ectopic expression of tissue restricted antigens (TRAs) necessary for negative selection. Among other functions, AIRE allows the expression of organ-specific antigens in the thymus such as insulin (Goldsby, 2006, Anderson et al., 2002).

Nonetheless, this process of T cell deletion cannot eliminate all potentially self-reactive cells from the T cell repertoire, as some escape to the periphery due to insufficient binding affinity for the self-antigen presented. Therefore, these cells must be inactivated in the periphery by the actions of regulatory T cells (Treg) (Goldsby, 2006).

1.2 Treg AND AUTOIMMUNITY

In autoimmune disease, autoreactive T cells escape thymic selection and can, therefore, trigger a cascade of immune responses. In the thymus, T cells undergo positive and negative selection in order to give rise to T cells that recognise self-major histocompatibility complex (MHC) molecules. Nonetheless, this process of T cell deletion cannot eliminate all potentially self-reactive cells from the T cell repertoire, as some escape to the periphery due to insufficient binding affinity for the self-antigen presented. These cells must thus be inactivated in the periphery by the actions of regulatory T cells (Treg) (Goldsby, 2006).

Treg is a subpopulation of T cells that maintain tolerance to self-antigens and actively suppress the immune system in the process to abrogate autoimmune disease. The transcription forkhead box P3 (FOXP3) was thought to be the exclusive intracellular marker for the identification of Tregs (Fontenot and Rudensky, 2005, Hori et al., 2003). To this day, two major classes of $CD4^+$ Treg cells have been described — $Foxp3^+$ Treg cells and $Foxp3^-$ Treg cells, classified into natural and induced Tregs. Natural Tregs are CD4+CD25+Foxp3+ T cells that develop in the thymus and migrate to the periphery to induce immune homeostasis (Nomura and Sakaguchi, 2005, Sakaguchi, 2000, Sakaguchi, 2005). Induced Tregs are CD4+ T cells that acquire the surface marker CD25 outside the thymus upon induction by inflammation or autoimmunity (Jonuleit and Schmitt, 2003, Sakaguchi et al., 1995). It is no more preferable to refer to these two subsets as thymus-derived Treg cell (tTreg cell) and peripherally derived Treg cell (pTreg cell) respectively (Abbas et al., 2013). Both subsets require the expression of the transcription factor Foxp3, which can be used to identify the cells.

Nevertheless, literature has revealed several other types of T cell with suppressive activity that does not express Foxp3. These include IL-10 producing type 1 regulatory cells (Tr1) (Groux et al., 1997, Roncarolo et al., 2006), and TGFβ-secreting T helper 3 cells (Th3) (Chen et al., 1994). These two subtypes arise from naive T cells in the periphery by the actions of Dendritic cells (DCs). Recently, Treg17, an IL-17 producing Treg cells, have been added to this list (Singh et al., 2013). Tr1 cells main mechanisms of action are the secretion of IL-10 and the release of Granzyme B that attack myeloid cells. The identification of this type of cells relies strongly on their unique cytokine profile. The high secretion of IL-10 is the main characteristic of Tr1 cells followed by low levels of IL-4, IL-17, IL-2, and variable levels of IFN-γ (Gregori et al., 2012). Similar to other T cell subsets, Tr1 cells can transiently express FOXP3 upon activation, nevertheless, in Tr1 cells FOXP3 expression is not constitutive and never reaches the high levels characteristic of $CD25^+Foxp3^+$ regulatory T cells. Similarly, induced Tregs that mature from CD4+ precursors in peripheral sites are influenced by TGFβ (Fu et al., 2004), and acquire the typical Treg markers CD25 and CTLA-4. Nevertheless, this subset of cells show different characteristics from 'traditional' Tregs. These cells are named Th3 cells and their differentiation depends upon IL-2 and TGF-beta. As demonstrated by Maynard et. al. (2007), $FOXP3^+IL\text{-}10^-$ Treg were mostly located in secondary lymphoid tissues, lung and liver, whereas the small and large intestine had enrichment of $FOXP3^-IL\text{-}10^+$ and $FOXP3^+IL\text{-}10^+$ respectively (Maynard et al., 2007). The same study also reached the conclusion

that $FOXP3^+$ and $FOXP3^-$ $CD4^+$ thymic precursors gave rise to IL-10 peripheral Tregs (pTregs), though only the $FOXP3^-$ precursor cells gave rise to all Treg subsets (Maynard et al., 2007). In addition to the $CD4^+$ Tregs, a specialised subset of $CD8^+$ T cells - the $CD8^+CD28^-$ T suppressor cell (Ts) - influence APCs in up-regulating inhibitory receptors ILT3 and ILT4 rendering them tolerogenic (Wang et al., 2013).

HLA-G binds to myeloid APCs through the ILT2 and ILT4 receptors, inhibiting their functions and differentiation, leading in improper T lymphocyte activation and to impaired NK cytotoxic activity (Gros et al., 2008, Ristich et al., 2007, Huang et al., 2010). Studies have shown that the presence of HLA-G did not inhibit myeloid APCs, but rather induced them into an alternative differentiation path with altered cytokine and chemokine expression (Apps et al., 2007, Gros et al., 2008, Liang et al., 2002, Li et al., 2009). Therefore, HLA-G interacts with ILT receptors found on APCs and DCs inducing their differentiation into regulatory cells (Zhang et al., 2008). Furthermore, APCs not only change function in the presence of HLA-G but can commonly express it. The expression of HLA-G on APCs is greatly enhanced by factors such as interferons (Lefebvre et al., 1999), IL-10 (Gregori et al., 2010, Moreau et al., 1999), IDO (Lopez et al., 2006, Chung et al., 2009, Munn et al., 2002), and CTLA4 (Bahri et al., 2009). Myeloid cells with up-regulated HLA-G expression were also detected in pathologic contexts, such as transplantation, cancer, viral infections, and inflammatory diseases (Carosella et al., 2008). HLA-G myeloid APCs may secrete or shed HLA-G molecules, contributing in the generation of a tolerogenic microenvironment by altering the functions of lymphocytes, but also of the HLA-G–expressing myeloid APCs themselves, in a feedback loop manner but also able to generate Tregs (LeMaoult et al., 2004). Tregs arise through activated lymphocytes via trogocytosis of HLA-G–containing APCs membranes (LeMaoult et al., 2007). Another way of inducing Tregs is through a new subset of DCs named DC-10 for their ability to produce IL-10 (Gregori et al., 2010). The expression of high levels of membrane bound HLA-G1, ILT4, and IL-10 by DC-10 is critical to the generation of Tr1 cells from DCs and T cells (Rossetti et al., 2010). By secreting IL-10 themselves, Tr1 cells aid the expression of HLA-G and its receptors in neighbouring DCs (Gregori et al., 2010). Thus, it is very possible that myeloid APCs, HLA-G, IL-10, and regulatory cells, which have been independently investigated, are all key components of a general tolerogenic mechanism centred around the HLA-G/DC interaction.

The precise mechanism in which Tregs enforce their actions is not fully elucidated although evidence portrays various mechanisms. These include secretion of

immunosuppressive factors such as IL-9, IL-10 and TGF-β (Levings et al., 2002, Taylor et al., 2006), cell to cell interaction that mediate regulation through TCR affinity (Haribhai et al., 2011), as well as other co-stimulatory molecules, such as CTLA-4 (Shevach et al., 2001), and cytolytic activity. Upon activation of the transcription factor Foxp3, Tregs initiate their suppressive effect that includes secretion of cytokines IL-10 (Davidson et al., 2000, Moore et al., 1993) and TGF-β that induce cell-cycle arrest and apoptosis of targeted immune cells (Grossman et al., 2004).

Due to the regulatory and tolerogenic ability of Tregs, their interactions with other components of immune inhibition and tolerance, such as HLA-G was greatly investigated. Therefore, studies focused on the function of HLA-G on myeloid APCs, the expression of HLA-G by APCs and the capability of HLA-G to characterize or induce regulatory cells. Nevertheless, it is yet to be determined whether the function or differentiation of HLA-G positive Treg cells is impaired in autoimmune diseases.

As aforementioned, loss of self-tolerance is the initiation point in T1DM pathogenesis. Environmental triggers, such as viral infections, can act upon genetic predisposition to the disease leading in the destruction of pancreatic beta cells. In healthy islets of Langerhans reside specialized antigen presenting cells and lymphocytes (Yin et al., 2012, Willcox et al., 2009) that are capable of recognizing beta-cell auto-antigens, such as proinsulin, GAD65 and IA-2 (Arif et al., 2004). However, it is possible that these are present in healthy individuals as well. What discriminates a healthy individual from a new-onset patient is the pro-inflammatory responses in disease. $CD4^+$ T cells produce and secrete interferon-γ (INF) and interleukin-17 (IL-17) when interacting with beta-cell in T1DM patients, whereas in healthy individuals the cells secrete IL-10.

Islet specific T cells are effective regulators of tissue-specific pro-inflammatory immune responses and act via a suppressive mechanism that includes lysis of the antigen presenting cells (Tree et al., 2010, Boissonnas et al., 2010). Therefore, in disease onset, IL-10 secreting T cells may be deficient in number and function where a regulation of initiating a pro-inflammatory response is required. These islet-specific cells that secrete IL-10 are identical to another cohort of T cell: the Tregs. (Buckner, 2010a, Ferraro et al., 2011). The idea of local immune-suppression by functional regulatory T cells modulating disease activity and progression is interesting from both an immune-pathogenetic, as well as from a therapeutic view.

A perspective less investigated in T1DM was first addressed by Brusko et. al (2005) that linked age with the function of Tregs (Brusko et al., 2005). In 2007, this team demonstrated that the frequency of $CD4^+CD25^+FOXP3^+$ cells was age independent, whereas $CD4^+CD25^+FOXP3^-$ cell frequency was strongly associated with age ($p<0.0001$) (Brusko et al., 2007). With increasing subject age, the FOXP3- T-cells population also increased, and this was noted independent of T1DM (Brusko et al., 2007). Therefore, since there is a correlation between Tregs and age, it will be interesting to investigate the Treg profile according to disease age of onset in order to reveal any impairment in the mechanism.

In therapeutic context and due to its important role, studies and clinical trials are exploring the possibility of Treg administration to improve immune tolerance in the periphery of T1DM patients (Marek-Trzonkowska et al., 2012). As aforementioned, only the $FOXP3^-$ precursor cells gave rise to all Treg subsets (Maynard et al., 2007). In the periphery, HLA-G positive Tregs of thymic origin exist in limited numbers and proliferating ability (Feger et al., 2007). Recently, it was demonstrated that hypomethylating agent azacitidine (Aza) induced FOXP3 negative HLA-G expressing immunomodulatory T cells and, therefore, acquiring the best of both worlds: the immune-modulatory effects of Tregs and HLA-G. This subtype of Tregs is a good candidate for explaining events in autoimmune disease and even a better candidate for Treg oriented therapy.

The initiation of an autoimmune disease is caused by an abnormal immune response to self-antigens, a normal immune response to abnormal self-antigens, or the abnormal immune response to abnormal self-antigens. The progression of the disease relies strongly on three main factor: the hereditary susceptibility, the triggering vehicle, such as a virus, and the immune system imbalance/malfunction.

1.3 IMMUNE BALANCE AND CYTOKINES IN T1DM

In addition to loss of immune tolerance in the thymus, abnormalities in the peripheral tolerance regulation is also of importance to the pathogenesis of T1DM. As indicated above, regulatory T cells are a critical part of peripheral tolerance maintenance. In peripheral blood and pancreatic lymph node, defects cause impairment of Treg cells. An imbalance exists between Th17 immunity and regulatory T cells (Buckner, 2010a, Ferraro et al., 2011) in T1DM patients. Due to its important role, studies and clinical trials are thus exploring the possibility of Treg administration to improve immune tolerance in the periphery of T1DM patients (Marek-Trzonkowska et al., 2012).

Homeostasis between T-helper 1 (Th1) and T-helper 2 (Th2) is essential for immune regulation. Th1 cells are responsible for cellular immunity type-1 pathway that eliminates viruses, pathogens and cancerous cells. Th2 cells are key players in the humoral immunity, up-regulate antibody production to destroy extracellular organisms and involved in the tolerance of fetus during pregnancy. These two types of immune cells act as immune system supervisors (Moser and Murphy, 2000, Upham, 2003, Moll, 2003).

The cells functional integration of the immune system is accomplished through cytokines that stimulate up or down-regulation of immune cells (BB, 1998, Balkwill, 2000) and chemokines that attract cells to migrate in a particular direction. Th1 cells induced by the transcription factor T-bet produce INF-γ, IL-2 and lymphotoxin (Szabo et al., 2000, Szabo et al., 2003). In contrast, Th2 cells are generated by the transcription factor GATA-3 to produce IL-4, IL-5 and IL-13 cytokines (Zheng and Flavell, 1997, Mosmann and Coffman, 1989).

Cytokines are small soluble immunomodulatory molecules responsible for cell-to-cell communication. These glycoproteins act as humoral regulators in an autocrine, paracrine or even endocrine manner that regulates functional activities of individual cells and tissues. Cytokines are grouped in regards to their function: hematopoiesis regulators, inflammation effectors and immune response regulators. In addition, cytokines include growth factors, interleukins, interferons, tumor-necrosis factor family, colony stimulating factors and a collection of chemokines that engage G-protein-coupled receptors (O'Shea et al., 2002).

One of the most known cytokine is the interleukin 10 (IL-10), which belongs to the interleukins family. The human IL-10 gene is located on chromosome 1q21-32, consists of 5 exons and 4 introns, and is about 4.7kb. The human IL-10 proximal promoter side is characterized by a TATA box -90bp upstream the translation start side and a CCAAT box located at -237bp upstream the translation start side. The transcription start side of this gene is approximately -57bp upstream the translational site. A number of transcription family members, including specificity protein (Sp), signal transducers and activators of transcription (STAT), interferon regulatory factors (IRF), activator protein (AP), cAMP response element binding protein (CREB), CCATT enhancer/ binding protein (C/EBP), c-musculoaponeurotic fibrosarcoma factor (c-MAF), and nuclear factor κ-B (NF-κB) have been characterized as essential or critical factors in IL-10 regulation (Brightbill et al., 2000, Ma et al., 2001, Brenner et al., 2003, Cuesta et al., 2003, Ziegler-Heitbrock et al., 2003, Zhang et al., 2006, Csoka et al., 2007).

IL-10 is an effective anti-inflammatory cytokine that plays an essential role in reversing inflammation and preventing autoimmune pathologies (Kuhn et al., 1993, Sabat et al., 2010). Immune response to pathogens involves rapid activation of pro-inflammatory cytokines that act to initiate host defence against pathogen invasion. Nevertheless, excess inflammation can give rise to metabolic, systemic and hemodynamic disturbances, which can eventually be harmful to the host. As a result, the immune system has evolved parallel anti-inflammatory mechanisms, such as the release of IL-10, that serve to reduce the production of pro-inflammatory molecules and limit tissue damage by restoring tissue homeostasis (Moore et al., 2001, Mosser and Zhang, 2008). Therefore, impairment in the IL-10 expression or signalling pathway can provoke an exaggerated inflammatory response, resulting in tissue damage and immunopathology (Li et al., 1999, Ejrnaes et al., 2006, Siewe et al., 2006, Sun et al., 2009). Aberrant expression or deficiencies of IL-10 can also enhance the inflammatory response leading to the development of autoimmune diseases (Gazzinelli et al., 1996, Sellon et al., 1998, O'Garra et al., 2008).

Since IL-10 is a key player in the host anti-inflammatory response, it has multiple cellular sources that make it more effective and target-oriented. IL-10 is widely described as a T helper 2 -derived cytokine; however, it is now accepted that IL-10 is not restricted to certain T cell subsets but rather it can be produced in almost all leukocytes (Kuhn et al., 1993). Some of the major sources of IL-10 include T helper (Th) cells, monocytes (MO), macrophages (MΦ) and dendritic cells (DC), although numerous immune effector cells are capable of producing IL-10 in certain contexts including B cells, cytotoxic T cells, NK cells, mast cells, neutrophils and eosinophils (Maloy and Powrie, 2001, Maynard et al., 2009, Ouyang et al., 2011).

IL-10 anti-inflammatory activity is facilitated by the hederodimeric IL-10 receptors, IL-10R1 and IL-10R2. The receptor complex is expressed at varying degrees in numerous cell types, but macrophages and monocytes appear to be the primary target for IL-10. Receptor coupling activates JAK/STAT signalling, causing large changes in the expression profile of immunomodulatory genes (Wills-Karp et al., 2010), that, in turn, inhibit the release of pro-inflammatory mediators, decrease antigen presentation and phagocytosis, and concomitantly enhance the inhibitory, tolerance, and scavenger functions of these cells.

Severe skewing of theTh1/Th2 cytokine balance is characteristic in various human diseases including autoimmune diseases such as T1DM (Elenkov, 2004, Elenkov and Chrousos, 1999, Mosmann and Sad, 1996). Autoimmune disease occurs as a result of the balance being weighed towards Th1 immune reactions and away from Th2 responses. In Th1-associated

autoimmune diseases, such as T1DM, there is an increased TNF-α, IL-12 and the pro-inflammatory transcription factor NF-β (c-Rel and p50) that are major inflammation mediators (Elenkov, 2004, Yadav and Sarvetnick, 2003).

The difference between Th1 versus Th2 cytokine profile in autoimmunity usually depends on disease progression. During primary inflammation and auto-antibodies production, the Th1 profile is more prevalent, signified by elevated IL-12 and IL-18 levels in patient sera. This expression is, however, reversed in disease-active patients to a Th2 nature as suggested by persistent IL-10 levels (Hase et al., 2001, Mok and Lau, 2003, Morimoto et al., 2001, Robak et al., 2002). In T1DM and the animal model NOD mice, therapeutic trials with bacille Calmette-Guerin (BCG) vaccination revealed some beneficial degree. In NOD mice, BCG injections have prevented the occurrence of diabetes by boosting IL-4, a Th2 cytokine (Singh et al., 1999). Nevertheless, direct administration of Th2 cells to NOD mice worsened the disease (Pakala et al., 1997). In summary, Th1 dominance is present in major autoimmune diseases, but efforts to restore balance with Th2 cells or Th2 cytokines may worsen the condition.

Disease severity and prevalence has been strongly associated with IL-10 promoter polymorphisms. Functional polymorphisms of the IL-10 gene have been shown to influence susceptibility of various diseases and IL-10 secretion variability has been conferred to genetic factors (Westendorp et al., 1997, Hohler et al., 2002). The IL-10 gene is located on chromosome 1q31-32 and includes 23 single nucleotide polymorphisms (SNPs) on its promoter region (Kang et al., 2010). This loci genetically confers susceptibility to a number of autoimmune diseases (Johanneson et al., 2002). Particularly, three SNPs in the promoter region have been shown to be of grave importance in regulating IL-10 activity. Situated at positions -592, -819 and -1082 relative to the translational start site, the three SNPs are in linkage disequilibrium, and therefore are inherited together. The three major haplotypes of these SNPs are GCC, ACC and ATA and translate into high, moderate and low production of IL-10 (Eskdale et al., 1998, Lazarus et al., 1997, Turner et al., 1997).

In addition, two microsatellites are also located in the promoter region: the IL-10R and IL-10G. These are positioned approximately 1.1 and 4.0 kb upstream the transcription start site (Eskdale and Gallagher, 1995, Eskdale et al., 1996). The IL-10R alleles carry 12 to 16 CA repeats and the IL-10G alleles span from 16 to 28 CA repeats. Such alleles are in linkage with the promoter SNPs and combine to form various haplotypes (Eskdale et al., 1999). However, SNP frequencies vary through ethnic groups making it difficult to determine an association between

SNP frequency and specific disease. Therefore, the combinations of polymorphisms haplotypes can explain IL-10 production and autoimmune disease. In some degree, researchers have demonstrated an association between IL-10 and systemic lupus erythematosus (SLE), rheumatoid arthritis (RE) and asthma (Lim et al., 1998, Lopez et al., 2010, Fu et al., 2011, Lin et al., 2003, Yang et al., 2000). From the list of autoimmune diseases, the most striking evidence of association comes from SLE studies. Whilst results are contradicting between various ethnic groups, no single SNP has been shown to have a significant association with SLE (Lazarus et al., 1997, Guarnizo-Zuccardi et al., 2007, Nath et al., 2005), though strong association with specific variants localized at the TNF locus has been demonstrated in SLE (Lee et al., 2006). TNF-α is also involved in leukocyte trafficking via the regulation of chemokine receptors (Jawa et al., 1999). The TNF-α expression effects are according to timing and course of the disease (Yadav and Sarvetnick, 2003) that leads to autoimmunity. When considering both TNF and IL-10 SNP genotypes, the low IL-10/high TNF genotype conferring individuals had the highest presence of auto-antibodies (Suarez et al., 2003). Hence, numerous factors, such as demographics, etiology of disease and other targets of IL-10, can mask the true associations between IL-10 SNPs and autoimmune disease.

Lastly, IL-23 cytokine that shares its p40 subunit with that of IL-12 has been shown to be involved in autoimmune diseases such as T1DM (Cua et al., 2003), through the actions of destructive Th-17 cells. Th-17 is an additional subset of effector T helper cells that produce IL-17A, IL-17F, IL-21 and IL-22. These cells have been described to be critical players in autoimmune tissue inflammation (Bettelli et al., 2008). Th-17 differentiation in induced by a combination of TGF-β1, IL-6 and IL-1 cytokines which induce RORγt transcription factor required for this cell generation (Mangan et al., 2006, Veldhoen et al., 2006, Ivanov et al., 2006). Th-17 exposure to IL-23 cytokine is crucial for the cells' stabilization in order to be able to induce autoimmune tissue inflammation (Awasthi et al., 2009, Cua et al., 2003, McGeachy et al., 2009). Polymorphisms in IL-23 receptor (IL23R) has been genetically linked to various autoimmune diseases (Duerr et al., 2006, Rahman et al., 2008). Once the Th-17 cells are exposed to IL-23, the concentration of anti-inflammatory cytokine IL-10 diminishes, making these cells pathogenic (McGeachy et al., 2007). Th17 as Th1 express T-bet (Yang et al., 2009) but are thought to be mostly pathogenic (Bettelli et al., 2008, Jager et al., 2009), though IL-17 producing cells that are non-pathogenic may also exist (Esplugues et al., 2011). The introduction mechanism of these cells going from non-pathogenic to pathogenic is still unclear,

but some studies have shown that granulocyte-macrophages colony-stimulating factor (GM-CSF), produced by Th-17 and trans-activated by RORγt, is required for their pathogenic transition (Codarri et al., 2011, McQualter et al., 2001).

1.4 GENETIC CAUSES OF T1DM

1.4.1 HLA regions associated with T1DM

Type 1 diabetes mellitus (T1DM) is a chronic autoimmune disease that eventually causes the complete loss of pancreatic beta-cells. Auto-antigens of pancreatic origin provoke a humoral and cellular immune response and are the main markers employed for disease diagnosis (Vehik et al., 2011, Roep and Peakman, 2011). The auto-antigen list includes insulin/proinsulin, glutamic acid decarboxylase (GAD65), the tyrosin phosphatise-like protein IA-2, the islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) and the cation efflux transporter ZnT8 (Vehik et al., 2011).

Over the years, genetic studies have identified over 40 susceptibility loci, many of which have been mapped to allelic variants of known genes (Pociot et al., 2010). T1DM show familial predisposition since family members have higher risk of acquiring the disease than the general population. This disease is mostly expressed in children, adolescents and young adults while studies reveal that autoimmunity can be triggered during early childhood (Barker et al., 2004). This evidence may suggest the existence of an autoimmune triggering mechanism early in development. Defections in central tolerance to pancreatic auto-antigens are the primary step towards T1DM. As mentioned earlier, tissue restricted antigens (TRAs) are presented in the thymus for conveying immunological self-tolerance in early development (Derbinski and Kyewski, 2010). TRAs in autoimmunity can evolve in auto-antigens through allelic variation, alternative splicing and epigenetic regulation of gene expression. These genetic mechanisms upon testing on mice revealed a linkage between self-tolerance impairment and a wide range of auto-antigens, including insulin (Fan et al., 2009). In humans, the insulin gene locus is one of the main susceptibility genes in T1DM and its expression in the thymus is susceptible to allelic variation and epigenetic effects (Pugliese et al., 1997, Vafiadis et al., 1997). The insulin gene transcription level in the thymus is also influenced by polymorphisms in the AIRA transcription factor (Sabater et al., 2005, Cai et al., 2011).

The TRAs are presented to the developing lymphocytes through a series of antigen-presenting molecules of the Major Histocompatibility Complex (MHC), the Human Leukocyte Antigen (HLA) complex. It is located on chromosome 6 and encompasses a large number of genes that are related to immune system function and the production of HLA antigens (Hauser and Oksenberg, 2006). In addition, MHC determines compatibility of donors for organ or bone marrow transplant as well as one's susceptibility to an autoimmune disease via cross-reacting immunization (Petersdorf and Malkki, 2005). The HLA system is one of the highest polymorphic systems known, since the combinations between MHC class I and II molecules are greatly diverse (Apanius et al., 1997). MHC molecules mediate interactions with leukocytes, specifically T helper cells, to activate a cascade of immune responses upon foreign stress, such as viruses and bacteria (Bjorkman et al., 1987a).

HLA antigens are divided into MHC classes according to their function. MHC class I antigens include HLA-A, HLA-B and HLA-C molecules that present peptides/viral peptides broken down in the proteasome from inside the cell. These peptides are usually small polymers, about 9 amino acids long, recognized by cytotoxic T cells as foreign and attract cytotoxic T-cells to destroy them. Class II molecules include HLA-DP, DM, DOA, DOB, DQ and DR that present antigens from outside the cell to T-lymphocytes. Once the antigen is attached to a class II molecule, it induces the multiplication of T-helper cells, which in turn stimulate B cells to produce IgG antibodies that have affinity for the specific antigen that first provoked T-cell activation (Marsh et al., 2005).

Furthermore, the structural characterization of the MHC class I and II receptors, as well as their specific peptide-binding complexes allowed researchers to re-evaluate the functional basis for the association to HLA autoimmune diseases. The presence of self-peptides that can be presented and reacted upon by T cells could explain the genetic association with MHC. MHC class I and class II antigens differences are in structure. MHC Class I proteins comply of an 'α' chain and a β2-micro-globulin. The 'α' chain of the class I molecule has two peptide-binding domain – α1 and α2, an immunoglobulin-like domain, α3, the trans-membrane region and the cytoplasmic tail (Bjorkman et al., 1987b). HLA class I molecules are expressed on the surface of almost all nucleated cells. The MHC Class II protein is made up by an 'α' and 'β' chains each one with two domains: α1 and α2, β1 and β2. Hence, each class II molecule has four domains: the peptide-binding domain (α1 or β1), the immunoglobulin-like domain (α2 or β2), the trans-membrane region, and the cytoplasmic tail (Brown et al., 1993). Class II molecules

are expressed only on B lymphocytes, antigen-presenting cells (monocytes, macrophages, and dendritic cells), and activated T lymphocytes.

HLA gene variants have been established as the primary susceptibility determinants of T1DM. These alleles confer approximately 50-60% of the overall risk of developing T1DM (Noble and Erlich, 2012). A set of class II haplotypes, HLA-DR3/DQ2 and HLA-DR4/DQ8, carry the strongest association to disease with up to 50% of patient carrying both haplotypes. This genotype presents such a high association to T1DM, probably due to an HLA-DQ heterodimer forming in trans, that presents the pancreatic islets auto-antigens in a manner that leads to a diabetic phenotype (van Lummel et al., 2012). In addition, this genotype is becoming common in younger children to whom the disease incidence rate increases dramatically (Hermann et al., 2003, Gillespie et al., 2004, Fourlanos et al., 2008, Vehik et al., 2008, Vehik and Dabelea, 2011). This increasing trend may be annotated to increased environmental pressure that is enhancing HLA-mediated genetic predisposition.

As mentioned earlier, the HLA complex is strongly involved in the selection of the T cell repertoire during immune system maturation in the thymus. Therefore, any malfunction of the system may lead in the auto-reactive T cell leakage from the thymus to the periphery. Several studies have revealed mechanisms on which weak interactions between T-cell receptor (TCR) and HLA, increase the probability of auto-reactive CD8 T cells escaping thymic selection and further become auto-reactive (Bulek et al., 2012). The mouse model for autoimmune diabetes, NOD, has provided insight on the mechanisms involved in the pathogenesis and studies through this have revealed that the mouse I-Ag7 MHC class II molecule has binding affinity to autoantigens, such as insulin peptide. This binding affinity results in poor negative selection of the auto-reactive T cells that are saved and included in the T cell repertoire (Mohan et al., 2011). The human equivalent of the I-Ag7 MHC class II molecule is the HLA-DQ8 as they share similar binding features and sequence specificity (Suri et al., 2005). Class II HLA alleles portray the greatest association with T1DM. Nevertheless, higher risk is determined via specific combinations of DRB1 and DQB1 alleles rather than individual loci. Genotypic combinations that phenotypically portray greater risk include DRB1*03:01-DQA1*05:01-DQB1*02:01, DRB1*04:xx-DQA1*03:01-DQB1*03:02 (DR3/DR4) (Erlich et al., 2008). Various studies have observed that DRB1*03:01-DQA1*05:01-DQB1*02:01 and DRB1*04:xx-DQA1*03:01-DQB1*03:02 heterozygotes in trans have a higher risk than homozygotes for either haplotypes. The DQ molecule is a heterodimer; therefore up to four DQ

antigens can be expressed on the cell surface and the trans-conferring risk haplotypes is never seen in cis (Erlich et al., 2008).

Analysis between DR4 high and low risk haplotypes differed at the DQB1 locus. High risk associated DQB1*03:02 and low risk associated DQB1*03:01. Sequence analysis revealed a difference of four amino acids between the two DQ alleles that alter codon 57. At this codon, DQB1*03:01 encodes an Asp whereas DQB1*03:02 an Ala. Structural analysis designated that the Asp at position 57 is a residue of the peptide-binding groove, in pocket P9, and contributes to a salt bridge that is absent in the presence of Ala, Ser or Val. These findings suggested a 'protective' role of Asp, whereas other amino acids at this position are associated to susceptible DQB1 alleles (Todd et al., 1987, Horn et al., 1988). The evidence of the correlation between the amino acid residue at DQB1 position 57 and T1D risk are striking.

Table 1: DRB1-DQA1-DQB1 haplotypes most strongly associated with T1DM in European populations. (Noble and Valdes, 2011). Summary of the most common predisposing haplotypes and the most protective haplotypes of T1D in European populations.

DRB1	*DQA1*	*DQB1*	Controls (%)	T1D (%)	OR	95% CI[a]
Predisposing haplotypes						
04:05	03:01	03:02	0.2	2.5	11.37	2.71-47.68
04:01	03:01	03:02	4.5	28.1	8.39	5.97-11.80
03:01	05:01	02:01	12.5	34.1	3.64	2.89-4.58
04:02	03:01	03:02	1.0	3.5	3.63	1.76-7.49
Protective haplotypes						
13:03	05:01	03:01	1.0	0.1	0.08	0.01-0.64
11:04	05:01	03:01	2.3	0.2	0.07	0.02-0.30
15:01	01:02	06:02	12.0	0.4	0.03	0.01-0.07
07:01	02:01	03:03	4.3	0.1	0.02	0.00-0.13
14:01	01:01	05:03	2.1	0.0	0.02	0.00-0.32

In addition to DR and DQ class II HLAs, the DP molecule encoded by the DPA1 and DPB1 genes is also in association with T1DM. Most of the DP variation is presented by the DPB1 gene (Noble et al., 2000, Cucca et al., 2001, Cruz et al., 2004, Stuchlikova et al., 2006, Baschal et al., 2007, Valdes et al., 2001, Varney et al., 2010). Studies revealed that DPB1*03:01 and *02:02 portray a positive association with T1DM, while DPB1*04:02 shows a negative one (Cruz et al., 2004, Cucca et al., 2001, Noble et al., 2000, Varney et al., 2010).

Studies on HLA involvement in T1DM predisposition also included an independent association with HLA class I alleles, such as HLA-A2, A24 and B39 (Noble and Erlich, 2012). The strongest HLA associations to T1DM are well established to be of class II genes. Nevertheless, class I A and B loci have been seen to affect susceptibility, playing particular role in age of onset of T1DM (Noble et al., 2010, Noble et al., 2002, Tait et al., 2003, Valdes et al., 2005, Nejentsev et al., 2007, Howson et al., 2009). In patients with highest risk DR3/DR4 genotype, who also carry the A*24:02 allele, have a significantly lower age of onset than those that are A*24 negative (Noble et al., 2002). Another highly predisposing allele is the B*39 class I allele, with the B39:06 allele being the most common associate with T1DM (Noble et al., 2010, Valdes et al., 2005).

The MHC class I genes are further divided into class 1a, which includes the HLA–A, –B and –C, and class 1b, which includes the HLA–E, –F and –G. One of the main differences between class 1a and 1b genes is that the former are highly polymorphic, while the latter have fewer variants. Class 1a antigens are membrane bound and expression is ubiquitous, whereas class 1b antigen expression may be tissue/organ-specific and conditional. HLA-G belongs to the class 1b group and is alternatively spliced into seven transcripts, four of which are predicted to encode membrane bound proteins and three to encode soluble proteins (Kovats et al., 1990, Ishitani and Geraghty, 1992). HLA G1-G4 have a cytoplasmic tail that bind them to the cell membrane, whereas G5-G6 are soluble forms due to the presence of intron 4 containing a premature stop codon that prevents the translation of the transmembrane and cytoplasmic tail. HLA-G7 has an intron 2 premature codon, making it soluble. The HLA-G gene consists of eight exons encoding a signal peptide (exon 1), the α1, α2 and α3 domains (exons 2, 3 and 4), the transmembrane domain (exon 5), and the intracellular domain (exons 6 and 7). Exon 7 is always absent from mature mRNA due to a stop codon in exon 6 and exon 8 that is never translated (Ishitani and Geraghty, 1992, Kuersten and Goodwin, 2003).

During pregnancy, the endometrial leukocyte populations change as a result of implantation. Following a short inflammatory reaction caused by the blastocyst breach of the uterine wall, local immune protection by the innate immune system is offered to the decidua T and B cells, which are now found mainly in the myometrium distal to fetal tissue, whereas natural killer (NK) cells and macrophages predominate in the decidual (Givan et al., 1997, Bulmer et al., 1988). In addition, IL-10 secreting Treg cells proliferate by estrogen stimulation (Polanczyk et al., 2004) and are approximately 14% of the CD4+ cells in the early decidua

(Heikkinen et al., 2004), critical for maintaining tolerance. During this period, immunomodulatory hormones, such as prolactin, chorionic gonadotropin and progesterone, are abundant and act as chemokines to operate as cell number and type modulators (Lysiak et al., 1995). Cytokines of a Th1- and Th2-nature are also present to modulate uterine networks (Simon et al., 1995, Hunt et al., 1992).

The fetus that arises from the inner cell mass of the blastocyst is protected by a shell of trophoblast cells that derive from the trophectoderm layer of the blastocyst. Therefore, the trophoblast cells are responsible for the appropriate interaction between fetus and mother protecting the first from any maternal immune attacks. To achieve this, trophoblast secretes high levels of complement regulatory proteins (Hsi et al., 1991) to contain antibody-mediated damages, express B7 family inhibitors (Petroff et al., 2003) to reduce cell-mediated immunity, and TNF family ligands (Chen et al., 1991) to induce apoptosis to immune cells. Fetal cells also produce immunosuppressive cytokines, chemokines and prostaglandins that reduce T cell proliferation. They also exhibit high levels of immune suppressive hormones, such as progesterone. Most importantly, these trophoblast cells regulate their own HLA gene expression and production of proteins. It is these proteins that when recognized as foreign by maternal immune cells would stimulate maternal CTL, capable of destroying HLA-expressing fetal cells (Le Bouteiller, 1994). Instead, the antigens expressed in trophoblast cells program maternal leukocytes into tolerance pathways.

HLA-G is recognized mainly by immunoglobulin-like transcript (ILT) receptors, also known as leukocyte immunoglobulin-like receptor subfamily B (LILRB), expressed by T cells, B cells, NK cells and mononuclear phagocytes (Allan et al., 2000). LILRB1s are expressed on the surface of several leukocytes, such as NK cells, and LILRB2s are mainly expressed on the surface of restricted cells including monocytes and dentritic cells (Brown et al., 2004). LILRB1 and LILRB2 have the ability to interact with classical HLA class I molecules, but show higher binding affinity to HLA-G (Shiroishi et al., 2003). LILRB2 is suggested to be the main HLA-G receptor, presented by monocytes/macrophages, one of the most abundant leukocyte populations in the human decidua (Bulmer et al., 1988). Literature shows the existence of HLA-G activated pathways which for ILT2/ILT4 include tyrosine phosphorylation, SHP-1 association and calcium regulation by expression of specific genes in leukocytes of the deciduas as needed for pregnancy-appropriate cell behavior.

One of the first identified functions of HLA-G was the inhibition of deciduas NK cell cytolysis by cyto-trophoblast cells that expressed HLA-G (Rouas-Freiss et al., 1997). HLA-G is an NK cell inhibitory receptor (KIR) ligand, and hence expression of this by trophoblast protects from NK cell-mediated death (Shiroishi et al., 2006, Gao et al., 2000). KIRs are transmembrane glycoproteins expressed by NK cells and subsets of T cells. The KIR2DL4 binds to the α1 domain of the HLA-G molecule (Yan and Fan, 2005). Studies further reveal that HLA-G plays a critical role in the maternal-fetal tolerance, by the expression of HLA-G throughout embryo development starting from the embryonic stem cells. HLA-G has an inhibitory function on multiple immune cells acting either directly or indirectly. Inhibition targets include the cytolytic functions of NK cells (Rouas-Freiss et al., 1997), the antigen-specific cytolytic functions of $CD8^+$ T cells (Riteau et al., 2001), the allo-proliferative responses of $CD4^+$ T cells (Lila et al., 2001), and the differentiation of dentritic cells (Liang et al., 2002). In addition, the continued proliferation of T cell and NK cells can also be inhibited (Bahri et al., 2006, Caumartin et al., 2007). HLA-G has also a less direct function through the generation of suppressor/regulatory cell (LeMaoult et al., 2004), implicating it in the re-modeling of the immune system in specific-antigens tolerance.

Apart from the function in pregnancy, HLA-G plays a role in other conditions, such as cancer (Paul et al., 1998), transplantation (Lila et al., 2000), autoimmune diseases (Mitsdoerffer et al., 2005) and viral pathologies (Onno et al., 2000). HLA-G is also involved in organ-specific autoimmunity, such as pancreatic islets. In the case of autoimmune disease, the involvement of HLA-G has been considered in multiple sclerosis (MS) (Wiendl et al., 2005), rheumatoid arthritis (Verbruggen et al., 2006), systemic lupus erythematosus (Rizzo et al., 2008), and psoriasis (Borghi et al., 2008, Aractingi et al., 2001). Multiple sclerosis is an autoimmune disease characterized by autoreactive Th1 cells, which cross the blood-brain barrier into the CNS after activation and after a cascade of immune responses destroy the myelin sheath of neurons. CSF samples of MS patients showed positive correlation between sHLA-G levels concentrations and IL-10, suggesting that CSF sHLA-G levels may act as anti-inflammatory under the control of IL-10 (Wiendl et al., 2005). Further studies demonstrated that IL-10 contributed to the suppression activity of HLA-G positive regulatory T cells (Tregs), as they were strongly present in CSF and inflammatory brain lesions of MS patients. HLA-G5, the prevalent isoform of HLA-G, is likely to be involved in the resolution of MS autoimmunity by acting as an anti-inflammatory molecule (Fainardi et al., 2007). sHLA-G inhibit the

proliferation of Th1 cells and the apoptotic elimination of activated T cytotoxic and NK cells in the brain through the Fas/FasL-dependent pathway. Furthermore, astrocytes secrete IL-10 that in turn increase production of HLA-G5 molecules leading in a shift of the Th1/Th2 balance toward Th2 direction leading to anti-inflammatory responses that are related to the remission of MS [78].

MHC class I expression is enhanced in the pancreatic islets in autoimmune diabetes and islet transplantation. Autoimmune diabetes is characterized by lymphoid infiltrates, such as T- and NK cells (Pankewycz et al., 1991, Poirot et al., 2004). Their destructive processes are counterbalanced by immunoregulatory mechanisms at sites of islet immunity. HLA-G is expressed by pancreatic islets and ducts and is up-regulated in response to pro-inflammatory cytokines (Cirulli et al., 2006). In the pancreatic endocrine cells, HLA-G is expressed constantly as β2-microglobulin-free heavy chain, at low levels, and remains mainly intracellular (Cirulli et al., 2006). This intracellular retention of HLA-G was also previously noted in trophoblast and transfected cell lines. Interestingly, this molecule was recycled from the golgi to the endoplasmic reticulum guided by signaling sequences of its cytoplasmic tail (Park et al., 2001, Park and Ahn, 2003). Through this mechanism, the HLA-G lifespan is prolonged but it also allows it to interact with multiple low-affinity peptides (Park and Ahn, 2003). Since low-affinity peptides may act as auto-antigens (Fairchild and Wraith, 1996), it is likely that intracellular HLA-G may function as a 'neutralizing' agent, aiding the immunologic ignorance of the islet by preventing self-antigen that escaped clonal deletion to be presented to the cell surface. HLA-G expression is regulated by inflammatory cytokines such as IFN-γ and IL-10, suggesting that the potential immune cell interactions are different than the ones occurring at the fetal/maternal interface. Furthermore, HLA-G can be exported on islet cells surface not only through the constitutive secretory pathway, but also through the regulated pathway by which insulin is secreted (Cirulli et al., 2006). Numerous auto-antigens in islet immunity are components of secretory granules (Nepom, 1995, Nakayama et al., 2005, Kent et al., 2005); therefore insulin exocytosis sites may be where the immunogenic ligands become exposed. Since the activation of autoreactive T cells depends upon the surface density of antigen/MHC complexes, this may lead to the activation of low-affinity cytotoxic T-cells. The presence of HLA-G at such granule exocytosis sites may therefore be a prevention mechanism to such aberrant activations.

In the case of rheumatoid arthritis (RA), it has been shown that sHLA-G levels were significantly lower compared to healthy subjects (Rizzo et al., 2008). RA is characterized by

joint destruction by immune cells in an unbalanced Th1 environment. In coeliac disease however, a disorder of abnormal immune response at the gut, sHLA-G in the serum of patients was increased as well as on intestinal biopsies, whereas sHLA-G molecules were totally absent from healthy biopsies (Rizzo et al., 2008). In SLE, results on HLA-G are contradicting. Rosado et al. (Rosado et al., 2008) reported higher HLA-G levels in patients, whereas Rizzo et al. (Rizzo et al., 2008) showed lower sHLA-G concentrations in SLE patients plasma and higher frequency of a 3' UTR polymorphism, the 14-bp insertion sequence and of the +14/+14-bp genotype.

Due to the presence of a stop codon in the exon 6 of HLA-G, the exon 7 is always absent from mature mRNA and the exon 8 is considered the 3'UTR region of this gene. This region contains a number of regulatory elements (Kuersten and Goodwin, 2003). The 3' UTR polymorphisms that can influence HLA-G expression include the insertion or deletion of a 14bp fragment that has been associated with mRNA stability, the SNP at position +3142 that may be a target for certain miRNAs (Bartel, 2009), and six additional SNPs located in putative binding sites for miRNAs (Castelli et al., 2009).

Studies have shown that the insertion of the 14bp leads in the formation of a cryptic breakpoint in the mRNA that lose the first 92bp of exon 8 (Hviid et al., 2003). It was concluded by many studies that the 14bp INS/INS genotype is associated with lower serum and plasma levels of sHLA-G compared to the INS/DEL and DEL/DEL genotypes (Chen et al., 2008, Hviid et al., 2004, Hviid et al., 2006). In addition, numerous SNPs have been identified within this region (Castelli et al., 2009) with the +3142 C/G SNP to be the most critical. This particular SNP was proposed to be involved in HLA-G regulation since it is associated with asthma (Tan et al., 2005). The G variant of a G/C SNP at position +3142 of the 3'UTR has been hypothesized to increase the affinity of the resulting mRNA for miR-148a, miR-148b and miR-152 (Veit and Chies, 2009). Among these miRNAs, only miR-152 has been demonstrated responsible for HLA-G post-transcriptional regulation. Over-expression of miR-152 in JEG-3 cell lines resulted in decreasing HLA-G expression and increased susceptibility to NK cell–mediated cytolysis (Zhu et al., 2010).

Analysis of the 3'UTR of the HLA-G locus exposed the presence of additional six SNPs located in putative binding sites for miRNAs (Castelli et al., 2009). The 14bp INS/DEL and the 7 SNPs arranged in different combinations generate eight distinct haplotypes of 3'UTR HLA-

G region (Castelli et al., 2010). Therefore, it has been hypothesized that the expression of HLA-G might be influenced by a combination of specific polymorphisms.

Most polymorphisms do not alter the amino acid sequence; the few that do alter it do not change secondary structures of the heavy chains. The short cytoplasmic tail retains HLA-G longer in the endoplasmic reticulum and prolongs the half-life of the molecule on the cell surface because of the lack of an endocytosis motif (Park et al., 2001, Park and Ahn, 2003). This permits multiple interactions with cells of the immune system. Nucleotide variability in the coding region of the HLA-G gene is evenly distributed throughout exon 2, 3 and 4, as well as introns (Donadi et al., 2011).

HLA-G is present in various different tissues even though under normal conditions, the HLA-G protein is detected only in trophoblasts cells, in some monocytes, T cells and in the thymus (Kovats et al., 1990, Crisa et al., 1997, Rebmann et al., 2003). The HLA-G molecule has an inhibitory effect on both cytotoxic T lymphocyte response and NK functions (Le Gal et al., 1999). Furthermore, antigen-presenting cells (APC) that have been transfected with HLA-G can prevent the proliferation of CD4+ T cells and hence direct these cells toward immunosuppression (LeMaoult et al., 2004). Finally, soluble (sHLA-G) expression seems to be able to induce CD8+ T cell apoptosis through the Fas/FasL pathway (Fournel et al., 2000, Contini et al., 2003). Therefore, HLA-G may have a role in the suppression of immune responses and contribute to long-term immune escape or tolerance (Carosella et al., 1999, Ishitani et al., 2003, LeMaoult et al., 2004).

The association between the 14bp polymorphism in the HLA-G 3'untranslated region and the genes expression, alternative splicing and concentration in sera has been widely explored (Hviid et al., 2003, O'Brien et al., 2001, Hviid et al., 2004, Hiby et al., 1999, Rousseau et al., 2003). Specific HLA-G haplotypes are associated with functional alterations in HLA-G expression. Consequently, this may uphold a role in relation to HLA-G expression during implantation and pregnancy (Fuzzi et al., 2002, Yie et al., 2005, Hylenius et al., 2004, Hviid et al., 2004), and in relation to some disorders with an immunological etiology, such as asthma, as well as autoimmune disease and in organ transplantation rejection (Lila et al., 2002, Nicolae et al., 2005, Rizzo et al., 2005b, Creput et al., 2003, Le Rond et al., 2004). Additionally, the genotype of the 14-bp polymorphism is also associated with differences in interleukin-10 (IL-10) secretion from peripheral blood mononuclear cells (PBMCs) after stimulation with lipopolysaccharide (LPS) (Rizzo et al., 2005a). In summary, the only HLA-G polymorphism

that was significantly associated with differences in HLA-G and IL-10 concentrations is the 14-bp polymorphism, with the highest IL-10 concentrations being portrayed by the INS/INS HLA-G genotype (Rizzo et al., 2005a). Different 14bp genotypes affects the soluble HLA-G levels in serum with the DEL/DEL being associated with no detectable serum-sHLA-G (33,34) and is also associated with differences in alternative splicing of HLA-G transcripts (Hviid et al., 2003, O'Brien et al., 2001, Hiby et al., 1999).

On the contrary, the effect of IL-10 on HLA-G expression was first described by Moreau et. al., 1999 (Moreau et al., 1999), who demonstrated that IL-10 enhances steady-state levels of HLA-G transcription in cultured trophoblast cells. This association was further investigated in peripheral blood monocytes (PBMCs) showing that IL-10 can up-regulate classical MHC class I molecules, including HLA-G, while MHC class II are down-regulated in monocytes following IL-10 treatment. Therefore, it could be concluded that HLA-G expression by IL-10 on monocytes may play an important role in down-regulating immune responses (Moreau et al., 1999).

Moreover, within the HLA encoding region, especially between class II and class I, lay genes commonly referred to as 'class III' region. This region includes immunologically relevant genes such as Tumor Necrosis factor Alpha (TNF-α) and the complement C4-encoding genes C4A and C4B. MIC-A is a class I-like molecule that is also found in this region. The TNFA gene has been associated with T1DM, especially for the -238 and -308 promoter SNPs. Nevertheless, publications are? in conflict where strong disease association is observed in cases where data is not adjusted to reflect LD with DR-DQ regions (Perez et al., 2004), and low significance in studies that LD was taken into account (Nishimura et al., 2003, Noble et al., 2006). A number of studies also reported T1DM association for C4A null patients and for C4B 'short' allele (Jenhani et al., 1992, Lhotta et al., 1996). The complement component 4 is a protein involved in the complement immune system and the C4 gene vary in copy number and usually this variation is associated with systemic lupus erythematosus (SLE) (Yang et al., 2007). MIC-A within the HLA region, encodes a highly polymorphic MHC class I related gene. The encoded protein is expressed on the cell surface and even though is similar to class I molecules, it does not seem to associate with beta-2-microglobulin and has no role in antigen presentation. MIC-A functions as a stress-induced antigen recognized by intestinal epithelial gamma delta T cells. Alternative splicing results in multiple transcript variants (Groh et al., 1998, Groh et al.,

2002). Various studies have associated MIC-A5 with T1DM as a susceptibility gene (Gambelunghe et al., 2000, Bilbao et al., 2002, Gupta et al., 2003).

1.4.2 The shared epitope hypothesis

Up to date, no single HLA gene has been identified as conferring disease risk. Nevertheless, the HLA's involvement in autoimmune pathogenesis and progression is clear through various association studies, specifically the role of class II molecules (Roark et al., 2014, du Montcel et al., 2005). A possible explanation for this involvement comes through the shared epitope hypothesis that assumes that structural differences of the binding groove of HLA class II molecules may influence the direct interaction with T cell or antigens involved in the process (Gregersen et al., 1987). Therefore, HLA susceptibility may not lie in one HLA gene, as various alleles may share peptide-binding motifs, also known as shared epitopes.

HLA epitopes are located in the highly-polymorphic antigen-binding groove of all HLA molecules. These consist of single or group of amino acids of both contiguous and non-contiguous nature. The HLA molecule binding groove is made up from pockets; class I has six distinct pockets and class II has four, all consisting of various combinations of polymorphic amino acids. Therefore, the range of peptides/antigens an HLA molecule bind is determined by the amino acids that line the pockets and can potentially combine to form millions of diverse HLA epitopes. Depending on these combinations, different HLA molecules may share similar epitopes, and therefore share affinity for the same peptides. In addition, the strong linkage disequilibrium between HLA-DR and HLA-DQ further complicates the deciphering of alleles rendering susceptibility or resistance.

1.5 Non-HLA regions associated with T1DM

The Human gene Nomenclature Committee catalogued the various genetic determinants associated with T1DM from IDDM1 to IDDM18. IDDM1 codes for HLA gene association. Genome-wide associations to T1DM are depicted in figure 1.

T1DM is highly specific to pancreatic beta cells; the insulin gene (INS) on chromosome 11p15.5 is therefore a strong candidate as a susceptibility locus since insulin may act as an auto-antigen (IDDM2). Upon studying this gene, a variable number of tandem nucleotide repeats (VNTR) upstream the insulin gene was identified that correlates to thymic insulin expression (Bennett et al., 1995, Undlien et al., 1995, Bennett and Todd, 1996). Larger VNTR, >100 repeats, show high mRNA expression and protection, whereas low number repeats (between 23-63), are associated with low thymic expression and susceptibility (Vafiadis et al., 1997, Pugliese et al., 1997, Cai CQ, 2011, Durinovic-Bello et al., 2010). Therefore, polymorphisms in the insulin may affect thymocyte selection, and hence the T cell repertoire in the periphery.

IDDM3 is located near the D15S107 marker on chromosome 15q26 (Field et al., 1994). Nevertheless, even though some association was established by some studies (Luo et al., 1995, Zamani et al., 1996), others failed to replicate (Cox et al., 2001). Furthermore, IDDM4 is located on chromosome 11q13, near the fibroblast growth factor 3 (FGF3) locus (Davies et al., 1994). The list of candidate genes near the FGF3 region include the gene for zinc finger protein 162 (ZFM1), a nuclear protein found in the pancreas (Sawicki et al., 1997), and the gene for Fas-associated death domain protein (FADD) that is involved in the cell signalling pathway for apoptosis. Interaction between the intracellular Fas 'death domain' and FADD is required for apoptosis and publications showed that binding of the Fas ligand on cytotoxic T cells to Fas expressed on insulin-producing beta cells may trigger their death (Signore et al., 1998, Mandrup-Poulsen, 2001).

IDDM 5, IDDM 8 and IDDM 15 are all located on chromosome 6q with varying degree of linkage. IDDM5 on 6q25 is a susceptibility region that has been replicate. The gene encoding manganese superoxide dismutase (SOD2) is located near this region and there is evidence to support that polymorphisms in this region are associated to T1DM (Pociot et al., 1994). The MnSOD protein holds structural variants with reduced activity that hypothesises to increase T1DM susceptibility. IDDM 8 is also located on chromosome 6, locus 6q27 (Davies et al., 1994, Luo et al., 1996). Cox et al., 2001 provided linkage evidence of this region to T1DM through a study on multiplex families (Cox et al., 2001). Lastly, IDDM15 is located on 6q21 in the region of D6S283-D6S1580, which is highly linked with HLA (Delepine et al., 1997). When studied in T1DM families, it showed high linkage of locus to disease. This region also holds the gene responsible for transient neonatal diabetes mellitus, increasing evidence that this region is of outmost importance in disease (Temple et al., 2000, Cave et al., 2000).

IDDM7 is located on chromosome 2q31 in the region of D2S152 and association has been shown in several populations (Copeman et al., 1995, Esposito et al., 1998, Kristiansen et al., 2000a). Candidate genes in this region include interleukin-1 gene cluster, IL1R1 (Bergholdt et al., 2000), IL1B (Kristiansen et al., 2000b) and IL1RN (Mandrup-Poulsen et al., 1994), HOX8 (Owerbach and Gabbay, 1995), GAD1 (Rambrand et al., 1997), Galnt3 (Kristiansen et al., 2000a), and NeuroD (Iwata et al., 1999, Hansen et al., 2000).

Some genetic IDDM locus failed to be replicated within studies and further investigation is required to confirm existing findings. Examples of this are the IDDM9 on chromosome 3q21-q25, which is strongly associated with HLA-DR3/4 heterozygous diabetic offspring 19 and IDDM10, the GAD2 gene encoding GAD56 (Mein et al., 1998, Rambrand et al., 1997, Reed et al., 1997). IDDM13 locus maps to 2q34 and the strongest evidence for linkage is obtained for the D2S137-D2S164 region 27. Candidate genes include the IA-2 (Esposito et al., 1998), IGFBP2 (Owerbach et al., 1997), IGFBP5 (Owerbach et al., 1997) and NRAMP1 (Esposito et al., 1998), but no disease associated mutations have been identified in these genes. Another locus that needs further confirmation in other populations is IDDM16, the immunoglobulin heavy chain (IGH) region on chromosome 14q32.3 that increases susceptibility to T1DM (Field et al., 2002). The effect of IGH on disease susceptibility is enhanced by interactions with HLA genes (Field et al., 2002). Genetic controlled differences in immunoglobulin structure will results in differential immune response to foreign and self-antigens, and therefore increase T1DM risk.

IDDM11 on the susceptibility locus 14q24.3-q31 with significant linkage to D14S67 (Field et al., 1996) enclose two candidate genes that are involved in the beta cell regulation and function. The ENSA gene encodes α-endosulfine, an endogenous regulator of beta-cell K (ATP) channels (Heron et al., 1999). This protein inhibits sulfonylurea binding on beta-cell membranes, reduce K (ATP) channel currents and stimulate insulin secretion (Heron et al., 1999). The second candidate gene is the SEL1L gene (Harada et al., 1999, Biunno et al., 2000), a negative regulator of the Notch pathway (Weinmaster, 2000), involved in the correct development of pancreatic endocrine cells (Apelqvist et al., 1999, Jensen et al., 2000b, Jensen et al., 2000a). Nonetheless, controversial studies that failed to identify association are also published for this gene (Larsen et al., 2001).

Lastly, next to HLA, there are a few identified susceptibility genes with striking evidence of increasing immune reactivity along with loss of control on T cell selection, activation and

differentiation into memory and regulatory phenotypes (Pociot et al., 2010). These identified loci are not disease specific as they have been involved in several autoimmune diseases, but are shown in various studies to be replicable. The most discussed genes in T1DM are the CTLA-4 (IDDM12), IDDM18, IL2RA and PTPN22. These genes influence basic pathways of T cell activation, function, and regulation.

Cytotoxic T Lymphocyte Antigen 4, CTLA-4, is expressed on the surface of $CD4^+$ T helper cells, bind to APC and transmit an inhibitory signal to other T cells (Dariavach et al., 1988, Magistrelli et al., 1999, Waterhouse et al., 1995). Intracellular CTLA-4 is also present in Treg cells and may play role in their function. CTLA-4 reverses the stop signal induced by TCR necessary for the firm interaction between T cells and APCs (Schneider et al., 2006, Rudd et al., 2009). There are three identified polymorphisms on CTLA-4; a C-T SNP in position -318 from the ATG start codon (Deichmann et al., 1996), the A49G in exon 1 (Kouki et al., 2000) and the dinucleotide repeat in the 3'-UTR. Another SNP in CTLA-4 intron 1 (C/T in position -819 from exon 2-start site) showed more significant association with type 1 diabetes than the A49G and the microsatellite (Marron et al., 2000). CTLA4 blockade with soluble anti-CTLA4 monoclonal antibody resulted in less augmentation in T cells from individuals possessing the susceptibility alleles (49 G/G) compared with those possessing protective alleles (49 A/A) (Kouki et al., 2000). Sequencing of cDNA of T and B lymphocytes from human and rodents revealed a CTLA-4 alternate transcript that lacks the trans-membrane domain (Magistrelli et al., 1999, Oaks et al., 2000) and results in a soluble form (sCTLA-4) detectable in the sera. It has also been demonstrated that serum levels of sCTLA-4 are higher in patients with autoimmune thyroid diseases compared to healthy subjects (Oaks and Hallett, 2000).

IDDM18 identified on chromosome 5q31.1-q33.1 is in proximity to the interleukin 12 gene, IL12B (Morahan et al., 2001, Nolsoe et al., 2000). IL-12p40 is involved in T cell responses, and therefore may play a role in T1DM susceptibility (Adorini, 2001). IL12 provokes T lymphocyte differentiation into the Th1 subset, leading in the production of cytokines for cell-mediated immunity (Liblau et al., 1995, Tian et al., 1998). In autoimmunity, IL12 induced autoreactive T cell are predispose to self-destructed immunity (Segal and Shevach, 1996, Segal et al., 1997). There are several polymorphisms reported on this locus (Huang et al., 2000, Hall et al., 2000), but a single base change on a 3' UTR show strong linkage to T1DM susceptibility (Morahan et al., 2001).

The interleukin 2 receptor alpha (IL2RA) region on chromosome 10p15 is associated with type 1 diabetes, as it encodes the alpha-chain of the IL-2 receptor complex (CD25) which binds to IL-2, a main component in Treg cell proliferation (Lowe et al., 2007). IL-2 is a key growth factor of lymphocytes and is involved in the initiation of immune responses by promoting proliferation and expansion of CD4 and CD8 T cells. This cytokine is also critical in the development, function and homeostasis of Treg cells (Malek and Castro, 2010). Polymorphisms on this gene reduce levels of soluble IL-2 receptor (Lowe et al., 2007), reduce STAT5a IL-2 responsiveness in antigen-experienced CD4 T cells, lower levels of regulatory T cell Foxp3 transcription factor and reduce the suppressive function of CD4+CD25+ regulatory T cells (Garg et al., 2012). Experimentation on NOD mice with IL-2 gene susceptibility revealed diabetes development and abnormal Treg function (Yamanouchi et al., 2007). Regulatory T cells within the islet express reduced amount of IL2RA, resulting in increased apoptosis and disease development (Tang et al., 2008). Low doses of IL-2 were introduced in the NOD mice reversing the onset of diabetes (Grinberg-Bleyer et al., 2010) and clinical trials show benefits in patients with graft-versus-host disease and hepatitits C virus-induced vasculitis (Saadoun et al., 2011, Koreth et al., 2011).

Lastly, in 2004, the protein tyrosine phosphetase non-receptor PTPN22 gene on chromosome 1p13 was found to be associated with T1DM susceptibility (Smyth et al., 2004). This gene encodes for a lymphoid protein tyrosine phosphatise (LYP) that is responsible for preventing spontaneous T cell activation by dephosphorylating and inactivating T cell receptors to avoid antigen binding (Smyth et al., 2004). A single nucleotide polymorphism (SNP) in the PTPN22 gene can lead to susceptibility to autoimmune diseases such as type 1 diabetes, since it decreases the negative regulation of hyper-reactive T cells. Sequencing of the PTPN22 gene for T1DM associated polymorphisms revealed a SNP at 1858bp in codon 620. Two alleles referred to as 1858C and 1858T were identified and the 1858T variant was shown to occur more often in type 1 diabetes patients (Tang et al., 2012, Fichna et al., 2010).

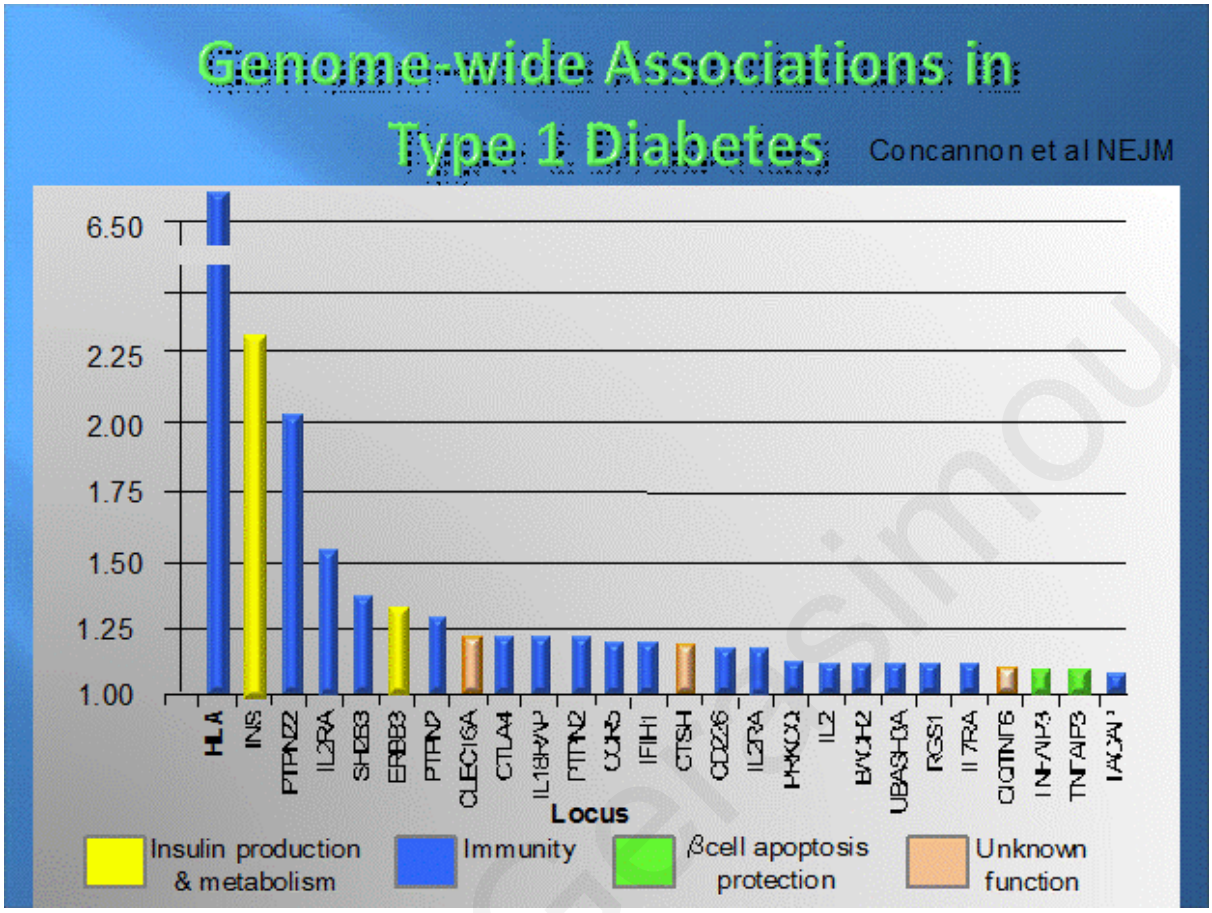

Figure 1: Genome-wide associations in Type 1 Diabetes. Genetic associations in T1DM. Odds ratios for a series of identified "genes/genetic loci" from genome screens and replication studies. In most cases the association is with a locus and not proven for the genes indicated (Concannon et al., 2005).

1.6 VIRAL MIMICRY AND PATHOPHYSIOLOGY OF T1DM

In immunology, molecular mimicry is the mechanism that may explain some forms of autoimmune disease, adequate to initiate activation of autoreactive T or B cells leading in tissue pathology (Kohm et al., 2003). The immune system attacks self-antigens that are structurally similar to non-self-antigens from bacteria or viruses. There is growing evidence for the role of viruses in the pathogenesis of T1DM through observations that indicate seasonal variations in diabetes onset correlated with the seasonality of enterovirus infections (Figure 2). Clinical studies on diabetes patients revealed increased antibody levels to Coxsackie virus B4 and explored the viral RNA association and immune responses to the enterovirus in relation to beta cell function and autoimmunity. Some studies have linked enterovirus infections in mothers with increased T1DM risk in their offspring (Viskari et al., 2012). Data suggest that the virus

can infect the thymus and alter thymic selection process; therefore, early life infections may lead to central tolerance impairment (Jaidane et al., 2012).

Pancreas was also tested for the presence of viral RNA and viral antigens showing a 10-fold increase of viral antigens in patients with T1DM compared to control groups (Richardson et al., 2009). Beta cells can be infected and damaged by enteroviruses that induce HLA class I antigen expression and alpha-interferon, provoking inflammation and triggering islet autoimmunity (Foulis et al., 1987, Dotta et al., 2007, Chapman and Kim, 2008). Furthermore, enterovirus can induce functional changes and influence beta-cell replication (Willcox et al., 2011, P., 2011).

In addition, the identification of IFIH1 susceptibility locus further supports the enterovirus involvement in T1DM (Chistiakov, 2010). The IFIH1 gene encodes the helicase C domain protein 1 induced interferon, also known as melanoma differentiation-associated protein 5 (MDA5). This helicase is responsible for cytoplasmic recognition of double-stranded RNA (dsRNA) generated during the replication of the virus in the host cells. Upon recognition, IFIH1 signals promote interferon and NFκB responses, followed by inflammatory cytokines production. In pancreatic beta-cells IFIH1 predisposing alleles might lead to up-regulation of HLA-class I expression, increasing the potential to present self-antigens and trigger autoimmunity (Chistiakov, 2010).

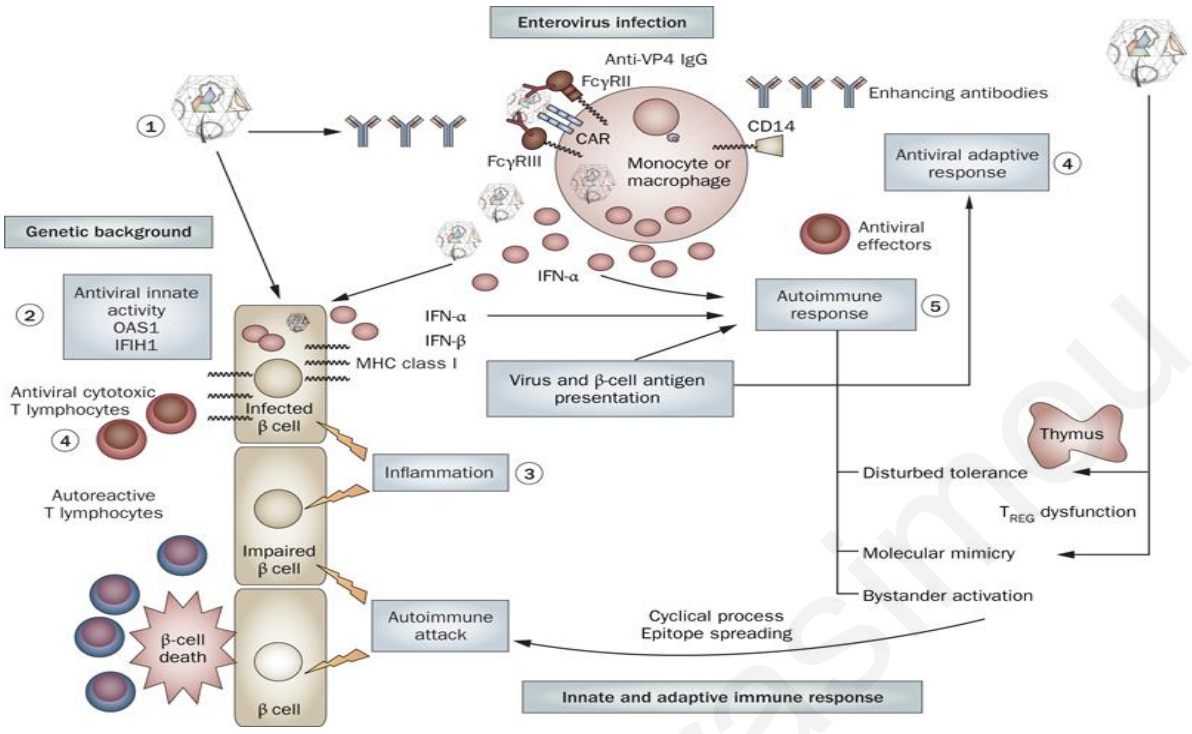

Figure 2: Pathophysiology of T1DM through a cascade of events following infection. Pathophysiology of T1DM through a cascade of events following infection. (1) Antibody dependent enhancement of infection of monocytes and macrophages could increase the spreading of viruses to pancreatic β cells and consequently the production of IFN-α able to stimulate autoimmunity. β cells can be persistently or repeatedly infected. (2) Genetic background influences the innate response to viruses. Variants of the OAS1 gene increase the risk of T1DM and rare alleles of IFIH1 polymorphisms protect from the disease. (3) Enterovirus infections activate the production of IFN-α and IFN-β and trigger apoptosis, and induce the expression of MHC class 1 antigens, and the production of chemokines, which attracts T cells producing pro-inflammatory cytokines. (4) Successive enteroviral infection consecutively and cumulatively trigger the production of adaptive immune response effectors: facilitating antibodies and antiviral cytotoxic T lymphocytes to disrupt β cells, consecutively contributing to β-cell antigen release. (5) Following enteroviral infections, the inflammation process together with the presentation of β-cell self-antigen stimulate the adaptive immune response. (Hober and Sauter, 2010)

T1DM is an organ-specific immune disease that attacks the insulin producing pancreatic cells. The pancreas is a glandular organ of the endocrine and digestive system. Endocrine function involves production and secretion of a series of hormones including insulin, glucagon, somatostatin and pancreatic polypeptide that maintain body homeostasis. As a digestive organ with exocrine function, it secretes pancreatic juice containing digestive enzymes that break down the proteins, carbohydrates and lipids into chyme. Staining of the pancreas expose two types of parenchymal tissue: the lightly stained clusters named islets of Langerhans and the darker stained acinar cells. The acinar cells secrete the digestive enzymes of the exocrine pancreas into the gut via connection to ducts (Fox, 2010).

The islets of Langerhans are the main component of the endocrine pancreas. Within these clusters of cells, four different populations are distinguished that can be classified

according to their hormone secretion ability. The α cells make up 33-46% of the islet cells and are responsible for elevating the blood glucose levels via the secretion of glucagon. When secreted, glucagon binds on hepatocyte receptors activating glycogen phosphorylase enzyme inside the cell to hydrolyse glycogen to glucose through a process known as glycogenolysis. High blood glucose levels stimulate the release of insulin by β cells that make up 65-80% of the islets. Insulin allows glucose to be taken up by insulin-depended organs and tissues, such as liver, skeletal muscles and fat tissue by inhibiting the release of glucagon. Therefore, glucagon and insulin are the key players in the maintenance of blood glucose homeostasis. The δ cells release the hormone somatostatin, which is a peptide hormone that regulates the endocrine system and influences neurotransmission and cell proliferation through binding with the G protein-coupled somatostatin receptors. Somatostatin also inhibits pancreatic exocrine secretion. Lastly, the PP cells of the islets make up around 3-5% of the cell load and function to self-regulate pancreatic secretion activities. The islets are arranged in clusters and are surrounded by a dense network of capillaries. A series of layers of endocrine cells line the inner part of the capillaries that come in direct contact with blood vessels (Brissova et al., 2005, Elayat et al., 1995).

As aforementioned, loss of self-tolerance is the initiation point in T1DM pathogenesis. Environmental triggers, such as viral infections, can act upon genetic predisposition to the disease leading in the destruction of pancreatic beta cells. In healthy islets of Langerhans reside specialized antigen presenting cells and lymphocytes (Yin et al., 2012, Willcox et al., 2009) that are capable of recognizing beta-cell autoantigens, such as proinsulin, GAD65 and IA-2 (Arif et al., 2004). Nevertheless, it is possible for these to be present in healthy individuals as well. What discriminates between healthy and new-onset patients are the pro-inflammatory responses in disease. CD4 T cells produce and secrete interferon-γ (INF) and interleukin-17 (IL-17) when interacting with beta-cell in T1DM patients, whereas in healthy individuals the cells secrete IL-10. Islet specific T cells are effective regulators of tissue-specific pro-inflammatory immune responses and act via a suppressive mechanism that includes lysis of the antigen presenting cells (Tree et al., 2010, Boissonnas et al., 2010). Therefore, in disease onset, IL-10 secreting T cells may be deficient in number and function where a regulation of initiating a pro-inflammatory response is required. These islet-specific cells that are secrete IL-10 are identical to another cohort of T cell, the Tregs.

Tregs are characterised by the intracellular expression of FoxP3 and its surface expression of high levels of CD25, a component of IL-2 receptor. These cells also secrete anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF)-β. As discussed above, polymorphisms in the IL-2 pathway genes portray higher risk for T1DM, possibly via affecting critical Treg pathways and function (Garg et al., 2012, Lawson et al., 2008, Lindley et al., 2005). Therefore, defects in IL-10 secreting, islet-specific CD4 T cells and Tregs may themselves be subtle, but when combined they would be able to generate beta cell destructive inflammatory responses. The above processes signify the initiation of beta-cell autoimmunity. Disease progression is strongly characterized by beta cell recognition by effector T and B cells.

Autoreactive B and T lymphocytes reside in secondary lymphoid organs and bone marrow where they produce islet cell specific autoantibodies leading to disease phenotype (Tiberti et al., 2011). These autoantibodies are used in disease prediction and classification as they survive for many years. B lymphocyte infiltration into the islets is shown to increase as insulitis becomes more aggressive (Pescovitz et al., 2009, Willcox et al., 2009). In clinical trials, the use of B-lymphocyte-depleting monoclonal antibody demonstrated significant ability to preserve C-peptide in T1DM patients (Yu et al., 2011), suggesting that depletion is beneficial as it impairs interactions between B and T cells needed for maintaining T cell responses. $CD4^+$ T cells that recognise beta-cell autoantigens are also a characteristic of T1DM (Mannering et al., 2010, Abreu et al., 2012, Huurman et al., 2012). In disease onset, autoreactive $CD4^+$ T cells are present in circulation (Peakman et al., 1999, Arif et al., 2004). These cells secrete cytokines, such as IFN-γ and IL-17, and are recognised into Th1 and Th17 effector T cells present in insulitic lesions in diagnosis (Arif et al., 2011, Planas et al., 2010). The chemokines and cytokines released via these cells play a critical role in beta-cell destruction. IFN-γ in particular contributes to rendering beta-cells more susceptible to damage by hyper-expressing HLA molecules on cell surface (Coppieters et al., 2012). This feature of insulitis is likely to prepare cells as targets for cytotoxic CD8 T cells. Furthermore, IFN-γ and TNF-α released by cytotoxic T cells and macrophages make beta-cells more susceptible to direct killing by other cytokines with IL-17 being an enhancer of this process (Arif et al., 2011, Honkanen et al., 2010).

The most prevalent mechanism for beta-cell destruction is the cytotoxic CD8 T lymphocytes (CTL). Normally, CTL function in virus-infected cells eradication. This immunological pathway relies on a sequence of events which activate APCs that engulfed viral particles display these antigens on their surface through complexes with HLA molecules. Virus-

specific $CD4^+$ T cells assist naive $CD8^+$ T cells to acquire a T-cell receptor that recognises the viral peptide-HLA complex. These CTLs then enter circulation, reach the infection site and destroy cells displaying the same peptide-HLA complex. The killing mechanisms include release of cytokines, surface proteins and release of lytic apoptotic granule content (Coppieters et al., 2012).

In T1DM pathophysiology, a heterogeneous in terms of antigens and epitopes CTL population infiltrates the islets (Coppieters et al., 2012, Velthuis et al., 2010). The major antigens presented to CTLs through HLA molecules on beta-cell surface are peptides of preproinsulin (Kronenberg et al., 2012, Skowera et al., 2008). In addition, antigen presentation is glucose sensitive, and therefore as dys-glycaemia develops, beta-cell destruction is accelerated. The pre-proinsulin peptide is not typically secreted outside the beta-cell, hence for CTLs to be sensitised against it, the whole beta-cell must be engulfed by the APCs. Antigen presenting dentritic cells have shown in vitro to engulf entire beta-cells in NOD mice (Skowera et al., 2008, Tang et al., 2006).

Nonetheless, the presence of autoreactive CTLs that recognise self-antigens, such as pre-proinsulin, signify that they are available in the naive T cell repertoire and have survived thymic selection processes. A theory exists that even though the INS gene is transcribed in the thymus in thymic epithelial cells, there is a reduced presentation via the HLA, leading to thymic escape. This is shown through the AIRE knockout mouse model that provides a model for the thymic environment, influencing autoantigen presentation (Guerder et al., 2012, Derbinski and Kyewski, 2010).

Following CTL infiltration of the islets, there are some cytotoxic mechanisms of beta-cell destruction. CTLs mediate a cytolytic reaction towards target cell that relies on cell-contact-dependent mechanisms. The two distinct destructive pathways are the cytotoxic de-granulation and TNF-family-related death receptors interaction. Perforin is released during the cytotoxic de-granulation process that aids in the entry and intra-target cell activity of co-released granzymes (Pipkin and Lieberman, 2007). Due to their serine protease activity, granzymes induce a rapid cell death when introduced into the target cell cytoplasm through independent mechanisms or by activating the caspase cascade (Lieberman, 2003, Coppieters et al., 2011). When employing the TNF-family-related death receptors, such as Fas ligand (FasL), that bind Fas expressed on the target cell surface, a series of intracellular pathways are initiated that again function via caspases leading in cell apoptosis (Itoh et al., 1997). The prevalence of these two

pathways is portrayed in both animal and human studies (Thomas et al., 2010). Therefore, the destructive mechanism requires cytotoxic granule component to be released onto the beta-cell during the cognate interaction that is dependent upon CTL recognition of the beta cell-specific target (Knight et al., 2013).

It was only very recently recognised by Delong et. al, 2016 that non-obese diabetic mice-CD4 T cell clones associated with diabetes, recognise epitopes formed by covalent cross-linking of pro-insulin peptides to other peptides present in beta-cell secretory granules. Therefore, these hybrid insulin peptides (HIPs) are a type of neo-antigens recognised by autoreactive T cells, and may elucidate how immune tolerance is lost in T1DM (Delong et al., 2016).

1.7 OBJECTIVES

The incidence rate of type 1 diabetes peaks at the ages of 2, 4-6 and 10-14 years due to alterations in the infections pattern or the increases in insulin resistance (Rewers et al., 2004). The age-distribution of type 1 diabetes onset is similar across geographic areas and ethnic group (Rewers et al., 1989). For these reasons, we decided to distribute patients in the age of onset groups of 1-6, 7-14 and >14 years old.

The cut off age of onset for T1DM for this study was set at 39, with mean age being 11. People over 40 years old were considered as Latent Autoimmune Diabetes in Adults (LADA) and were not included in the final cohort. In many autoimmune studies, a female predominance is observed suggesting that girls have a higher risk of developing an autoimmune disease than boys. According to Soltesz et al. (2007), male and female under the age 15 have relative equal rates of T1DM, while for Europeans aged 15-40, there is a clear male predominance (Soltesz et al., 2007). The incidence rate of T1DM worldwide showed no difference between genders, but it is rising in children under the age of 15 (2006). In Cyprus, the overall mean annual incidence from 1990 to 2009 is 12.46 per 100,000 (Skordis et al., 2012). A comparison of the incidence between 1990-1999 and 2000-2009 portrayed a rising trend, from 10.80 per 100,000 person-years during the first decade to 14.44 per 100,000 person-years during the second decade. Furthermore, a ratio of 1.05 male to female predominance was observed, agreeing with the male predominance in T1DM patients under 15 years old. This trend was reversed in older ages (Skordis et al., 2012). Our sample agrees with previous publications and is representative to Greek-Cypriot T1DM as previously described by Skordis et.al, 2012.

HLA gene variants in T1DM have been established as primary susceptibility determinants that confer as much as 50-60% of the overall disease risk (Noble and Erlich, 2012). Class II haplotypes, HLA-DR3/DQ2.5 and HLA-DR4/DQ8, carry the strongest association to disease with up to 50% of patient carrying both haplotypes (van Lummel et al., 2012). Nevertheless, disease association MHC haplotypes vary through populations. The disease associated DRB1*04:01 is absent from some populations such as the Jewish (Martinez-Laso et al., 1996), but is in higher frequencies in Northern Europeans (Arnaiz-Villena et al., 1995, Morales et al., 1991). Therefore, the absence of an HLA-DR antigen that is associated with diabetes in the Cypriot population will provide a unique opportunity to dissect the different MHC loci in the pathogenesis of diabetes. Patients were also asked to provide information with regards to their family background since the genetic involvement in T1D is well established (Hyttinen et al., 2003).

In addition, HLA-G has also been recently investigated in regards to its involvement in inflammatory and autoimmune disease. The HLA-G receptor functions towards immune inhibition and tolerance through direct immune-inhibitory functions by effector cells blocking, regulatory cell generation through indirect immune-inhibitory functions and other HLA-G functions that result in immune-inhibition. Based on the above information, it is evident that HLA-G has an important immune-modulatory function both in physiology, i.e. maternal-fetal tolerance, but also in pathophysiology, i.e. inflammatory and autoimmune diseases. In the later context, HLA-G may be functioning to counteract disease-driven immune responses but failing due to defects in inhibition pathway or specific genotype that conveys susceptibility to autoimmunity, in this study's case T1DM.

Hence, this study examines the MHC haplotype associated to disease in the Cypriot population in an attempt to reveal novel association of the disease to other classical and non-classical MHC loci. The study will specifically compare T1DM patients against healthy individuals in order to expose any discrepancies. Therefore, we hypothesize that immunomodulatory elements in the MHC locus including non-classical HLA class I are associated with Type I diabetes either in conveying susceptibility or by contributing to the overall pathogenesis.

In order to be able to investigate the hypothesis, the following objectives have been identified:

Receive research approval from the National Bioethics Committee.

Select a cohort of consented Greek-Cypriots from the bone marrow registry.

Recruit a cohort of consented Greek Cypriot patients with Type I diabetes and collect whole blood genomic material.

Genotype the patient cohort and control group for the following MHC loci; HLA-A, -B, -C, -DRB1, -DQA, -DQB. –DPB1 and -G.

Compare the observed antigen frequency of the HLA loci in the patients with T1DM to the Greek Cypriot bone marrow donors.

Group patients according to their HLA-DRB1 antigens (based on the presence of DR3/DR4) and compare other HLA loci (including HLA-G) to HLA-DRB1 matched individuals.

Investigate the cytokine involvement in autoimmune disease and Th1/Th2 skewness.
Investigate other genetic susceptibility to T1DM loci such as CTLA-4 and PTPN22.

<u>Scientific objectives to be met are as follows:</u>

Report the HLA allele frequencies and haplotypes in the Cypriot diabetic population.
Suggest alternative explanation for the T1DM patients that do not carry the risk associated allele.
Re-evaluate the HLA-class I association to T1DM.
Decipher the already described MHC telomeric region involvement in T1DM by investigating the involvement of non-classical HLA-G.
Present for the first time the HLA-G profile of the Cypriot population.
Investigate what other genetic immunomodulatory factors may be involved in the pathogenesis and progression of T1DM.
Propose a potential mechanism where immune monitoring fails to eliminate autoimmune responses.

CHAPTER 2

MATERIALS AND METHODS

2.1 THE COHORT

In collaboration with Dr. Skordis, the pedoendocrinologist of Pedi Clinic, we collected information for all patients that display T1DM in regards to ethnicity, family background, and

age of onset. For the purpose of this study, a cohort of 170 Greek-Cypriot patients (89 females, 81 males) with juvenile T1DM were recruited. The cut-off age of onset for T1DM was set to 39 years, with the average age being 11 years. People over 40 years were considered as Latent Autoimmune Diabetes in Adults (LADA) and were not included in the final cohort. Information regarding demographics, gender, ethnicity, and age of onset was also collected for each patient. Patient age of onset groups were allocated into three tertiles: 59 individuals into Age group 1 with ages 0 to 8 years, 60 individuals into Age group 2 with ages 9 to 13 years, and 51 individuals into Age group 3 with age 14 to 39 years. A random population control group of 192 (78 females, 114 males) healthy individuals was also recruited from the Cypriot Bone Marrow registry, for comparison purposes. Written informed consent was obtained from all participants and the study was reviewed and approved by the Cyprus National Bioethics Committee. In the case of minors/children, a written informed consent was obtained from parents or legal guardians.

2.1.1 Extraction of Total human genomic DNA

HLA typing methodologies involve the incorporation of DNA that is most commonly obtained through peripheral blood DNA extraction protocols. This study uses the commercially available QIAGEN genomic DNA extraction kit (Cat. no. 51106) that is designed to directly isolate DNA from whole blood. The cohort samples and random population samples were transferred on 96 well blocks and extracted according to the manufacturer's instructions.

2.1.2 Quantification of DNA

The concentration of DNA in solution was determined spectrophotometrically at wavelength 280 nm in a Thermo Scientific NanoDrop^{TM} 2000 Spectrophotometer. For pure DNA the ratio of (OD_{260}/OD_{280}) was between 1.8 and 2.0.

2.2 HLA TYPING

2.2.1 Classical HLA typing

Classical-HLA class I and class II molecules (A, B, C, DRB1, DQB1, DQA1 and DPB) for both diabetic and control cohorts were typed by HistoGenetics. HistoGenetics is the global leader in HLA sequence based typing (SBT). The main sequencing platform employed is Next Generation Sequencing (NGS) technology using Illumina MiSeq. Recently, HistoGenetics has introduced a 3rd generation sequencing technology with Pacific Biosciences RS II for routine HLA testing to resolve ambiguities that are not resolved using the Illumina approach, and also to characterize novel alleles.

2.2.2 HLA-G genotyping

Genotyping of the HLA-G 14bp insertion/deletion polymorphism

The HLA-G 14bp *insertion (INS)/deletion (DEL)* polymorphism *(rs371194629)* was studied using the sequence-specific polymerase chain reaction (SSP-PCR) method. Genomic DNA was extracted from whole blood with the use of the commercially available QIAGEN® genomic DNA extraction kit. All reactions were prepared in 25 μl final volume, in the presence of 1X reaction buffer, 200 mM of each dNTP, 1.5-2.0 mM $MgCl_2$, 5U *Taq* DNA polymerase (QIAGEN), 20 pmole of each primer and ≥25 ng of genomic DNA. Sense and anti-sense primers (5'- GTGATGGGCTGTTTAAAGTGTCACC- 3' and 5'- GGAAGGAATGCAGTTCAGCATGA- 3' respectively) were employed as previously described (Fabris et al., 2011). Reactions were carried out with an initial denaturation 95°C for 5 min, 35 cycles of denaturing at 95°C for 30 sec, annealing at 60°C for 1 min and extension at 72°C for 2 min, followed by a final extension step at 72°C for 10 min. The product sizes observed were 210 bp for the *DEL/DEL* genotype and 224 bp for the *INS/INS* genotype, whereas the heterozygous *INS/DEL* genotype portrayed both.

HLA-G allele, promoter and 3'UTR Genotyping

In order to achieve the complete genotyping of HLA-G, two amplified products of the molecule spanning from the promoter to the '3UTR were produced. All T1D patients and control group where amplified to produce an HLA-G ~3kb product, spanning from +3 to

+3110bp and an HLA-G ~2kb URR product, spanning from -1612 to +244bp. The primers employed were house designed and sequences were as follows.

Table 2: Primers designed for the amplification of the HLA-G gene.

Primer Name	Sequence
HLA-G 2kb Forward Primer	'5-ACATTCTAGAAGCTTCACAAGAATG-'3
HLA-G 2kb Reverse Primer	5'-CACGGAACACCAAGGCCCA-'3
HLA-G 3kb Forward Primer	'5-GGTCGGGCGGGTCTCAA-'3
HLA-G 3kb Reverse Primer	'5-TGGAAGACATGAGAACTTTCCA-'3

All reactions were prepared in 25 μl final volume in the presence of 1X reaction buffer, 200 mM of each dNTP, 1.5-2.0 mM $MgCl_2$, 5U *Taq* DNA polymerase (QIAGEN), 20 pmole of each primer and ≥25 ng of genomic DNA. Sense and anti-sense primers were employed as previously described. Reactions for the HLA-G 3kb product were carried out with an initial denaturation 95°C for 5 min, 40 cycles of denaturing at 95°C for 30 sec, annealing at 60°C for 45sec and extension at 72°C for 1 min, followed by a final extension step at 72°C for 10 min. Reactions for the HLA-G 2kb product were carried out with an initial denaturation 95°C for 5 min, 40 cycles of denaturing at 95°C for 30 sec, annealing at 56°C for 45 sec and extension at 72°C for 2 min, followed by a final extension step at 72°C for 10 min.

Following amplification, the products are prepared for Next Generation Sequencing (NGS) with the Nextera XT® DNA Sample Preparation Index Kit (96 Indices, 384 Samples) catalogue number FC-131-1002. The protocol provided by the manufacturer explains how to prepare up to 96 indexed paired-end libraries from a variety of input DNA for subsequent cluster generation and DNA sequencing using the reagents provided in the Illumina Nextera®XT DNA Sample Preparation Kit. Through this protocol the amplicon is fragmented and adapter sequences are added onto template DNA with a single tube tagmentation reaction to generate multiplexed sequencing libraries. This process is achieved via an engineered transposome that simultaneously fragments and tags ("tagment") input DNA, adding unique adapter sequences in the process followed by a limited-cycle PCR reaction uses these adapter sequences to amplify the insert DNA. The dual indexing strategy uses two 8 base indices, Index 1 (i7) adjacent to the P7 sequence, and Index 2 (i5) adjacent to the P5 sequence. Dual indexing is enabled by adding

a unique Index 1 (i7) and Index 2 (i5) to each sample from 12 different Index 1 (i7) adapters (N701–N712) and 8 different Index 2 (i5) adapters (S501–S508) for the 96 sample Nextera XT Index Kit.

Subsequent to this PCR reaction, a product clean-up step, using AMPure XP beads (cat. no. A63880), is performed to purify the DNA library and remove very short library fragments from the product. A second set of magnetic beads provided by the Nextera XT® DNA Sample Preparation Index Kit, normalizes the quantity of each library to ensure more equal library representation in the pooled sample and in preparation for cluster generation and sequencing, equal volumes of normalized library are combined, diluted in hybridization buffer, and heat denatured prior to MiSeq sequencing. Prior loading pooled library to the MiSeq NGS system, the library's final concentration is measured using the KAPA Library Quantification Kit (cat.no. KK4824) in an RQ-PCR manner according to the manufacturer's instructions.

The HLA-G genotyping was interpreted by the SBTengine® Software (GenDX), whereas the promoter and 3'UTR SNPs of interest as previously described (Castelli et al., 2010, Castelli et al., 2014b) were interpreted using the GeneTalk Analyze human sequence variants platform. The HLA-G results obtained through our analysis were shared with Dr. Castelli's Laboratory for re-analysis and confirmation.

2.2.3 HLA Epitope Analysis

The Major Histocompatibility Complex (MHC) is a highly polymorphic and gene-rich region that requires a more thorough investigation into its composing elements. To achieve such dissection of the HLA region, a Java based application, the SKDM HLA Tool, was utilized to test HLA allele differences between two populations and, by retrieving amino acid sequences, perform amino acid analysis in case-control datasets. This tool was also used to examine zygosity and strongest association among amino acid epitopes of the same HLA molecule or between HLA isotypes (Kanterakis et al., 2008).

All P-values were corrected for multiple testing and were denoted as p-corr.

2.3 CYTOKINE GENOTYPING

The ONE LAMBDA® Cytokine Genotyping Primer Pack (cat. no. PCYTGEN) was utilized to genotype the following cytokine factors: **TNF-alpha** -308 A>G, **IL-10** -592 A>C, -819 C>T and -1082 A>G, **TGF-beta** codon 10 C>T and codon 25 C>G, **IL-6** -174 C>G and **IFN-gamma** +874 A>T. The method was based on sequencing specific primers (SSP)-PCR where perfectly matching oligonucleotide primers are used in amplifying the target allele or group of alleles of interest, while mismatched primer pairs do not yield an amplified result. Following PCR amplification according to the manufacturer's instructions, the amplified product is separated by agarose gel electrophoresis and visualised under ultraviolet light following ethidium bromide staining.

The final result interpretation was based on the presence or absence of a specific amplified DNA fragment while an internal control primer pair (Human β-globin gene) was included in every PCR reaction to assure that the amplification was successful. The specific cytokine products that were successfully amplified and therefore positive were smaller than the internal control product,. hence they could be easily distinguished on the gel.

2.4 PTPN22 AND CTLA4 GENOTYPING

Two genes that were associated through publication with T1DM are the protein tyrosine phosphatase nonreceptor 22 gene (PTPN22) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4). For the course of this study, these two genes were investigated for the PTPN22 +1858 C>T SNP and CTLA-4 +49 A>G SNP using the restriction fragment length polymorphism (RFLP) technique. The RFLP method was based on the principle that homologous DNA sequences have differences (SNPs) that can be detected and digested with specific restriction endonucleases. The resulting DNA fragments were then separated by length through agarose gel electrophoresis.

All reactions were prepared in 25 μl final volume in the presence of 1X reaction buffer, 200 mM of each dNTP, 1.5-2.0 mM $MgCl_2$, 5U *Taq* DNA polymerase (QIAGEN), 20 pmole of each primer and ≥25 ng of genomic DNA. The PTPN22 sense and anti-sense primers (5'-ACTGATAATGTTGCTTCAACGG- 3' and 5'- TCACCAGCTTCCTCAACCAC - 3', respectively), were employed as previously described (Zhebrun et al., 2011). Reactions were carried out with an initial denaturation 94°C for 5 min, 30 cycles of denaturing at 94°C for 30 sec, annealing at 62°C for 1 min and extension at 72°C for 1min, followed by a final extension step at 72°C for 5 min. The products were then restriction digested by *RsaI* enzyme. The

CTLA-4 sense and anti-sense primers (5'- CAAGGCTCAGCTGAACCTGGGT - 3' and 5'- TACCTTTAACTTCTGGCTTTG - 3', respectively), were employed as previously described (Imen Sfar et al., 2010). Reactions were carried out with an initial denaturation 94°C for 5 min, 30 cycles of denaturing at 94°C for 30 sec, annealing at 62°C for 1 min and extension at 72°C for 1min, followed by a final extension step at 72°C for 5 min. The products were then restriction digested by *Kpnl* enzyme.

2.5 HLA-G+ TREG INDUCTION

The induction of HLA-G^+ Treg using hypomethylating agent Azacitidine (Aza-C) protocol was first described by Dr. Spyridonidis Laboratory at Petra University (Vittoraki et al., 2013). After personal communication with Dr. Spyridonidis, the improved protocol was shared for the course of this study and is described below.

2.5.1 Subjects and sample collection

Peripheral blood sample was obtained from healthy individuals. Informed consent was obtained from all volunteers prior to sample collection in accordance with the Cyprus Bioethics Committee. Peripheral blood mononuclear cell (PBMC) isolation was carried out with the use of a separation medium (HISTOPAQUE-1077, Sigma Cat. No.10771). After reaching room temperature, 3mL of the separation medium was poured into a Leucosep® tube and centrifuged for 30 seconds at 1000g in order for the medium to set below the porous barrier. 3-9mL of peripheral blood was then poured directly into the tube and centrifuged 10 minutes at 1000g with brakes off. After centrifugation, the sequence of layers occurs as seen in the picture below.

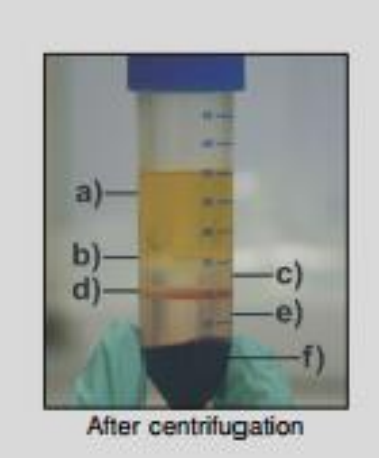

Figure 3: PBMCs isolation. After centrifugation of sample in the Leucosep® tube the following layers are observed; a) Plasma, b) PBMC's, c) Separation medium, d) Porous barrier, e) Separation medium and f) Pellet (eruthrocytes and granulocytes) (Picture from Leucosep® instructions manual)

The plasma layer fraction is discarded and the PBMC's layer is harvested into a 50mL Falcon tube where it is washed twice with 10mL of phosphate-buffered saline for 5 minutes at 1500rpm. Cell pellet is then re-suspended in 5mL RPMI.

2.5.2 Cell culture

All manipulations were performed in aseptic conditions under a laminar air flow hood workplace. Cultures were maintained in a humidified incubator at 37°C and 5% CO2. Cells were counted using a haemocytometer (Neubauer).

At least $1x10^6$ PBMCs were activated for 3 days in the presence of anti-CD3/CD28 beads (bead:cell 1:1; Dynabeads®, Invitrogen) in RPMI 1640 (GIBCO, Invitrogen, Paisley, UK) medium supplemented with L-glutamine (4mM), 10% FBS and 100U/ml penicillin–streptomycin. Cell proliferation and activation was assessed by labelling with fluorescein diacetate succinimidyl ester (CFSC Cell Proliferation Kit) at a final concentration of 6μM and flow cytometry. The activated T cells were then be incubated in the presence of human recombinant IL-2 (50U/mL, Peprotech) and 5-aza-2′-deoxycytidine, Aza-C (Sigma) (2μM) or 50U/mL rhIL-2 PBS as control for an additional 72 hours.

2.5.3 Antibodies and flow cytometry

Flow cytometry is a technology that simultaneously measures and then analyzes multiple physical characteristics of single cells, as they flow in a fluid stream through a beam of light of a single wavelength. A number of detectors are aimed at the point where the stream passes through the light beam: one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter or SSC), and one or more fluorescent detectors. FSC correlates with the cell volume and SSC depends on the inner complexity of the particle. Therefore, the properties measured include a particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity. These characteristics are determined using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence.

For this study, the following monoclonal antibodies from AbD Serotec, BioLegend, exBio, eBiosciences and BD Biosciences, conjugated with FITC, PE, PE Cy5, PerCP Cy5.5, APC or Alexa 647 were used in standard staining conditions for flow cytometric analysis on FACS Verse^{TM} cytometer: anti-human HLA-G, CD4, CD8, CD3, CD25, CD127 and FoxP3. Isotype control antibodies from BD Biosciences will be used to subtract non-specific signals. This methodology was employed to assess the differences in number between the naturally occurring HLA-G positive cells and the induced HLA-G positive Tregs.

2.6 HLA-G EXPRESSION STUDY

2.6.1 RNA extraction and cDNA synthesis

QIAamp RNA Blood Mini Kit was used in the preparation of total cellular RNA from up to 1.5 ml of human whole blood (cat. no. 52304). Patient whole blood was erythrocytes lysed and washed with PBS. Remaining leukocytes were re-suspended in RLT/BME for RNA extraction and cDNA synthesis. The kit includes the QIAamp spin columns that have selective binding properties of a silica–based membrane. A specialized high-salt buffering system allows RNA species longer than 200 bases to bind to the QIAamp membrane. Contaminants are washed away and total RNA is eluted in RNase-free water for direct use in any downstream application. RNA concentration was assessed by Nanodrop2000 and maximum 100ng/μl was used for cDNA synthesis.

For the cDNA synthesis from the extracted RNA, we employed the Invitrogen SuperScript™ II Reverse Transcriptase (RT) (Cat. No. 18064-022) that is used to synthesize first-strand cDNA. cDNA was synthesised according to the manufacturer's instruction.

2.6.2 Taqman Assay for HLA-G expression study

This Applied Biosystem TaqMan® Gene Expression Assay was used to assess the HLA-G expression in patient samples from all three age of onset groups. Gene Expression Assays consist of a pair of unlabeled PCR primers and a TaqMan® probe with a FAM™ dye label on the 5' end, and minor groove binder (MGB) non-fluorescent quencher (NFQ) on the 3' end. 5µl 2x TaqMan® Gene expression master mix was used with 0.5µl HLA-G primer probe mix (Hs00365950_g1 catalogue number 4331182) and 2µl cDNA in a total reaction of 10µl. The conserved gene ABL was also used as a normal gene marker to be used in delta Ct normalization of samples. For each patient, a duplicate reaction for HLA-G and ABL was run on the BIORAD CFX-96 RQ-PCR.

A summary of Ct results, Delta Ct (ΔCt) and Delta delta Ct (ΔΔCt) is found in Appendix I. The fold change between the three age groups was also calculated.

2.7 STATISTICAL ANALYSIS

In order to interpret the results acquired with the above methodologies, the IBM SPSS Statistical software package was utilized to calculate statistical significance. Cross tabulations of category (T1D Vs CTLs) and by age of onset group were calculated and P-values were obtained by Fisher's Excact Test.

Through the course of this study, we suggest that since Type 1 diabetes is not a monofactorial disease but rather the combination of parameters and events that lead to pathogenesis, we propose that calculated P-values and Odd Ratio (OR) through the traditional analytical methods, i.e the Fisher's Excact Test, carry a degree of bias as it is asssumed that the one factor being questioned at a time is the solemn contributor to the disease. Since factors that affect the pathogenesis and course of the disease come into clusters that interplay with each other, we have also tested our dataset with a **Logit analysis.** This type of analysis calculates

the significance of each parameter in concordance with the other parameters in question. The Logit function is the inverse of the sigmoid "logistic" function used in statistics. When the function's parameter represents a probability *p*, the Logit function gives the log-odds, or the logarithm of the odds. All P-values were corrected for multiple testing and were denoted as p-corr.

p/(1 − p). **The logit of a number *p* between 0 and 1 is given by the formula:**

$$\operatorname{logit}(p) = \log\left(\frac{p}{1-p}\right) = \log(p) - \log(1-p) = -\log\left(\frac{1}{p} - 1\right).$$

Dr. Castelli's laboratory that re-analysed our HLA-G raw data in order to confirm the associations between the HLA-G promoter haplotype, genotype and '3-UTR haplotypesutalised GWASpi tool to perform Genome-Wide Association Studies. This tool is written in Java and utilizes command line analysis.

CHAPTER 3

RESULTS

3.1 T1DM AND CONTROL COHORTS

For the course of this study, information was collected for patients that display T1DM, in the content of ethnicity, as this is a population study and, therefore, it is very important that the cohort includes only Greek Cypriots. This information further aided in the purity of our cohort since only one individual from each family was included in the final cohort in order to minimize bias effects of similar results due to inheritance. Finally, the age of onset was either stated by each patient or calculated through the date of birth and date of diagnosis. This was to further assist our result stratification according to age of onset and draw novel conclusions as to the involvement of HLA-G and other genetic factors on the disease age of appearance.

A cohort of 182 (98 females, 84 males) patients with T1DM were recruited for the purpose of this study. The age of onset was recorded only for 170 patients. Patient age of onset groups were allocated into three tertiles: 59 individuals into Age group 1 with ages 0 to 8 years, 60 individuals into Age group 2 with ages 9 to 13 years and 51 individuals into Age group 3 with age 14 to 39 years. 192 healthy individuals (78 females, 114 males) of Greek-Cypriot origin were enrolled in this study. Written informed consent was obtained from all participants and the study was reviewed and approved by the Cyprus National Bioethics Committee. In the case of minors, a written informed consent was obtained from parents or legal guardians.

3.2 HLA TYPING

3.2.1 The European Ancestral Haplotype

HLA A*0101 -Cw*0701 - B*0801 -DRB1*0301 -DQA1*0501 - DQB1*0201 (**HLA A1-B8-DR3-DQ2 haplotype)** is the most common haplotype at 5-12% in European Caucasians (Table 3) (Gragert et al., 2013). This haplotype is a multi-gene haplotype that covers the majority of the HLA locus as the result of common ancestry. Therefore, this haplotype is also referred as the European ancestral haplotype.

Table 3: Frequency of the most common European haplotype in different European populations. The most common haplotype at frequency 5-12% in European Caucasians is HLA A1-B8-DR3-DQ2 haplotype (Gragert et al., 2013). The table lists the frequency of this haplotype among different European populations.

HLA-A	HLA-B	HLA-DR	Country	Frequency	Ranking
1	8	3	Ireland	0.118113	1
1	8	3	UK	0.090778	1
1	8	3	Denmark	0.082071	1
1	8	3	Netherlands	0.081384	1
1	8	3	Norway	0.074874	1
1	8	3	Australia	0.072456	1
1	8	3	Hungary	0.060234	1
1	8	3	Poland	0.0596	1
1	8	3	Serbia	0.058396	1
1	8	3	Czechia	0.055996	1
1	8	3	Sweden	0.055636	1
1	8	3	Germany	0.053718	1
1	8	3	Slovenia	0.052959	1
1	8	3	Croatia	0.051613	1
1	8	3	Belgium	0.051003	1
1	8	3	Slovakia	0.050142	1

Table 4: The most common European haplotype in the Cypriot population. The most common haplotype at frequency 5-12% in European Caucasians is HLA A1-B8-DR3-DQ2 haplotype, which ranks 87th in the Cypriot population with frequency of 0.2%.

# ID	A	B	C	DRB1	DQB1	FREQ_1
15	24:02:00	08:01	07:02	03:01	02:01	0.0067
21	03:01	35:08:00	04:01	03:01	02:01	0.0052
30	02:01	41:01:00	07:01	03:01	02:01	0.0046
64	03:01	08:01	07:01	03:01	02:01	0.0029
86	24:02:00	35:02:00	04:01	03:01	02:01	0.0023
87	**01:01**	**08:01**	**07:01**	**03:01**	**02:01**	**0.0023**
92	02:01	41:01:00	17:01	03:01	02:01	0.0023

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Figure 4: The genetic Heterogeneity of the Cypriot Population. The cumulative incidence of the 50 most common HLA haplotypes among Cypriot, Finnish, European American, German and Czech populations taken from the frequencies in the respective registries. The cumulative incidence of the 50 most common HLA haplotypes accounts for only around 30% of the Cypriot population in contrast to much higher percentages for the other populations presented here suggesting that as compared to these populations Cypriots are highly heterogeneous genetically.

In the Cypriot population though, the ancestral haplotype ranks 87th, with a frequency of 0.2%. (Table 4).

The Cypriot population MHC is highly polymorphic. As seen in Figure 4, the cumulative incidence rate of the 50 first HLA haplotypes when added give the percentage of population that is covered. The first 50 HLA Cypriot haplotypes barely cover 30% of the Cypriot population. Therefore, the combination of HLA alleles are very heterogeneous in our population that can either aid in the confirmation of the already proposed T1DM susceptibility alleles, or identify other MHC susceptibility loci.

3.2.2 The HLA typing and Amino Acid analysis of the T1DM cohort and the Control Population by locus

Genotypic combinations that phenotypically portray greater risk for diabetes include DRB1*03:01-DQA1*05:01-DQB1*02:01, DRB1*04:xx-DQA1*03:01-DQB1*03:02 (DR3/DR4) (Erlich et al., 2008). Therefore, the T1DM cohort and the control population were HLA typed for the following loci: HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DQA1, HLA-DQB1 and HLA-DPB1. The T1DM cohort and the control population were also tested for the Shared Epitope Hypothesis. Epitope amino acid analysis was performed to unmask possible associations with other HLA alleles that are not included in the risk haplotype but may share similar risk conferring epitope amino acids in their binding domain. The SKDM software was utilized to analyse each HLA locus amino acid composition. The amino acids in question were divided into pocket and non-pocket amino acids. HLA-A, -B and –C pocket amino acids include the following: Pocket A: 63, 66, 99, 163, 167, 171; Pocket B: 9, 63, 66, 67, 70, 99; Pocket C: 9, 70, 74, 97; Pocket D: 99, 114, 155, 156; Pocket E: 97, 114, 152, 156; and Pocket F: 77, 80, 116. The HLA-DRB1 and DQB included the following pocket amino acids: Pocket 4: 13, 71, 78, 70, 74, 26; Pocket 6: 9, 11, 30; Pocket 7: 28, 61, 71, 47, 67; and Pocket 9: 9, 60, 57, 37, 38. HLA-DQA1 pocket amino acids included Pocket 1: 34, 46, 56, 35, 55, 57, 27, Pocket 4: 11, 65,

14, Pocket 6: 14, 68, 66, 69, Pocket 7: 68, 72, and Pocket 9: 75, 76, 72, 79. Lastly, the HLA-DPB1 pocket amino acid tested were Pocket 1: 87, 84, Pocket 4: 13, 69, 76, 68, 72, 24, Pocket 6: 9, 11, 28, Pocket 7: 26, 59, 69, 45, 65, and Pocket 9: 9, 58, 55, 35, 36.

HLA epitopes associated with susceptibility and resistance show gene-dose effect. Another SKDM tool output seen above is an assessment of zygosity, where for each previously identified AA, it is indicated whether a homozygote or heterozygote condition differentiates susceptibility to disease. Three tests are performed here and the p-value correction is 3. A synopsis of the most significant correlations ($p<0.05$) between homozygote or a heterozygote condition and susceptibility or resistance is depicted in the following tables.

HLA-A locus

Table 5: Allele frequency analysis for HLA-A, in the Cypriot population. The HLA-A alleles of the two populations. The table includes presence in the population and frequency, allele number and frequency, delta difference between the T1D and CTL population frequencies, a corrected P-value and the Odds Ratio (OR).

HLA-A locus											
Allele	Pop (T1D)	Freq (T1D)	Pop (CTL)	Freq (CTL)	Allele (T1D)	Freq (T1D)	Allele (CTL)	Freq (CTL)	Delta	p^corr	OR
24:02	71	41.76%	52	27.08%	76	22.35%	54	14.06%	14.68%	0.1492	1.92
25:01	14	8.24%	3	1.56%	14	4.12%	3	0.78%	6.68%	0.1805	5.02
30:02	7	4.12%	1	0.52%	7	2.06%	1	0.26%	3.60%	1	5.86
01:03	6	3.53%	1	0.52%	6	1.76%	1	0.26%	3.01%	1	5.04
01:01	29	17.06%	27	14.06%	30	8.82%	28	7.29%	3.00%	1	1.25
02:05	8	4.71%	4	2.08%	8	2.35%	4	1.04%	2.63%	1	2.19
30:01	12	7.06%	9	4.69%	12	3.53%	9	2.34%	2.37%	1	1.52
23:01	8	4.71%	5	2.60%	9	2.65%	5	1.30%	2.11%	1	1.78
29:02	8	4.71%	6	3.13%	8	2.35%	6	1.56%	1.58%	1	1.5
02:02	4	2.35%	2	1.04%	4	1.18%	2	0.52%	1.31%	1	2.06
03:01	25	14.71%	26	13.54%	25	7.35%	27	7.03%	1.17%	1	1.1
68:02	4	2.35%	3	1.56%	4	1.18%	3	0.78%	0.79%	1	1.46
29:10	2	1.18%	2	1.04%	2	0.59%	2	0.52%	0.14%	1	1.13
66:01	1	0.59%	0	0.00%	1	0.29%	0	0.00%	0.59%	1	3.41
80:01	1	0.59%	0	0.00%	1	0.29%	0	0.00%	0.59%	1	3.41
30:04	4	2.35%	4	2.08%	4	1.18%	4	1.04%	0.27%	1	1.13
02:17	2	1.18%	2	1.04%	2	0.59%	2	0.52%	0.14%	1	1.13
29:01	10	5.29%	10	5.21%	9	2.65%	10	2.60%	0.08%	1	1.02
02:06	0	0.00%	1	0.52%	0	0.00%	1	0.26%	-0.52%	1	0.37
11:xx	0	0.00%	1	0.52%	0	0.00%	1	0.26%	-0.52%	1	0.37
24:26	0	0.00%	1	0.52%	0	0.00%	1	0.26%	-0.52%	1	0.37
24:xx	0	0.00%	1	0.52%	0	0.00%	1	0.26%	-0.52%	1	0.37
29:12	0	0.00%	1	0.52%	0	0.00%	1	0.26%	-0.52%	1	0.37
29:62	0	0.00%	1	0.52%	0	0.00%	1	0.26%	-0.52%	1	0.37
30:02	0	0.00%	1	0.52%	0	0.00%	1	0.26%	-0.52%	1	0.37
32:02	0	0.00%	1	0.52%	0	0.00%	1	0.26%	-0.52%	1	0.37
74:03	0	0.00%	1	0.52%	0	0.00%	1	0.26%	-0.52%	1	0.37
69:01	1	0.59%	4	2.08%	1	0.29%	4	1.04%	-1.49%	1	0.37
02:93	0	0.00%	3	1.56%	0	0.00%	3	0.78%	-1.56%	1	0.16
03:02	2	1.18%	6	3.13%	2	0.59%	6	1.56%	-1.95%	1	0.43
31:01	1	0.59%	5	2.60%	1	0.29%	5	1.30%	-2.01%	1	0.3
33:03	0	0.00%	5	2.60%	0	0.00%	5	1.30%	-2.60%	1	0.1
24:03	1	0.59%	8	4.17%	1	0.29%	8	2.08%	-3.58%	1	0.19
68:01	6	3.53%	14	7.29%	6	1.76%	14	3.65%	-3.76%	1	0.49
02:01	51	30.00%	65	33.85%	54	15.88%	72	18.75%	-3.85%	1	0.84
11:01	18	10.59%	28	14.58%	18	5.29%	29	7.55%	-3.99%	1	0.7
33:01	8	4.71%	17	8.85%	8	2.35%	17	4.43%	-4.14%	1	0.52
32:01	17	10.00%	30	15.63%	17	5.00%	32	8.33%	-5.63%	1	0.61
26:01	9	5.29%	21	10.94%	10	2.94%	22	5.73%	-5.65%	1	0.47

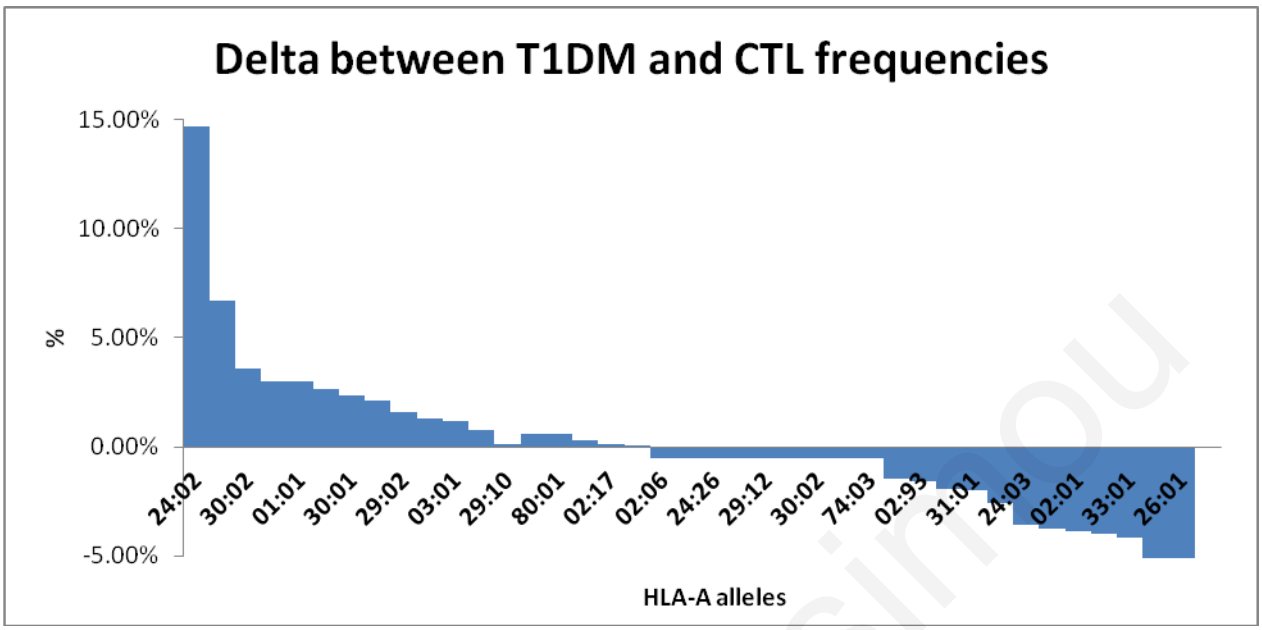


Figure 5: The difference (delta) between T1DM and CTL population frequencies for the HLA-A alleles. Each HLA-A allele is plotted against its delta difference between the two populations. Positive % denotes higher presence in the T1DM group, while negative % shows higher presence in the control group.

The HLA-A allele profile of T1DM cohort and random control population is depicted in Table 5 above. The HLA-A*24:02 and HLA-A*25:01 is prevalent in the diabetic population with a frequency difference between the two populations of 14.68% and 6.68% respectively (Figure 5). On the contrary, HLA-A*32:01 and HLA-A*11:01 were mostly associated with the control population. Nevertheless, regardless of the huge difference in allele frequency between the two groups, statistical analysis failed to reveal significance (pcorr.<0.05).

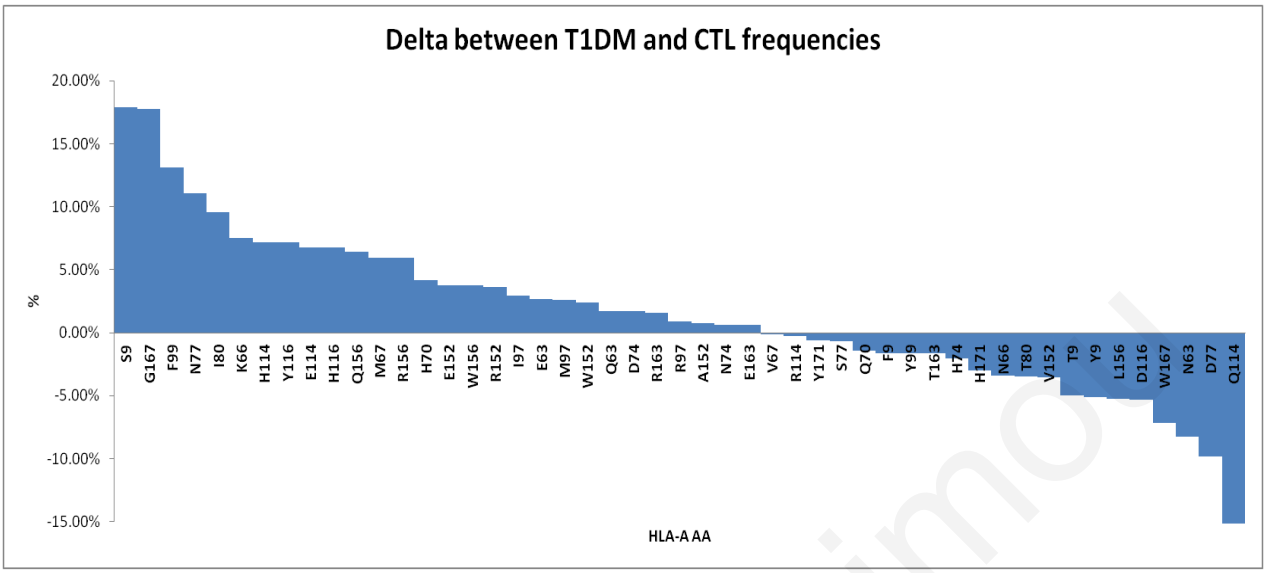


Figure 6: The difference (delta) between T1DM and CTL population frequencies for the HLA-A pocket amino acids. Each HLA-A amino acid is plotted against its delta difference between the two populations. Positive % denotes higher presence in the T1DM group while negative % shows association to the control group.

The HLA-A pocket amino acid profile of T1DM cohort and random control population is depicted above in Table 6 and the non-pocket amino acids are depicted in Table 7. No significant difference between the T1D cohort and the control population was observed for any of the amino acids interrogated. Nevertheless, Figure 6 portrays the amino acids that had great difference between the two groups. The amino acids observed on the left hand side cluster of the figure represent the amino acids associated with T1D, and the ones on the left hand side cluster were mostly recognised in the control population group.

Table 6: The HLA-A pocket Amino Acid typing of the two populations. The HLA-A pocket amino acid typing of the two populations. The table includes presence in the population and frequency, AA number and frequency, delta difference between the T1D and CTL population frequencies, a corrected P-value and the Odds Ratio (OR).

HLA-A							
AA at Position	**Population (T1D)**	**Freq (T1D)**	**Population (CTL)**	**Freq (CTL)**	**Delta**	**p^corr**	**OR**
S9	96	56.47%	74	38.54%	17.93%	9.71E-02	2.06
G167	101	59.41%	80	41.67%	17.75%	1.41E-01	2.041
F99	79	46.47%	64	33.33%	13.14%	1.74E+00	1.731
N77	126	74.12%	121	63.02%	11.10%	3.21E+00	1.673
I80	96	56.47%	90	46.88%	9.60%	9.76E+00	1.467
K66	119	70.00%	120	62.50%	7.50%	1.96E+01	1.396
H114	122	71.76%	124	64.58%	7.18%	2.32E+01	1.39
Y116	122	71.76%	124	64.58%	7.18%	2.32E+01	1.39
E114	23	13.53%	13	6.77%	6.76%	4.69E+00	2.118
H116	23	13.53%	13	6.77%	6.76%	4.69E+00	2.118
Q156	88	51.76%	87	45.31%	6.45%	3.26E+01	1.293
M67	34	20.00%	27	14.06%	5.94%	2.10E+01	1.521
R156	34	20.00%	27	14.06%	5.94%	2.10E+01	1.521
H70	162	95.29%	175	91.15%	4.15%	1.94E+01	1.906
E152	48	28.24%	47	24.48%	3.76%	6.24E+01	1.213
W156	48	28.24%	47	24.48%	3.76%	6.24E+01	1.213
R152	7	4.12%	1	0.52%	3.60%	3.75E+00	5.856
I97	82	48.24%	87	45.31%	2.92%	7.90E+01	1.124
E63	163	95.88%	179	93.23%	2.65%	4.72E+01	1.64
M97	116	68.24%	126	65.63%	2.61%	8.64E+01	1.124
W152	12	7.06%	9	4.69%	2.37%	4.93E+01	1.523
Q63	18	10.59%	17	8.85%	1.73%	7.89E+01	1.217
D74	164	96.47%	182	94.79%	1.68%	8.05E+01	1.456
R163	70	41.18%	76	39.58%	1.59%	1.10E+02	1.068
R97	83	48.82%	92	47.92%	0.91%	1.21E+02	1.037
A152	50	29.41%	55	28.65%	0.77%	1.20E+02	1.038
N74	1	0.59%	0	0.00%	0.59%	1.32E+02	3.407
E163	1	0.59%	0	0.00%	0.59%	1.32E+02	3.407
V67	168	98.82%	190	98.96%	-0.13%	1.32E+02	0.885
R114	89	52.35%	101	52.60%	-0.25%	1.32E+02	0.99
Y171	169	99.41%	192	100.00%	-0.59%	6.20E+01	0.294
S77	29	17.06%	34	17.71%	-0.65%	1.18E+02	0.958
Q70	79	46.47%	92	47.92%	-1.45%	1.10E+02	0.944
F9	115	67.65%	133	69.27%	-1.62%	1.08E+02	0.928
Y99	161	94.71%	185	96.35%	-1.65%	6.03E+01	0.687
T163	161	94.71%	185	96.35%	-1.65%	6.03E+01	0.687
H74	62	36.47%	74	38.54%	-2.07%	9.83E+01	0.916
H171	10	5.88%	17	8.85%	-2.97%	5.26E+00	0.192
N66	135	79.41%	159	82.81%	-3.40%	5.57E+01	0.802
T80	149	87.65%	175	91.15%	-3.50%	1.32E+02	0.693
V152	148	87.06%	174	90.63%	-3.57%	4.17E+01	0.7
T9	27	15.88%	40	20.83%	-4.95%	3.67E+01	0.722
Y9	55	32.35%	72	37.50%	-5.15%	4.25E+01	0.799
L156	123	72.35%	149	77.60%	-5.25%	3.61E+01	0.757
D116	122	71.76%	148	77.08%	-5.32%	3.65E+01	0.757
W167	149	87.65%	182	94.79%	-7.14%	2.99E+00	0.4
N63	40	23.53%	61	31.77%	-8.24%	1.32E+01	0.664
D77	117	68.82%	151	78.65%	-9.82%	5.41E+00	0.602
Q114	43	25.29%	79	41.15%	-15.85%	2.34E-01	0.487

Table 7: The HLA-A non-pocket Amino Acid typing of the two populations. The HLA-A non-pocket amino acid typing of the two populations. The table includes presence in the population and frequency, AA number and frequency, delta difference between the T1D and CTL population frequencies, a corrected P-value and the Odds Ratio (OR).

HLAA							
AA at Position	**Population (T1D)**	**Freq (T1D)**	**Population (CTL)**	**Freq (CTL)**	**Delta**	**p^corr**	**OR**
D166	101	59.41%	80	41.67%	17.75%	1.41E-01	2.041
L95	87	51.18%	68	35.42%	15.76%	3.81E-01	1.905
E76	107	62.94%	95	49.48%	13.46%	1.46E+00	1.728
E62	78	45.88%	64	33.33%	12.55%	2.32E+00	1.691
G65	77	45.29%	64	33.33%	11.96%	3.08E+00	1.651
P193	136	80.00%	133	69.27%	10.73%	2.92E+00	1.763
I194	136	80.00%	133	69.27%	10.73%	2.92E+00	1.763
R79	96	56.47%	90	46.88%	9.60%	9.76E+00	1.467
A81	96	56.47%	90	46.88%	9.60%	9.76E+00	1.467
L82	96	56.47%	90	46.88%	9.60%	9.76E+00	1.467
R83	96	56.47%	90	46.88%	9.60%	9.76E+00	1.467
S17	23	13.53%	13	6.77%	6.76%	4.69E+00	2.118
K44	34	20.00%	27	14.06%	5.94%	2.10E+01	1.521
V150	34	20.00%	27	14.06%	5.94%	2.10E+01	1.521
V158	34	20.00%	27	14.06%	5.94%	2.10E+01	1.521
K127	125	73.53%	130	67.71%	5.82%	3.29E+01	1.321
P184	140	82.35%	147	76.56%	5.79%	2.57E+01	1.421
R56	24	14.12%	18	9.38%	4.74%	2.49E+01	1.577
R43	12	7.06%	6	3.13%	3.93%	1.26E+01	2.263
A76	60	35.29%	61	31.77%	3.52%	6.65E+01	1.171
H151	164	96.47%	179	93.23%	3.24%	3.14E+01	1.903
I142	163	95.88%	178	92.71%	3.17%	3.44E+01	1.771
R145	163	95.88%	178	92.71%	3.17%	3.44E+01	1.771
D90	71	41.76%	76	39.58%	2.18%	9.87E+01	1.094
F109	170	100.00%	188	97.92%	2.08%	1.66E+01	8.141
S105	154	90.59%	170	88.54%	2.05%	8.02E+01	1.236
L62	18	10.59%	17	8.85%	1.73%	7.89E+01	1.217
G107	164	96.47%	182	94.79%	1.68%	8.05E+01	1.456
Q62	102	60.00%	112	58.33%	1.67%	1.10E+02	1.071
T149	23	13.53%	23	11.98%	1.55%	9.93E+01	1.149
K144	154	90.59%	171	89.06%	1.53%	9.62E+01	1.174
E161	170	100.00%	190	98.96%	1.04%	6.61E+01	4.475
M12	4	2.35%	3	1.56%	0.79%	9.38E+01	1.463
S31	1	0.59%	0	0.00%	0.59%	1.32E+02	3.407
Q35	1	0.59%	0	0.00%	0.59%	1.32E+02	3.407
E56	1	0.59%	0	0.00%	0.59%	1.32E+02	3.407
K177	1	0.59%	0	0.00%	0.59%	1.32E+02	3.407
R17	170	100.00%	191	99.48%	0.52%	1.32E+02	2.671
V12	170	100.00%	192	100.00%	0.00%	1.32E+02	0.886
T31	170	100.00%	192	100.00%	0.00%	1.32E+02	0.886
R35	170	100.00%	192	100.00%	0.00%	1.32E+02	0.886
Q43	170	100.00%	192	100.00%	0.00%	1.32E+02	0.886

Table 7 (continue): The HLA-A non-pocket Amino Acid typing of the two populations.

HLAA							
AA at Position	**Population (T1D)**	**Freq (T1D)**	**Population (CTL)**	**Freq (CTL)**	**Delta**	**p^corr**	**OR**
D102	170	100.00%	192	100.00%	0.00%	1.32E+02	0.886
R111	170	100.00%	192	100.00%	0.00%	1.32E+02	0.886
E177	170	100.00%	192	100.00%	0.00%	1.32E+02	0.886
G56-	169	99.41%	191	99.48%	-0.07%	1.32E+02	0.885
K186	169	99.41%	191	99.48%	-0.07%	1.32E+02	0.885
A158	168	98.82%	190	98.96%	-0.13%	1.32E+02	0.885
R44	168	98.82%	190	98.96%	-0.13%	1.32E+02	0.885
A149	168	98.82%	190	98.96%	-0.13%	1.32E+02	0.885
A150	168	98.82%	190	98.96%	-0.13%	1.32E+02	0.885
D161	27	15.88%	31	16.15%	-0.26%	1.32E+02	0.983
H102	9	5.29%	11	5.73%	-0.44%	1.32E+02	0.928
L50	0	0.00%	1	0.52%	-0.52%	1.32E+02	0.374
A79	0	0.00%	1	0.52%	-0.52%	1.32E+02	0.374
H111	0	0.00%	1	0.52%	-0.52%	1.32E+02	0.374
T73	169	99.41%	192	100.00%	-0.59%	6.20E+01	0.294
N127	130	76.47%	148	77.08%	-0.61%	1.19E+02	0.966
A90	161	94.71%	185	96.35%	-1.65%	6.03E+01	0.687
R151	68	40.00%	80	41.67%	-1.67%	1.10E+02	0.934
W107	63	37.06%	75	39.06%	-2.00%	9.84E+01	0.919
G62	62	36.47%	74	38.54%	-2.07%	9.83E+01	0.916
I95	134	78.82%	156	81.25%	-2.43%	7.91E+01	0.859
R65	161	94.71%	187	97.40%	-2.69%	3.62E+01	0.499
R186	10	5.88%	17	8.85%	-2.97%	5.26E+00	0.192
Q144	86	50.59%	103	53.65%	-3.06%	7.90E+01	0.885
G79	149	87.65%	175	91.15%	-3.50%	4.04E+01	0.693
L81	149	87.65%	175	91.15%	-3.50%	1.32E+02	0.693
R82	149	87.65%	175	91.15%	-3.50%	1.32E+02	0.693
G83	149	87.65%	175	91.15%	-3.50%	1.32E+02	0.693
P105	84	49.41%	103	53.65%	-4.23%	6.09E+01	0.845
T142	72	42.35%	91	47.40%	-5.04%	4.53E+01	0.816
H145	72	42.35%	91	47.40%	-5.04%	4.53E+01	0.816
A184	111	65.29%	136	70.83%	-5.54%	3.45E+01	0.776
V95	52	30.59%	70	36.46%	-5.87%	3.51E+01	0.77
L109	17	10.00%	32	16.67%	-6.67%	8.84E+00	0.563
E166	149	87.65%	182	94.79%	-7.14%	2.99E+00	0.4
R62	40	23.53%	61	31.77%	-8.24%	1.32E+01	0.664
I73	9	5.29%	27	14.06%	-8.77%	1.00E+00	0.354
V76	117	68.82%	151	78.65%	-9.82%	5.41E+00	0.602
A193	117	68.82%	151	78.65%	-9.82%	5.41E+00	0.602
V194	117	68.82%	151	78.65%	-9.82%	5.41E+00	0.602

HLA-B locus

Table 8: Allele frequency analysis for HLA-B, in the Cypriot population. The HLA-B alleles of the two populations. The table includes presence in the population and frequency, allele number and frequency, delta difference between the T1D and CTL population frequencies, a corrected P-value and the Odds Ratio (OR).

HLA-B locus											
Allele	**Pop (T1D)**	**Freq (T1D)**	**Pop (CTL)**	**Freq (CTL)**	**Allele (T1D)**	**Freq (T1D)**	**Allele (CTL)**	**Freq (CTL)**	**Delta**	**p^corr**	**OR**
08:01	33	19.41%	12	6.25%	35	10.29%	12	3.13%	13.16%	0.00945	3.52
50:01	27	15.88%	6	3.13%	28	8.24%	6	1.56%	12.75%	0.00149	5.5
41:01	26	15.29%	7	3.65%	29	8.53%	7	1.82%	11.64%	0.00826	4.54
44:03	18	10.59%	6	3.13%	19	5.59%	6	1.56%	7.46%	0.26729	3.48
15:01	13	7.65%	3	1.56%	13	3.82%	3	0.78%	6.09%	0.41425	4.64
73:01	8	4.71%	3	1.56%	8	2.35%	3	0.78%	3.15%	1	2.83
39:24	7	4.12%	3	1.56%	7	2.06%	3	0.78%	2.56%	1	2.48
39:06	5	2.94%	1	0.52%	5	1.47%	1	0.26%	2.42%	1	4.24
14:02	25	14.71%	25	13.02%	26	7.65%	27	7.03%	1.69%	1	1.15
45:01	2	1.18%	0	0.00%	2	0.59%	0	0.00%	1.18%	1	5.71
27:02	4	2.35%	3	1.56%	4	1.18%	3	0.78%	0.79%	1	1.46
44:02	10	5.88%	10	5.21%	10	2.94%	10	2.60%	0.67%	1	1.14
53:01	2	1.18%	1	0.52%	2	0.59%	1	0.26%	0.66%	1	1.89
44:21	1	0.59%	0	0.00%	1	0.29%	0	0.00%	0.59%	1	3.41
47:01	1	0.59%	0	0.00%	1	0.29%	0	0.00%	0.59%	1	3.41
35:02	13	7.65%	14	7.29%	13	3.82%	14	3.65%	0.36%	1	1.06
18:01	28	16.47%	31	16.15%	28	8.24%	33	8.59%	0.32%	1	1.03
15:03	2	1.18%	2	1.04%	2	0.59%	2	0.52%	0.14%	1	1.13
15:18	1	0.59%	1	0.52%	1	0.29%	1	0.26%	0.07%	1	1.13
27:05	1	0.59%	1	0.52%	1	0.29%	1	0.26%	0.07%	1	1.13
27:12	1	0.59%	1	0.52%	1	0.29%	1	0.26%	0.07%	1	1.13
15:08	0	0.00%	1	0.52%	0	0.00%	1	0.26%	0.07%	1	1.13
51:08	5	2.94%	6	3.13%	5	1.47%	6	1.56%	-0.19%	1	0.95
07:02	4	2.35%	5	2.60%	4	1.18%	5	1.30%	-0.25%	1	0.92
44:05	4	2.35%	5	2.60%	4	1.18%	6	1.56%	-0.25%	1	0.92
14:01	0	0.00%	1	0.52%	0	0.00%	1	0.26%	-0.52%	1	0.37
15:09	0	0.00%	1	0.52%	0	0.00%	1	0.26%	-0.52%	1	0.37
15:18	0	0.00%	1	0.52%	0	0.00%	1	0.26%	-0.52%	1	0.37
27:05	0	0.00%	1	0.52%	0	0.00%	1	0.26%	-0.52%	1	0.37
37:01	0	0.00%	1	0.52%	0	0.00%	1	0.26%	-0.52%	1	0.37
40:06	0	0.00%	1	0.52%	0	0.00%	1	0.26%	-0.52%	1	0.37
41:06	0	0.00%	1	0.52%	0	0.00%	1	0.26%	-0.52%	1	0.37
58:01	6	3.53%	8	4.17%	6	1.76%	8	2.08%	-0.64%	1	0.86
40:01	4	2.35%	6	3.13%	4	1.18%	6	1.56%	-0.78%	1	0.78
39:01	3	1.76%	5	2.60%	3	0.88%	5	1.30%	-0.84%	1	0.71
27:07	1	0.59%	3	1.56%	1	0.29%	3	0.78%	-0.97%	1	0.48
47:03	0	0.00%	2	1.04%	0	0.00%	2	0.52%	-1.04%	1	0.22
51:07	0	0.00%	2	1.04%	0	0.00%	2	0.52%	-1.04%	1	0.22
49:01	7	4.12%	10	5.21%	7	2.06%	10	2.60%	-1.09%	1	0.8
57:01	1	0.59%	4	2.08%	1	0.29%	4	1.04%	-1.49%	1	0.37
15:17	0	0.00%	3	1.56%	0	0.00%	3	0.78%	-1.56%	1	0.16
40:02	2	1.18%	6	3.13%	2	0.59%	6	1.56%	-1.95%	1	0.43
13:02	6	3.53%	11	5.73%	6	1.76%	11	2.86%	-2.20%	1	0.62
41:02	0	0.00%	6	3.13%	0	0.00%	6	1.56%	-3.13%	1	0.08
55:01	11	6.47%	19	9.90%	11	3.24%	19	4.95%	-3.43%	1	0.64
38:01	1	0.59%	10	5.21%	1	0.29%	10	2.60%	-4.62%	0.60438	0.15
35:08	6	3.53%	17	8.85%	6	1.76%	17	4.43%	-5.32%	1	0.4
07:05	1	0.59%	12	6.25%	1	0.29%	12	3.13%	-5.66%	0.1785	0.13
52:01	1	0.59%	13	6.77%	1	0.29%	13	3.39%	-6.18%	0.09621	0.12
35:03	7	4.12%	20	10.42%	7	2.06%	20	5.21%	-6.30%	1	0.39
51:01	11	6.47%	26	13.54%	11	3.24%	27	7.03%	-7.07%	1	0.45
35:01	22	12.94%	42	21.88%	23	6.76%	43	11.20%	-8.94%	1	0.54

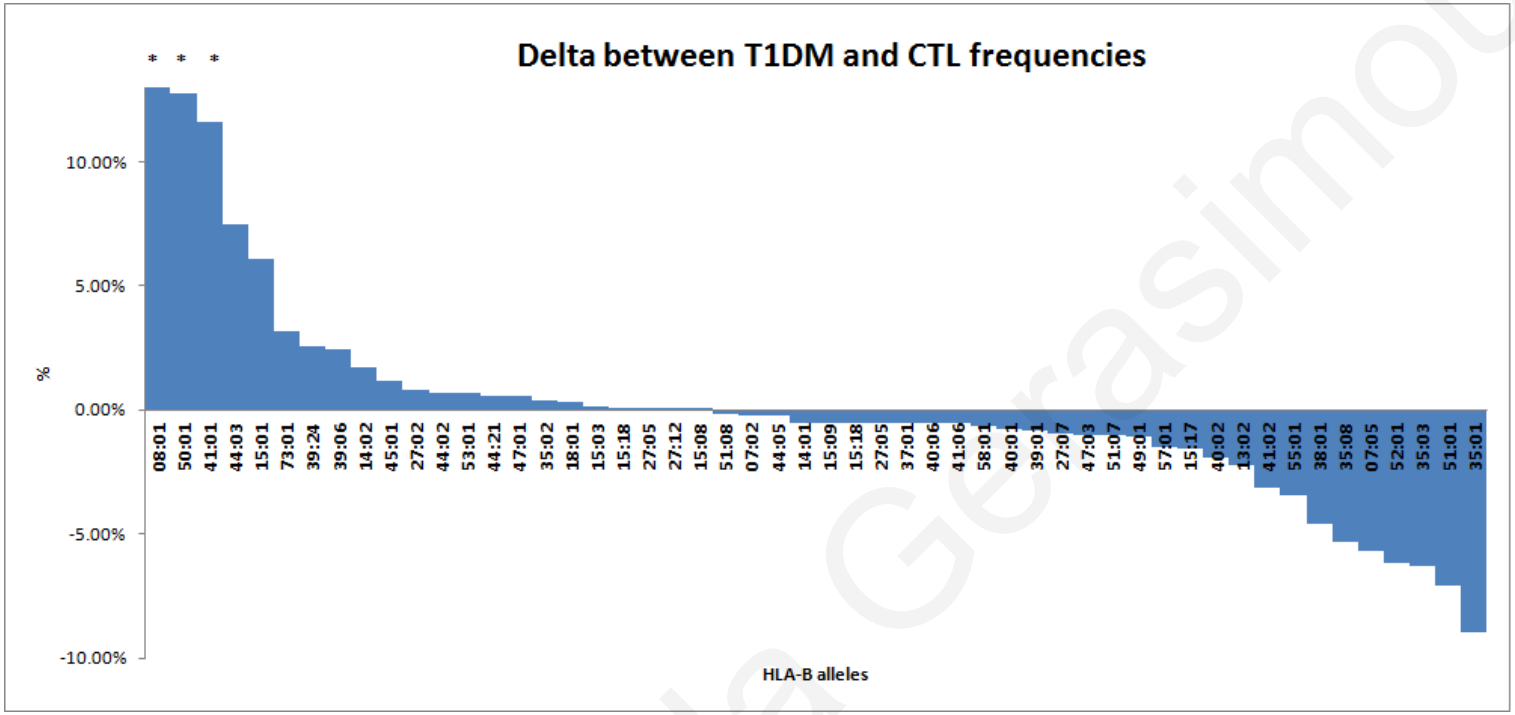


Figure 7: The difference (delta) between T1DM and CTL population frequencies for the HLA-B alleles. Each HLA-B allele is plotted against its delta difference between the two populations. Positive % denotes higher presence in the T1DM group while negative % show association to the control group. Significance is denoted with * ($p<0.05$).

The HLA-B allele profile of T1DM cohort and random control population is depicted above in Table 6. The HLA-B*08:01, HLA-B*50:01and HLA-B*41:01 is prevalent in the diabetic population with a significant frequency difference (pcorr.<0.05) and great OR values. Opposing these three alleles that were strongly associated with the diabetic cohort, HLA-B*35:01, *51:01 and *35:03 are on the other far end depicting their strong presence within the control population, without showing though any statistical significance.

Table 9: The HLA-B pocket Amino Acid typing of the two populations. The HLA-B pocket amino acid typing of the two populations. The table includes presence in the population and frequency, AA number and frequency, delta difference between the T1D and CTL population frequencies, a corrected P-value and the Odds Ratio (OR).

HLA-B							
AA at Position	**Population (T1D)**	**Freq (T1D)**	**Population (CTL)**	**Freq (CTL)**	**Delta**	**p^corr**	**OR**
D156	71	41.76%	44	22.92%	18.85%	2.22E-02	2.398
D9	37	21.76%	12	6.25%	15.51%	2.38E-02	3.518
E63	113	66.47%	100	52.08%	14.39%	9.30E-01	1.817
S67	117	68.82%	106	55.21%	13.62%	1.17E+00	1.784
T163	116	68.24%	107	55.73%	12.51%	2.17E+00	1.7
H9	88	51.76%	80	41.67%	10.10%	7.32E+00	1.499
N114	153	90.00%	154	80.21%	9.79%	1.54E+00	2.186
D74	90	52.94%	84	43.75%	9.19%	1.16E+01	1.444
L116	52	30.59%	43	22.40%	8.19%	1.18E+01	1.523
D116	35	20.59%	25	13.02%	7.57%	8.23E+00	1.721
S167	31	18.24%	21	10.94%	7.30%	6.56E+00	1.801
Y171	166	97.65%	175	91.15%	6.50%	1.46E+00	3.689
R97	148	87.06%	156	81.25%	5.81%	1.91E+01	1.539
W156	13	7.65%	4	2.08%	5.56%	2.82E+00	3.59
S97	47	27.65%	44	22.92%	4.73%	4.19E+01	1.283
V152	155	91.18%	167	86.98%	4.20%	3.03E+01	1.527
G77	9	5.29%	3	1.56%	3.73%	1.55E+01	2.832
T80	38	22.35%	36	18.75%	3.60%	5.47E+01	1.246
K70	14	8.24%	10	5.21%	3.03%	3.69E+01	1.61
N70	169	99.41%	186	96.88%	2.54%	1.59E+01	3.938
C67	51	30.00%	53	27.60%	2.40%	8.10E+01	1.124
S77	152	89.41%	169	88.02%	1.39%	9.34E+01	1.143
W97	25	14.71%	26	13.54%	1.16%	9.63E+01	1.101
N80	154	90.59%	172	89.58%	1.00%	1.08E+02	1.113
N97	6	3.53%	5	2.60%	0.93%	9.59E+01	1.347
D114	114	67.06%	127	66.15%	0.91%	1.15E+02	1.041
I66	170	100.00%	192	100.00%	0.00%	1.26E+02	0.886
Y99	170	100.00%	192	100.00%	0.00%	1.26E+02	0.886
F99	1	0.59%	2	1.04%	-0.45%	1.26E+02	0.674
S99	0	0.00%	1	0.52%	-0.52%	1.26E+02	0.374
D77	3	1.76%	5	2.60%	-0.84%	9.17E+01	0.712
H114	7	4.12%	10	5.21%	-1.09%	1.01E+02	0.797
V97	1	0.59%	4	2.08%	-1.50%	4.74E+01	0.371
W167	165	97.06%	191	99.48%	-2.42%	1.30E+01	0.236
Y116	91	53.53%	109	56.77%	-3.24%	7.52E+01	0.878
E152	90	52.94%	108	56.25%	-3.31%	7.52E+01	0.876
N66	7	4.12%	15	7.81%	-3.69%	2.35E+01	0.525
M67	7	4.12%	15	7.81%	-3.69%	2.35E+01	0.525
S70	7	4.12%	15	7.81%	-3.69%	2.35E+01	0.525
Y74	153	90.00%	180	93.75%	-3.75%	3.08E+01	0.607
H171	75	44.12%	95	49.48%	-5.36%	4.32E+01	0.807
F116	48	28.24%	66	34.38%	-6.14%	2.71E+01	0.753
S116	77	45.29%	99	51.56%	-6.27%	3.12E+01	0.779
L163	124	72.94%	153	79.69%	-6.75%	1.74E+01	0.689
N63	140	82.35%	172	89.58%	-7.23%	6.16E+00	0.547
F67	83	48.82%	108	56.25%	-7.43%	2.16E+01	0.743
E163	33	19.41%	52	27.08%	-7.67%	1.34E+01	0.652
Y67	16	9.41%	34	17.71%	-8.30%	4.01E+00	0.491
Q70	16	9.41%	34	17.71%	-8.30%	4.01E+00	0.491
L156	146	85.88%	182	94.79%	-8.91%	7.63E-01	0.344
T97	44	25.88%	71	36.98%	-11.10%	3.07E+00	0.598
R156	11	6.47%	34	17.71%	-11.24%	1.61E-01	0.331
N77	62	36.47%	101	52.60%	-16.13%	2.77E-01	0.519
Y9	132	77.65%	181	94.27%	-16.62%	5.77E-04	0.218
I80	35	20.59%	75	39.06%	-18.47%	1.90E-02	0.408

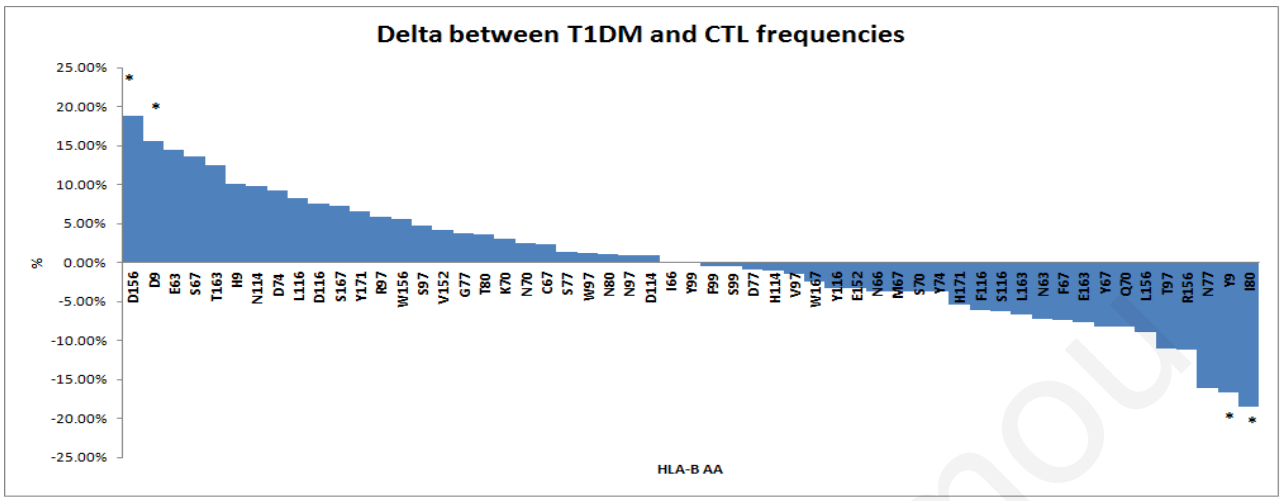


Figure 8: The difference (delta) between T1DM and CTL population frequencies for the HLA-B pocket amino acids. Each HLA-B amino acid is plotted against its delta difference between the two populations. Positive % denotes higher presence in the T1DM group, while negative % shows association to the control group. Significance is denoted with * ($p<0.05$).

The HLA-B pocket amino acid profile of T1DM cohort and random control population is depicted above in Table 9 and the non-pocket amino acids are depicted in Table 10. Figure 8 portrays the amino acids that had great difference between the two groups. The amino acids observed on the left hand side cluster of the figure represent the amino acids associated with T1D and the ones on the right hand side cluster were mostly recognised in the control population group. As indicated in both Table 9 and Figure 8, the two most commonly observed AA in the T1D cohort are the Aspartic Acid (D) at position 156 ($p=0.02$, OR=2.4) and at position 9 ($p=0.02$, OR=3.5). Opposing the association to T1D, a Tyrosine (Y) at position 9 is strongly found in the control group ($p=0.0.0006$, OR= 0.2) along with Isoleucine (I) at position 80 ($p=0.02$, OR=0.4).

Table 10: The HLA-B non-pocket Amino Acid typing of the two populations. The HLA-B non-pocket amino acid typing of the two populations. The table includes presence in the population and frequency, AA number and frequency, delta difference between the T1D and CTL population frequencies, a corrected P-value and the Odds Ratio (OR).

HLA-B							
AA at Position	**Population (T1D)**	**Freq (T1D)**	**Population (CTL)**	**Freq (CTL)**	**Delta**	**p^corr**	**OR**
T143	170	100.00%	192	100.00%	0.00%	1.26E+02	0.886
R145	170	100.00%	192	100.00%	0.00%	1.26E+02	0.886
W147	170	100.00%	192	100.00%	0.00%	1.26E+02	0.886
T178	170	100.00%	192	100.00%	0.00%	1.26E+02	0.886
T158	15	8.82%	17	8.85%	-0.03%	1.26E+02	1
S143	4	2.35%	6	3.13%	-0.77%	9.51E+01	0.775
L147	4	2.35%	6	3.13%	-0.77%	9.51E+01	0.775
A199	166	97.65%	190	98.96%	-1.31%	5.36E+01	0.486
G62	7	4.12%	12	6.25%	-2.13%	6.05E+01	0.662
L145	6	3.53%	11	5.73%	-2.20%	5.75E+01	0.624
S131	161	94.71%	187	97.40%	-2.69%	3.46E+01	0.499
E177	162	95.29%	189	98.44%	-3.14%	1.55E+01	0.353
Q180	162	95.29%	189	98.44%	-3.14%	1.55E+01	0.353
V12	78	45.88%	95	49.48%	-3.60%	6.65E+01	0.866
R65	7	4.12%	15	7.81%	-3.69%	2.35E+01	0.525
L103	98	57.65%	121	63.02%	-5.37%	4.19E+01	0.8
H113	151	88.82%	181	94.27%	-5.45%	1.06E+01	0.492
S11	58	34.12%	76	39.58%	-5.47%	4.11E+01	0.792
A41	150	88.24%	181	94.27%	-6.04%	7.36E+00	0.465
K178	9	5.29%	23	11.98%	-6.69%	3.39E+00	0.424
Q32	150	88.24%	183	95.31%	-7.08%	2.36E+00	0.38
A69	36	21.18%	55	28.65%	-7.47%	1.45E+01	0.672
A71	36	21.18%	55	28.65%	-7.47%	1.45E+01	0.672
I94	82	48.24%	108	56.25%	-8.01%	1.77E+01	0.726
I95	82	48.24%	109	56.77%	-8.54%	1.44E+01	0.711
A81	62	36.47%	101	52.60%	-16.13%	2.77E-01	0.519
T45	93	54.71%	137	71.35%	-16.65%	1.85E-01	0.487
L82	65	38.24%	106	55.21%	-16.97%	1.93E-01	0.504
R83	65	38.24%	106	55.21%	-16.97%	1.93E-01	0.504
A24	84	49.41%	136	70.83%	-21.42%	4.94E-03	0.404
V194	68	40.00%	118	61.46%	-21.46%	7.47E-03	0.42
A46	20	11.76%	22	11.46%	0.31%	1.26E+02	1.032
E46	170	100.00%	192	100.00%	0.00%	1.26E+02	0.886
R62	170	100.00%	192	100.00%	0.00%	1.26E+02	0.886
Q65	170	100.00%	192	100.00%	0.00%	1.26E+02	0.886
E76	170	100.00%	192	100.00%	0.00%	1.26E+02	0.886
A90	170	100.00%	192	100.00%	0.00%	1.26E+02	0.886
M98	170	100.00%	192	100.00%	0.00%	1.26E+02	0.886
L109	170	100.00%	192	100.00%	0.00%	1.26E+02	0.886
F109	170	100.00%	192	100.00%	0.00%	1.26E+02	1.055
T143	170	100.00%	192	100.00%	0.00%	1.26E+02	0.886

Table 10 (continue): The HLA-B non-pocket Amino Acid typing of the two populations

HLA-B							
AA at Position	**Population (T1D)**	**Freq (T1D)**	**Population (CTL)**	**Freq (CTL)**	**Delta**	**p^corr**	**OR**
R145	170	100.00%	192	100.00%	0.00%	1.26E+02	0.886
W147	170	100.00%	192	100.00%	0.00%	1.26E+02	0.886
T178	170	100.00%	192	100.00%	0.00%	1.26E+02	0.886
T158	15	8.82%	17	8.85%	-0.03%	1.26E+02	1
S143	4	2.35%	6	3.13%	-0.77%	9.51E+01	0.775
L147	4	2.35%	6	3.13%	-0.77%	9.51E+01	0.775
A199	166	97.65%	190	98.96%	-1.31%	5.36E+01	0.486
G62	7	4.12%	12	6.25%	-2.13%	6.05E+01	0.662
L145	6	3.53%	11	5.73%	-2.20%	5.75E+01	0.624
S131	161	94.71%	187	97.40%	-2.69%	3.46E+01	0.499
E177	162	95.29%	189	98.44%	-3.14%	1.55E+01	0.353
Q180	162	95.29%	189	98.44%	-3.14%	1.55E+01	0.353
V12	78	45.88%	95	49.48%	-3.60%	6.65E+01	0.866
R65	7	4.12%	15	7.81%	-3.69%	2.35E+01	0.525
L103	98	57.65%	121	63.02%	-5.37%	4.19E+01	0.8
H113	151	88.82%	181	94.27%	-5.45%	1.06E+01	0.492
S11	58	34.12%	76	39.58%	-5.47%	4.11E+01	0.792
A41	150	88.24%	181	94.27%	-6.04%	7.36E+00	0.465
K178	9	5.29%	23	11.98%	-6.69%	3.39E+00	0.424
Q32	150	88.24%	183	95.31%	-7.08%	2.36E+00	0.38
A69	36	21.18%	55	28.65%	-7.47%	1.45E+01	0.672
A71	36	21.18%	55	28.65%	-7.47%	1.45E+01	0.672
I94	82	48.24%	108	56.25%	-8.01%	1.77E+01	0.726
I95	82	48.24%	109	56.77%	-8.54%	1.44E+01	0.711
A81	62	36.47%	101	52.60%	-16.13%	2.77E-01	0.519
T45	93	54.71%	137	71.35%	-16.65%	1.85E-01	0.487
L82	65	38.24%	106	55.21%	-16.97%	1.93E-01	0.504
R83	65	38.24%	106	55.21%	-16.97%	1.93E-01	0.504
A24	84	49.41%	136	70.83%	-21.42%	4.94E-03	0.404
V194	68	40.00%	118	61.46%	-21.46%	7.47E-03	0.42

As seen in Table 10, the amino acids found in the non-pocket region and are strongly associated with the diabetic group include Lysine (K) at position 45, Leucine (L) at position 32, Threonine (T) at position 24 and position 14 with a significant p-value of <0.05. In contrast, an alanine (A) at position 24 (p=0.004, OR=0.4) is most common in the control population, as well as a valine (V) at position 194 (p=0.007, OR=0.4).

The HLA-B pocket and non-pocket amino acid zygosity analysis is summarised in Table 11. The only amino acid that showed a higer OR when in heterozygous is the aspartic acid (D) in position 9. Summarizing tables 12 and 13 show the HLA-B alleles and their shared amino acid, at each significant pocket and non-pocket position.

Table 11: The HLA-B pocket amino acid zygosity analysis. Homozygous and heterozygous inheritance of shared HLA-B epitopes associated with T1DM. Association with T1DM is designated with red and resistance with blue.

Locus	HLA-B	HLA-B
Location	9	9
Epitope	D	Y
P corr value (Homozygous)	NA	NA
OR (Homozygous)	NA	NA
P corr value (Heterozygous)	5.7E-4	8.4E-4
OR (Heterozygous)	3.52	0.28

Table 12: The HLA-B pocket amino acids that are significant ($p<0.05$) and are either associated with susceptibility to T1D (red) or resistance (blue).

HLA LOCUS	HLA-B	HLA-B	HLA-B	HLA-B
Location	9	9	80	156
EPITOPE	D	Y	I	D
PATIENT (N=170)	37	132	35	71
CONTROL (N=192)	12	181	75	44
Pcorr. Value	2.4E-4	5.8E-4	1.8E-2	2.22E-2
OR	3.5	0.2	0.41	2.4
Associated alleles	08:01	44:03, 15:01, 39:24, 39:06, 14:02, 44:02, 53:01, 44:21, 47:01, 35:02, 15:03, 15:18, 51:08, 07:02, 44:05, 14:01, 15:08, 15:09, 58:01, 39:01, 47:03, 51:07, 57:01, 15:17, 13:02, 55:01, 38:01, 07:05, 52:01, 35:03, 51:01, 35:01	27:02, 53:01, 51:08, 58:01, 51:07, 49:01, 57:01, 15:17, 38:01, 52:01, 51:01	08:01, 41:01, 45:01, 44:02, 44:21, 51:08, 44:05, 37:01, 41:06, 41:02

Table 13: The HLA-B non- pocket amino acids that are significant ($p<0.05$) and are either associated with susceptibility to T1D (red) or resistance (blue).

HLA LOCUS	HLA-B	HLA-B	HLA-B	HLA-B	HLA-B	HLA-B
Location	24	24	32	45	194	194
EPITOPE	T	A	L	K	I	V
PATIENT (N=170)	103	84	94	89	164	68
CONTROL (N=192)	77	136	66	59	159	118
Pcorr. Value	1.8E-2	4.9E-3	1.1E-2	5.5E-3	3.3E-3	7.5E-3
OR	2.3	0.4	2.4	2.5	5.3	0.42
Associated alleles	50:01,41:01, 44:03,73:01, 45:01,27:02, 44:02,44:21, 47:01,27:05, 27:12,44:05, 40:06,41:06, 40:01,27:07, 47:03,49:01, 40:02,13:02, 41:02	15:01,53:01, 35:02,51:08, 15:08,58:01, 51:07,57:01, 15:17,55:01, 35:08,52:01, 35:03,51:01, 35:01	50:01,41:01, 44:03,45:01, 27:02,44:02, 44:21,47:01, 27:05,27:12, 44:05,40:06, 41:06,40:01, 27:07,47:03, 49:01,40:02, 41:02	50:01,41:01, 44:03,45:01, 44:02,44:21, 47:01,44:05, 40:06,41:06, 40:01,47:03, 49:01,40:02, 41:02	08:01,50:01,41:01, 44:03,15:01,73:01, 39:24,39:06,14:02, 45:01,27:02,44:02, 47:01,18:01,15:03, 15:18,27:05,27:12, 07:02,44:05,14:01, 15:08,15:09,37:01, 40:06,40:01,39:01, 27:07,49:01,57:01, 15:17,40:02,13:02, 41:02,55:01,38:01, 07:05	53:01,35:02, 51:08,51:07, 35:08,52:01, 35:03,51:01, 35:01

HLA-C locus

Table 14: Allele frequency analysis for HLA-C in the Cypriot population. The HLA-C alleles of the two populations. The table includes presence in the population and frequency, allele number and frequency, delta difference between the T1D and CTL population frequencies, a corrected P-value and the Odds Ratio (OR).

HLA-C locus											
Allele	Pop (T1D)	Freq (T1D)	Pop (CTL)	Freq (CTL)	Allele (T1D)	Freq (T1D)	Allele (CTL)	Freq (CTL)	Delta	p^corr	OR
06:02	35	20.59%	22	11.46%	37	10.88%	23	5.99%	9.13%	0.51405	1.99
07:02	26	15.29%	13	6.77%	27	7.94%	13	3.39%	8.52%	0.26578	2.44
14:03	16	9.41%	2	1.04%	17	5.00%	2	0.52%	8.37%	0.00833	8.14
17:01	23	13.53%	12	6.25%	25	7.35%	12	3.13%	7.28%	0.52835	2.3
07:01	39	22.94%	33	17.19%	39	11.47%	35	9.11%	5.75%	1	1.43
12:03	38	22.35%	36	18.75%	41	12.06%	37	9.64%	3.60%	1	1.25
02:08	2	1.18%	0	0.00%	2	0.59%	0	0.00%	1.18%	1	5.71
08:02	25	14.71%	26	13.54%	26	7.65%	28	7.29%	1.17%	1	1.1
05:01	9	5.29%	8	4.17%	9	2.65%	8	2.08%	1.12%	1	1.28
16:01	5	2.94%	4	2.08%	5	1.47%	4	1.04%	0.86%	1	1.39
16:04	3	1.76%	2	1.04%	3	0.88%	2	0.52%	0.72%	1	1.59
03:04	5	2.94%	5	2.60%	5	1.47%	5	1.30%	0.34%	1	1.13
03:03	11	6.47%	13	6.77%	11	3.24%	13	3.39%	-0.30%	1	0.96
05:18	0	0.00%	1	0.52%	0	0.00%	1	0.26%	-0.52%	1	0.37
12:xx	0	0.00%	1	0.52%	0	0.00%	2	0.52%	-0.52%	1	0.37
03:02	3	1.76%	5	2.60%	3	0.88%	5	1.30%	-0.84%	1	0.71
16:02	9	5.29%	13	6.77%	9	2.65%	13	3.39%	-1.48%	1	0.78
07:04	3	1.76%	7	3.65%	3	0.88%	7	1.82%	-1.89%	1	0.52
15:05	9	5.29%	15	7.81%	9	2.65%	15	3.91%	-2.52%	1	0.67
02:02	10	5.88%	17	8.85%	10	2.94%	19	4.95%	-2.97%	1	0.66
14:02	3	1.76%	10	5.21%	3	0.88%	10	2.60%	-3.45%	1	0.36
01:02	2	1.18%	10	5.21%	2	0.59%	10	2.60%	-4.03%	0.9896	0.26
15:02	7	4.12%	16	8.33%	7	2.06%	16	4.17%	-4.21%	1	0.49
12:02	1	0.59%	13	6.77%	1	0.29%	13	3.39%	-6.18%	0.04811	0.12
04:01	45	26.47%	80	41.67%	46	13.53%	91	23.70%	-15.20%	0.06921	0.51

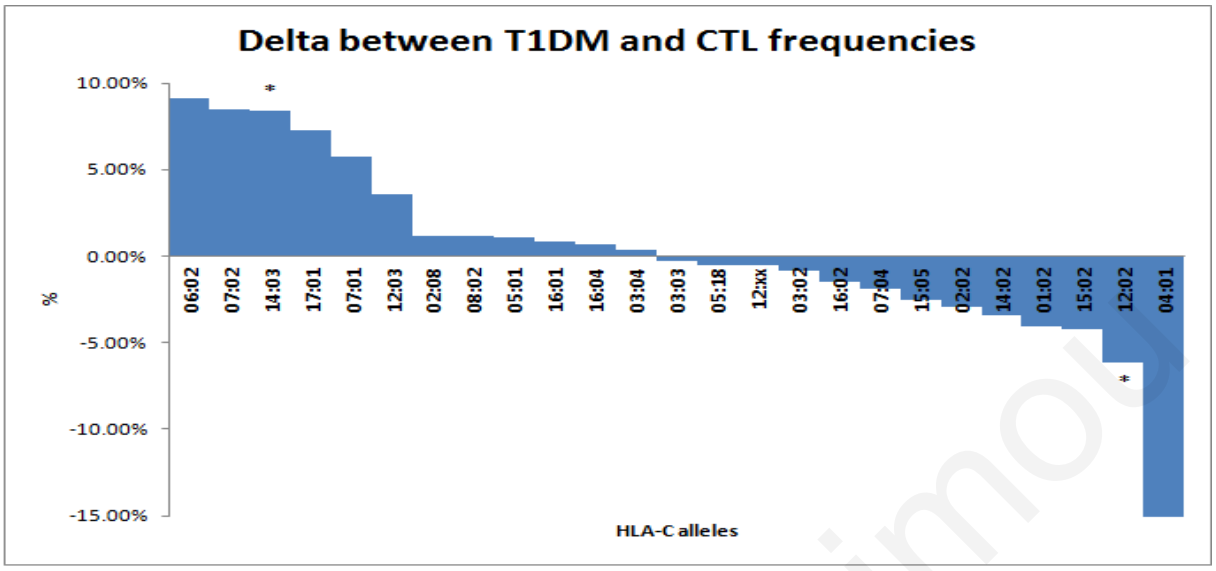


Figure 9: The difference (delta) between T1DM and CTL population frequencies for the HLA-C alleles. Each HLA-C allele is plotted against its delta difference between the two populations. Positive % denotes higher presence in the T1DM group, while negative % shows association to the control group. Significance is denoted with * ($p<0.05$).

The HLA-C allele profile of T1DM cohort and random control population is depicted above in Table 7. The HLA-C*06:02, *07:02, 14:03 an *17:01 alleles portray the highest allele frequencies difference with only HLA-C*14:03 reaching a significance of pcorr.=0.008 and OR=8.14. HLA-C*04:01 also showed the biggest allele frequency difference (15.20%) with strong association to the control group but escaping significance with a pcorr. = 0.069. The HLA-C*12:02 showed the second highest frequency in the control population, generating a delta difference of -6.18% and a significant pcorr. of 0.048. (Figure 9).

Table 15: The HLA-C pocket Amino Acid typing of the two populations. The HLA-C pocket amino acid typing of the two populations. The table includes presence in the population and frequency, AA number and frequency, delta difference between the T1D and CTL population frequencies, a corrected P-value and the Odds Ratio (OR).

HLA-C							
AA at Position	**Population (T1D)**	**Freq (T1D)**	**Population (CTL)**	**Freq (CTL)**	**Delta**	**p^corr**	**OR**
D9	90	52.94%	70	36.46%	16.48%	0.173964	1.953
S116	142	83.53%	131	68.23%	15.30%	0.076203	2.338
W97	99	58.24%	83	43.23%	15.01%	0.385644	1.825
L156	106	62.35%	96	50.00%	12.35%	1.67076	1.651
S99	29	17.06%	13	6.77%	10.29%	0.893004	2.438
D114	155	91.18%	159	82.81%	8.36%	1.70478	2.107
A152	75	44.12%	70	36.46%	7.66%	13.64135	1.374
W156	82	48.24%	80	41.67%	6.57%	20.50381	1.303
S77	135	79.41%	140	72.92%	6.50%	14.72713	1.426
N80	135	79.41%	140	72.92%	6.50%	14.72713	1.426
Y99	157	92.35%	167	86.98%	5.37%	10.22549	1.776
E163	34	20.00%	29	15.10%	4.90%	22.38944	1.401
K66	168	98.82%	186	96.88%	1.95%	24.43913	2.349
T163	164	96.47%	186	96.88%	-0.40%	84	0.882
N66	53	31.18%	61	31.77%	-0.59%	76.4647	0.974
Q156	14	8.24%	17	8.85%	-0.62%	71.67636	0.929
R97	152	89.41%	173	90.10%	-0.69%	72.51308	0.926
L163	19	11.18%	23	11.98%	-0.80%	73.0984	0.928
D156	3	1.76%	7	3.65%	-1.88%	61.1205	0.712
N114	91	53.53%	108	56.25%	-2.72%	56.45791	0.896
L116	8	4.71%	16	8.33%	-3.63%	7.91532	0.363
Y9	123	72.35%	146	76.04%	-3.69%	46.0756	0.849
E152	159	93.53%	187	97.40%	-3.87%	10.23355	0.407
Y116	18	10.59%	28	14.58%	-4.00%	22.87488	0.7
F9	2	1.18%	10	5.21%	-4.03%	26.94182	0.656
C99	2	1.18%	10	5.21%	-4.03%	26.94182	0.656
F116	101	59.41%	123	64.06%	-4.65%	32.49196	0.822
N77	119	70.00%	147	76.56%	-6.56%	15.90935	0.716
K80	117	68.82%	147	76.56%	-7.74%	15.90935	0.716
R156	86	50.59%	116	60.42%	-9.83%	6.000624	0.672
S9	60	35.29%	87	45.31%	-10.02%	4.58892	0.66
F99	60	35.29%	87	45.31%	-10.02%	4.58892	0.66

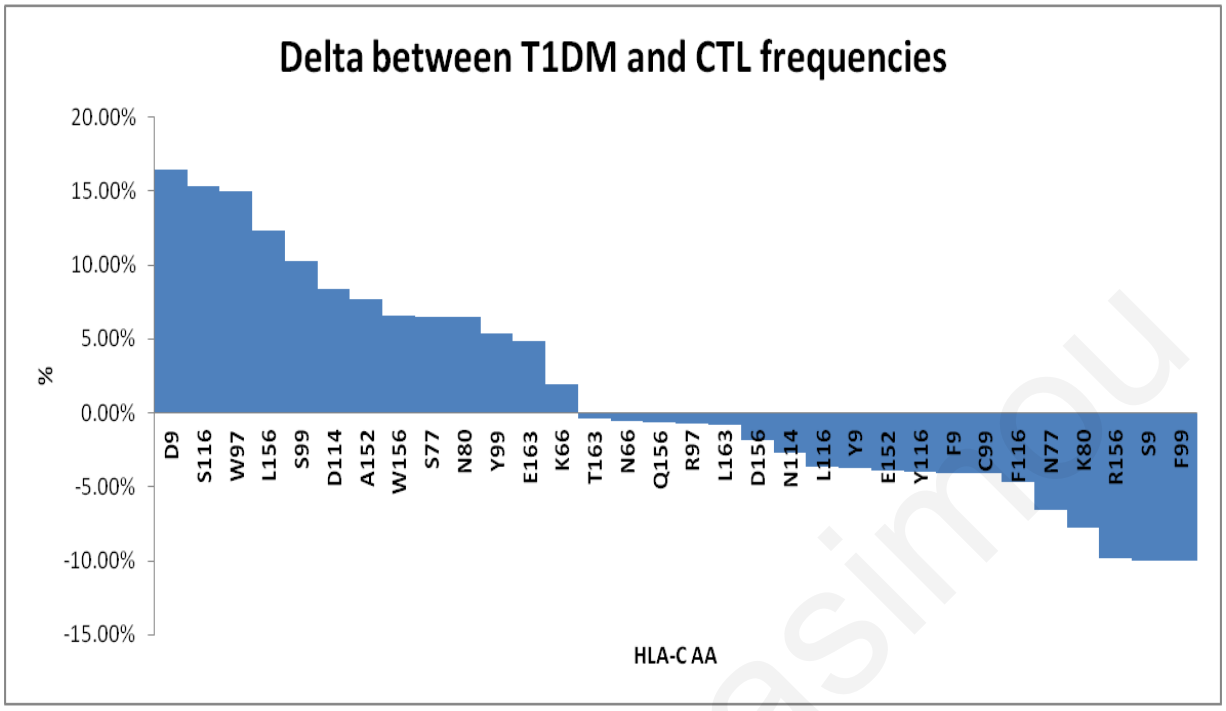


Figure 10: The difference (delta) between T1DM and CTL population frequencies for the HLA-C pocket amino acids. Each HLA-C amino acid is plotted against its delta difference between the two populations. Positive % denotes higher presence in the T1DM group, while negative % shows association to the control group. Significance is denoted with * ($p<0.05$).

The HLA-C pocket amino acid profile of T1DM cohort and random control population is depicted in Table 15 and the non-pocket amino acids are depicted in Table 16. No significant difference between the T1D cohort and the control population was observed for any of the amino acids interrogated. Nevertheless, Figure 10 portrays the amino acids that had great difference between the two groups. The amino acids observed on the left hand side cluster of the figure represent the amino acids associated with T1D, and the ones on the left hand side cluster were mostly recognised in the control population group.

Table 16: The HLA-C non-pocket Amino Acid typing of the two populations. The HLA-C non-pocket amino acid typing of the two populations. The table includes presence in the population and frequency, AA number and frequency, delta difference between the T1D and CTL population frequencies, a corrected P-value and the Odds Ratio (OR).

HLA-C							
AA at Position	**Population (T1D)**	**Freq (T1D)**	**Population (CTL)**	**Freq (CTL)**	**Delta**	**p^corr**	**OR**
L147	83	48.82%	61	31.77%	17.05%	0.102396	2.04
S24	91	53.53%	78	40.63%	12.90%	1.292676	1.679
P184	64	37.65%	52	27.08%	10.56%	2.757132	1.621
L194	64	37.65%	52	27.08%	10.56%	2.757132	1.621
S143	27	15.88%	12	6.25%	9.63%	1.775256	2.301
G170	27	15.88%	12	6.25%	9.63%	1.775256	2.301
R184	27	15.88%	12	6.25%	9.63%	1.775256	2.301
C1	159	93.53%	168	87.50%	6.03%	6.18324	2.017
A11	163	95.88%	173	90.10%	5.78%	3.43854	2.45
R14	169	99.41%	180	93.75%	5.66%	0.29988	7.825
A49	169	99.41%	180	93.75%	5.66%	0.29988	7.825
Q35	33	19.41%	33	17.19%	2.22%	49.457604	1.16
K138	33	19.41%	33	17.19%	2.22%	49.457604	1.16
A73	148	87.06%	163	84.90%	2.16%	54.615372	1.191
G16	170	100.00%	189	98.44%	1.56%	21.0588	6.298
E177	168	98.82%	187	97.40%	1.43%	38.194632	1.977
R21	164	96.47%	183	95.31%	1.16%	51.241512	1.31
R35	168	98.82%	188	97.92%	0.91%	57.825516	1.609
T94	168	98.82%	188	97.92%	0.91%	57.825516	1.609
T138	168	98.82%	188	97.92%	0.91%	57.825516	1.609
K177	36	21.18%	39	20.31%	0.86%	75.334812	1.055
R6	170	100.00%	191	99.48%	0.52%	84	2.671
G91	170	100.00%	191	99.48%	0.52%	84	2.671
R91	170	100.00%	191	99.48%	0.52%	84	0.959
L103	170	100.00%	191	99.48%	0.52%	84	2.671
E173	170	100.00%	191	99.48%	0.52%	84	2.671
Y113	169	99.41%	191	99.48%	-0.07%	84	0.885
P193	169	99.41%	191	99.48%	-0.07%	84	0.885
T143	168	98.82%	190	98.96%	-0.13%	84	0.885
R170	168	98.82%	190	98.96%	-0.13%	84	0.885
I95	50	29.41%	57	29.69%	-0.28%	84	0.988
L193	16	9.41%	19	9.90%	-0.48%	84	0.95
V103	19	11.18%	23	11.98%	-0.80%	73.098396	0.928
K173	19	11.18%	23	11.98%	-0.80%	73.098396	0.928
V194	164	96.47%	188	97.92%	-1.45%	44.119572	0.604
D90	117	68.82%	135	70.31%	-1.49%	68.80062	0.932
L95	162	95.29%	186	96.88%	-1.58%	49.283976	0.666
S16	12	7.06%	17	8.85%	-1.80%	47.54526	0.791
F95	3	1.76%	7	3.65%	-1.88%	61.1205	0.712
H21	56	32.94%	67	34.90%	-1.95%	62.092296	0.917
W147	159	93.53%	184	95.83%	-2.30%	29.720712	0.639
A90	135	79.41%	157	81.77%	-2.36%	50.03754	0.86
H184	158	92.94%	184	95.83%	-2.89%	21.483	0.584
K6	2	1.18%	10	5.21%	-4.03%	26.941824	0.656
A24	153	90.00%	181	94.27%	-4.27%	14.057064	0.556
T73	99	58.24%	122	63.54%	-5.31%	27.857844	0.801
G1	80	47.06%	104	54.17%	-7.11%	17.32752	0.753
I94	33	19.41%	51	26.56%	-7.15%	11.271204	0.669
H113	15	8.82%	31	16.15%	-7.32%	3.39864	0.511
S11	61	35.88%	95	49.48%	-13.60%	0.898968	0.573
W14	47	27.65%	91	47.40%	-19.75%	10.98174	0.491
E49	47	27.65%	91	47.40%	-19.75%	10.98174	0.491

HLA-DRB1

Table 17: Allele frequency analysis for HLA-DRB1 in the Cypriot population. The HLA-DRB1 alleles of the two populations. The table includes presence in the population and frequency, allele number and frequency, delta difference between the T1D and CTL population frequencies, a corrected P-value and the Odds Ratio (OR).

HLA-DRB1 locus											
Allele	Pop (T1D)	Freq (T1D)	Pop (CTL)	Freq (CTL)	Allele (T1D)	Freq (T1D)	Allele (CTL)	Freq (CTL)	Delta	p^corr	OR
03:01	83	48.82%	24	12.50%	101	29.71%	24	6.25%	36.32%	1.06E-12	6.56
04:05	74	43.53%	18	9.38%	82	24.12%	18	4.69%	34.15%	2.00E-12	7.28
04:02	19	11.18%	6	3.13%	19	5.59%	6	1.56%	8.05%	0.09413	3.69
04:01	12	7.06%	1	0.52%	12	3.53%	1	0.26%	6.54%	0.02762	10.07
04:04	1	0.59%	0	0.00%	1	0.29%	0	0.00%	0.59%	1	3.41
08:04	1	0.59%	0	0.00%	1	0.29%	0	0.00%	0.59%	1	3.41
04:08	1	0.59%	1	0.52%	1	0.29%	1	0.26%	0.07%	1	1.13
13:05	1	0.59%	1	0.52%	1	0.29%	1	0.26%	0.07%	1	1.13
16:05	1	0.59%	1	0.52%	1	0.29%	1	0.26%	0.07%	1	1.13
13:02	6	3.53%	7	3.65%	6	1.76%	7	1.82%	-0.12%	1	0.98
01:02	20	11.76%	23	11.98%	20	5.88%	23	5.99%	-0.22%	1	0.98
16:01	39	22.94%	45	23.44%	39	11.47%	50	13.02%	-0.50%	1	0.97
04:07	0	0.00%	2	1.04%	0	0.00%	2	0.52%	-1.04%	1	0.22
11:02	0	0.00%	2	1.04%	0	0.00%	2	0.52%	-1.04%	1	0.22
15:06	0	0.00%	2	1.04%	0	0.00%	2	0.52%	-1.04%	1	0.22
01:01	8	4.71%	12	6.25%	8	2.35%	12	3.13%	-1.54%	1	0.76
11:03	0	0.00%	3	1.56%	0	0.00%	3	0.78%	-1.56%	1	0.16
12:01	0	0.00%	3	1.56%	0	0.00%	3	0.78%	-1.56%	1	0.16
15:02	1	0.59%	6	3.13%	1	0.29%	6	1.56%	-2.54%	1	0.25
04:03	8	4.71%	14	7.29%	10	2.94%	14	3.65%	-2.58%	1	0.64
13:03	1	0.59%	10	5.21%	1	0.29%	10	2.60%	-4.62%	0.36263	0.15
15:01	6	3.53%	16	8.33%	6	1.76%	16	4.17%	-4.80%	1	0.42
13:01	0	0.00%	11	5.73%	0	0.00%	11	2.86%	-5.73%	0.03057	0.05
07:01	12	7.06%	28	14.58%	12	3.53%	29	7.55%	-7.52%	0.8505	0.46
11:01	8	4.71%	27	14.06%	9	2.65%	27	7.03%	-9.35%	0.1142	0.31
16:02	1	0.59%	20	10.42%	1	0.29%	21	5.47%	-9.83%	6.77E-04	0.07
10:01	2	1.18%	24	12.50%	2	0.59%	24	6.25%	-11.32%	5.06E-04	0.1
11:04	6	3.53%	37	19.27%	6	1.76%	39	10.16%	-15.74%	7.26E-05	0.16
14:01	0	0.00%	31	16.15%	0	0.00%	31	8.07%	-16.15%	4.29E-08	0.02

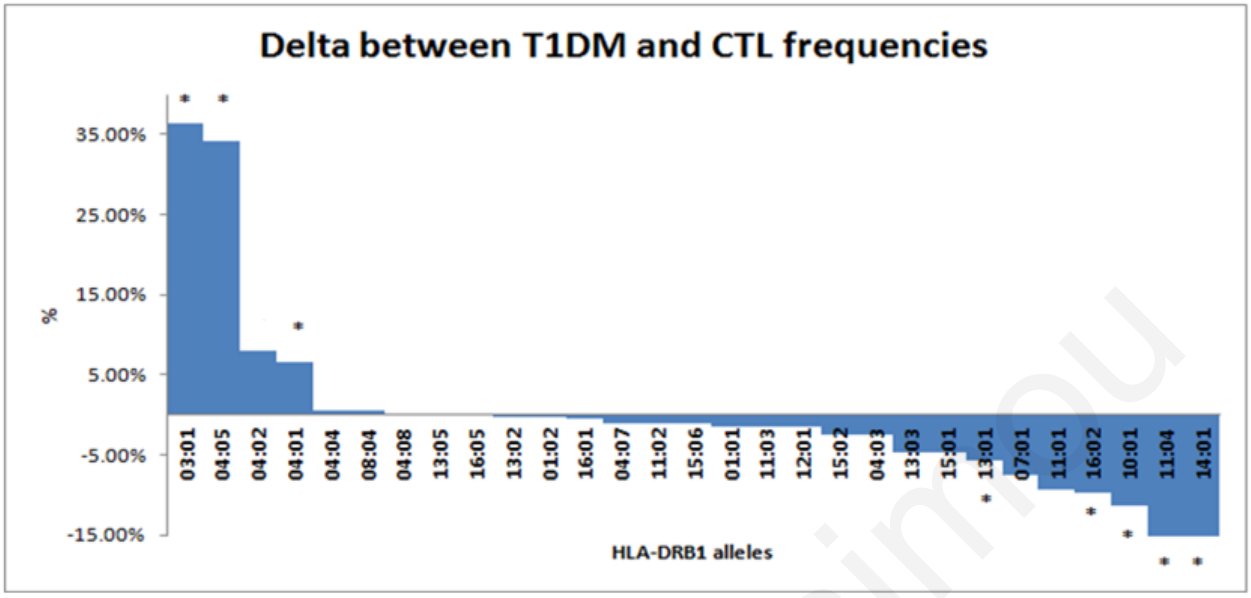


Figure 11: The difference (delta) between T1DM and CTL population frequencies for the HLA-DRB1 alleles. Each HLA-DRB1 allele is plotted against its delta difference between the two populations. Positive % denotes higher presence in the T1DM group, while negative % shows association to the control group. Significance is denoted with * ($p<0.05$).

The HLA-DRB1 allele profile of T1DM cohort and random control population is depicted above in Table 17. The two dominant HLA-DRB1 alleles with a significant frequency difference of 36.32% and 34.15%, a pcorr. of $1.06x10^{12}$ and $2.0x10^{-12}$ and an OR of 6.56 and 7.28 respectively are the HLA-DRB1*03:01 and *04:05. HLA-DRB1*04:01 portrays the highest OR of 10.07 and a significant pcorr. of 0.027. Lastly, HLA-DRB1*04:02 also showed a significant allele frequency difference and prevalence in the patient group. The HLA-DRB1 alleles that were most common in the control population than the diabetic group are the DRB1*14:01, *11:04, *10:01, *16:02 and *13:01 (Figure 11).

Table 18: The HLA-DRB1 pocket Amino Acid typing of the two populations. The HLA-DRB1 pocket amino acid typing of the two populations. The table includes presence in the population and frequency, AA number and frequency, delta difference between the T1D and CTL population frequencies, a corrected P-value and the Odds Ratio (OR).

DRB1							
AA at Positio	Population (T1D)	Freq (T1D)	Population (CTL)	Freq (CTL)	Delta	p^corr	OR
Y26	106	62.35%	24	12.50%	49.85%	4.20748E-12	6.563
R74	106	62.35%	24	12.50%	49.85%	4.20748E-12	6.563
H13	110	64.71%	37	19.27%	45.44%	8.82016E-17	7.574
Q70	158	92.94%	97	50.52%	42.42%	3.59023E-18	12.42
K71	91	53.53%	33	17.19%	36.34%	3.01784E-11	5.48
V11	110	64.71%	61	31.77%	32.94%	4.41847E-08	3.905
N37	88	51.76%	42	21.88%	29.89%	4.95599E-07	3.799
S57	74	43.53%	28	14.58%	28.95%	1.50714E-07	4.456
L67	158	92.94%	142	73.96%	18.98%	0.000147905	4.494
E9	164	96.47%	151	78.65%	17.82%	2.40344E-05	6.932
Y37	117	68.82%	117	60.94%	7.89%	14.793366	1.411
D28	168	98.82%	179	93.23%	5.59%	0.918918	5.069
Y30	168	98.82%	179	93.23%	5.59%	0.918918	5.069
Y60	170	100.00%	187	97.40%	2.60%	7.506044	10.003
L74	1	0.59%	0	0.00%	0.59%	55.883947	3.407
Y78	170	100.00%	191	99.48%	0.52%	119	2.671
V85	170	100.00%	191	99.48%	0.52%	119	2.671
V38	170	100.00%	192	100.00%	0.00%	119	0.886
E71	25	14.71%	29	15.10%	-0.40%	119	0.971
L11	28	16.47%	33	17.19%	-0.72%	105.772198	0.952
C30	28	16.47%	33	17.19%	-0.72%	105.772198	0.952
G13	1	0.59%	3	1.56%	-0.97%	74.45949	0.479
A85	20	11.76%	25	13.02%	-1.26%	89.457417	0.895
H30	0	0.00%	3	1.56%	-1.56%	29.8333	0.159
L37	0	0.00%	3	1.56%	-1.56%	29.8333	0.159
L38	0	0.00%	3	1.56%	-1.56%	29.8333	0.159
V86	116	68.24%	136	70.83%	-2.60%	77.025844	0.885
F47	104	61.18%	124	64.58%	-3.41%	61.255845	0.865
S13	101	59.41%	122	63.54%	-4.13%	53.474078	0.84
S11	102	60.00%	125	65.10%	-5.10%	39.134816	0.805
D57	156	91.76%	186	96.88%	-5.11%	4.644808	0.376
Y47	144	84.71%	175	91.15%	-6.44%	8.689618	0.544
G11	14	8.24%	29	15.10%	-6.87%	0.121261	0.046
Y13	14	8.24%	29	15.10%	-6.87%	0.121261	0.046
L30	14	8.24%	29	15.10%	-6.87%	0.121261	0.046
Q74	14	8.24%	29	15.10%	-6.87%	0.121261	0.046
V78	14	8.24%	29	15.10%	-6.87%	0.121261	0.046
A74	147	86.47%	182	94.79%	-8.32%	1.132523	0.361
A71	7	4.12%	24	12.50%	-8.38%	0.541926	0.315
G86	122	71.76%	154	80.21%	-8.44%	7.654675	0.629
V57	12	7.06%	30	15.63%	-8.57%	1.572347	0.42
S60	12	7.06%	30	15.63%	-8.57%	1.572347	0.42
F13	30	17.65%	54	28.13%	-10.48%	2.903957	0.552
R30	3	1.76%	24	12.50%	-10.74%	0.002685949	0.074
A38	3	1.76%	24	12.50%	-10.74%	0.002685949	0.074
L26	30	17.65%	56	29.17%	-11.52%	1.55771	0.524
F26	142	83.53%	184	95.83%	-12.30%	0.01744778	0.23
S37	72	42.35%	105	54.69%	-12.33%	2.469488	0.61
W9	82	48.24%	120	62.50%	-14.26%	0.948192	0.561
P11	48	28.24%	83	43.23%	-14.99%	0.377587	0.519
R13	48	28.24%	83	43.23%	-14.99%	0.377587	0.519
A57	0	0.00%	31	16.15%	-16.15%	0.000287944	0.164
H60	0	0.00%	31	16.15%	-16.15%	0.000287944	0.164
E28	40	23.53%	78	40.63%	-17.10%	0.08541106	0.453
I67	45	26.47%	84	43.75%	-17.28%	0.07811755	0.466
R71	132	77.65%	185	96.35%	-18.71%	5.12009E-06	0.139
E74	8	4.71%	46	23.96%	-19.25%	1.36529E-05	0.165
F37	12	7.06%	56	29.17%	-22.11%	4.39895E-06	0.191
F67	54	31.76%	104	54.17%	-22.40%	0.002459016	0.396
R70	2	1.18%	52	27.08%	-25.91%	8.2398E-12	0.04
D70	84	49.41%	157	81.77%	-32.36%	7.42703E-09	0.22

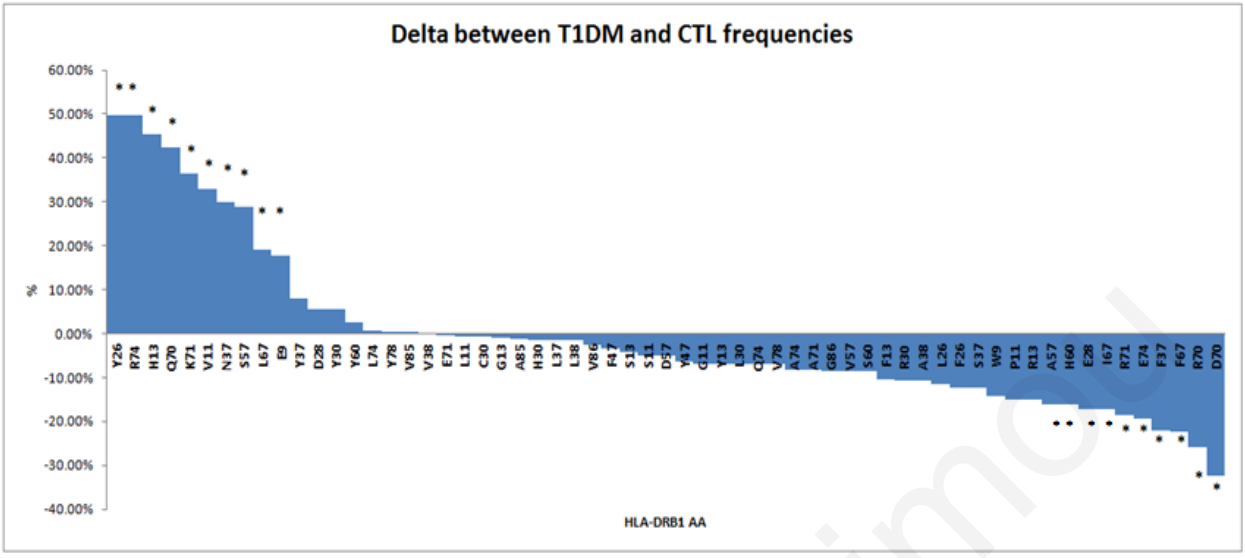


Figure 12: The difference (delta) between T1DM and CTL population frequencies for the HLA-DRB1 pocket amino acids. Each HLA-DRB1 amino acid is plotted against its delta difference between the two populations. Positive % denotes higher presence in the T1DM group, while negative % shows association to the control group. Significance is denoted with * ($p<0.05$).

The HLA-DRB1 pocket amino acid profile of T1DM cohort and random control population is depicted in Table 18 and the non-pocket amino acids are depicted in Table 19. Figure 12 portrays the amino acids that had great difference between the two groups. The amino acids observed on the left hand side cluster of the figure represent the amino acids associated with T1D, and the ones on the left hand side cluster were mostly recognised in the control population group. Interestingly, the pocket amino acid with the highest OR ($p=3.59x10^{-18}$, OR=12.4) that was highly associated with the diabetic patients, Glutamine (Q) at position 70, was also the same amino acid position that portrayed the lowest OR when substituted with an Arginine (R) ($p=8.2x10^{-12}$, OR=0.04) or an aspartic acid (D) ($p=7.4x10^{-9}$, OR=0.22). See Appendix I for a detailed description of these amino acids and associated alleles.

In summary, the non-pocket amino acids that have a high OR, a significant p-value and therefore strong presence in the diabetic group include: N77, H33, Y96, L180, E98, A104, N120, G73, T140, R133 and V142. The non-pocket amino acids of the HLA-DRB1 molecule that are not part of the binding pockets include: A73, T77, E10, V31, Y40, Q166, K98, S104, Q4, L133, M142, A140, Y112, M181, Q96 and E58. Summarizing tables 22 and 23, show the HLA-B alleles and their shared amino acid, at each significant pocket and non-pocket position.

Table 19: The HLA-DRB1 non-pocket Amino Acid typing of the two populations. The HLA-DRB1 non-pocket amino acid typing of the two populations. The table includes presence in the population and frequency, AA number and frequency, delta difference between the T1D and CTL population frequencies, a corrected P-value and the Odds Ratio (OR).

DRB1							
AA at Position	Population (T1D)	Freq (T1D)	Population (CTL)	Freq (CTL)	Delta	p^corr	OR
N77	106	62.35%	24	12.50%	49.85%	4.20748E-12	6.563
H33	110	64.71%	37	19.27%	45.44%	8.82016E-17	7.574
Y96	110	64.71%	37	19.27%	45.44%	8.82016E-17	7.574
L180	110	64.71%	37	19.27%	45.44%	8.82016E-17	7.574
E98	114	67.06%	65	33.85%	33.20%	4.29412E-08	3.945
A104	114	67.06%	65	33.85%	33.20%	4.29412E-08	3.945
N120	110	64.71%	61	31.77%	32.94%	4.41847E-08	3.905
G73	94	55.29%	50	26.04%	29.25%	2.04621E-06	3.486
T140	164	96.47%	151	78.65%	17.82%	2.40344E-05	6.932
Q10	147	86.47%	145	75.52%	10.95%	1.306739	2.049
R133	170	100.00%	178	92.71%	7.29%	0.01541645	27.7
V142	170	100.00%	178	92.71%	7.29%	0.01541645	27.7
H32	90	52.94%	92	47.92%	5.02%	41.125091	1.221
K12	147	86.47%	159	82.81%	3.66%	45.621268	1.318
Q149	146	85.88%	159	82.81%	3.07%	56.067921	1.256
F31	170	100.00%	187	97.40%	2.60%	7.506044	10.003
A58	169	99.41%	188	97.92%	1.50%	44.765777	2.698
T181	170	100.00%	190	98.96%	1.04%	59.555573	4.475
R4	170	100.00%	191	99.48%	0.52%	119	2.671
E14	170	100.00%	191	99.48%	0.52%	119	2.671
R25	170	100.00%	191	99.48%	0.52%	119	2.671
H16	170	100.00%	192	100.00%	0.00%	119	0.886
F40	170	100.00%	192	100.00%	0.00%	119	0.886
V50	170	100.00%	192	100.00%	0.00%	119	0.886
H112	170	100.00%	192	100.00%	0.00%	119	0.886
V164	170	100.00%	192	100.00%	0.00%	119	0.886
R166	170	100.00%	192	100.00%	0.00%	119	0.886
I31	28	16.47%	33	17.19%	-0.72%	105.772198	0.952
E96	28	16.47%	33	17.19%	-0.72%	105.772198	0.952
Y16	1	0.59%	3	1.56%	-0.97%	74.45949	0.479
A50	0	0.00%	2	1.04%	-1.04%	59.555573	0.223
F164	0	0.00%	2	1.04%	-1.04%	119	0.374
Y10	102	60.00%	125	65.10%	-5.10%	39.134816	0.805
T12	102	60.00%	125	65.10%	-5.10%	39.134816	0.805
H149	102	60.00%	125	65.10%	-5.10%	39.134816	0.805
N33	155	91.18%	188	97.92%	-6.74%	0.527527	0.239
V180	155	91.18%	188	97.92%	-6.74%	0.527527	0.239
K14	14	8.24%	29	15.10%	-6.87%	0.121261	0.046
Q25	14	8.24%	29	15.10%	-6.87%	0.121261	0.046
H96	112	65.88%	140	72.92%	-7.03%	20.182043	0.719
Y32	150	88.24%	183	95.31%	-7.08%	2.230298	0.38
S120	153	90.00%	188	97.92%	-7.92%	0.162078	0.209
A73	151	88.82%	189	98.44%	-9.61%	0.01318282	0.143
T77	152	89.41%	192	100.00%	-10.59%	8.88073E-05	0.021
E10	3	1.76%	24	12.50%	-10.74%	0.002685949	0.074
V31	3	1.76%	24	12.50%	-10.74%	0.002685949	0.074
Y40	3	1.76%	24	12.50%	-10.74%	0.002685949	0.074
Q166	3	1.76%	24	12.50%	-10.74%	0.002685949	0.074
K98	147	86.47%	187	97.40%	-10.93%	0.01286866	0.184
S104	147	86.47%	187	97.40%	-10.93%	0.01286866	0.184
Q4	1	0.59%	29	15.10%	-14.52%	0.121261	0.046
L133	47	27.65%	81	42.19%	-14.54%	0.503251	0.526
M142	47	27.65%	81	42.19%	-14.54%	0.503251	0.526
A140	81	47.65%	120	62.50%	-14.85%	0.690676	0.548
Y112	0	0.00%	31	16.15%	-16.15%	0.000287944	0.164
M181	14	8.24%	51	26.56%	-18.33%	0.000527111	0.255
Q96	49	28.82%	99	51.56%	-22.74%	0.001340297	0.383
E58	14	8.24%	67	34.90%	-26.66%	6.22453E-08	0.172

Table 20: The HLA-DRB1 pocket amino acid zygosity analysis. Homozygous and heterozygous inheritance of shared HLA-DRB1 pocket epitopes associated with T1DM. Association with T1DM is designated with red and resistance with blue.

Locus	HLA-DRB1	HLA-DRB1	HLA-DRB1	HLA-DRB1	HLA-DRB1	HLA-DRB1	HLA-DRB1	HLA-DRB1	HLA-DRB1	HLA-DRB1	HLA-DRB1
Location	26	37	37	57	67	67	70	70	71	71	74
Epitope	F	N	F	S	L	F	Q	D	K	R	E
P corr value (Homozygous)	1.2E-5	NA	NA	NA	9.4E-8	NA	1.4E-17	6.1E-10	NA	3.9E-9	NA
OR (Homozygous)	0.16	NA	NA	NA	7.71	NA	20.1	0.11	NA	0.1	NA
P corr value (Heterozygous)	NA	1.8E-8	5.9E-7	4.0E-9	1.2E-4	3.0E-4	5.0E-15	1.8E-7	2.6E-12	4.7E-5	3.4E-7
OR (Heterozygous)	NA	3.8	0.2	4.4	3.73	0.43	10.2	0.27	5.5	0.2	0.16

Table 21: The HLA-DRB1 non-pocket amino acid zygosity analysis. Homozygous and heterozygous inheritance of shared HLA-DRB1 non-epitopes associated with T1DM. Association with T1DM is designated with red and resistance with blue.

Locus	HLA-DRB1	HLA-DRB1	HLA-DRB1	HLA-DRB1	HLA-DRB1
Location	73	73	77	96	181
Epitope	G	A	T	Q	M
P corr value (Homozygous)	NA	NA	1.9E-8	2.1E-4	NA
OR (Homozygous)	NA	NA	0.01	0.03	NA
P corr value (Heterozygous)	2.5E-8	4.3E-6	NA	5.8E-4	2.2E-5
OR (Heterozygous)	3.6	0.09	NA	0.44	0.26

Inheritance of two copies of the HLA-DRB1 Q^{70} susceptibility epitope was also strongly associated with T1DM ($p= 1.4x10^{-17}$, OR 20.1) and probability decreased with inheritance of one copy ($p= 5x10^{-15}$, OR 10.2). Among the two alternative resistance epitopes identified for this position inheritance of R^{70} appears to be more protective than inheritance of D^{70}; inheritance of one copy of R^{70} had a lower probability of disease ($p= 9.44x10^{-13}$, OR 0.04) than inheritance of one ($p= 1.8x10^{-7}$, OR 0.27) or two copies of D^{70} ($p= 6.1x10^{-10}$, OR 0.11). The probability of disease in the case of inheritance of two copies of R^{70} was not calculated as this genotype was not present in the population.

Table 22: The HLA-DRB1 pocket amino acids that are significant ($p<0.05$) and are either associated with susceptibility to T1D (red) or resistance (blue).

HLA LOCUS	DRB1	DRB1	DRB1	DRB1	DRB1	DRB1	DRB1	DRB1	DRB1	DRB1	DRB1
Location	9	11	13	26	26	30	37	37	38	57	57
EPITOPE	E	V	H	Y	F	R	N	F	A	S	A
PATIENT (N=170)	164	110	110	106	142	3	88	12	3	74	0
CONTROL (N=192)	151	61	37	24	184	24	42	56	24	28	31
Pcorr. Value	2.4E-05	4.41E-8	8.8E-17	4.2E-12	0.02	0.003	5.0E-7	4.4E-6	0.003	1.5E-7	0.0003
OR	6.93	3.91	7.57	6.56	0.23	0.07	3.8	0.2	0.07	4.5	0.16
Associated alleles	03:01,04:05, 04:02, 04:01, 04:04, 08:04, 04:08, 13:05, 13:02, 04:07, 11:02, 11:03, 12:01, 04:03, 13:03, 13:01, 11:01, 10:01, 11:04, 14:01	04:05, 04:02, 04:01, 04:04, 04:08, 04:07, 04:03, 10:01	04:05, 04:02, 04:01, 04:04, 04:08, 04:07, 04:03	03:01	04:05, 04:02, 04:01, 04:04, 08:04, 04:08, 13:05, 16:05, 13:02, 16:01, 04:07, 11:02, 15:06, 11:03, 15:02, 04:03, 13:03, 15:01, 13:01, 07:01, 11:01, 16:02, 11:04, 14:01,	10:01	03:01, 13:05, 13:02, 13:01	07:01, 14:01	10:01	04:05, 13:03	14:01

Table 22 (continue): The HLA-DRB1 pocket amino acids that are significant ($p<0.05$) and are either associated with susceptibility to T1D (red) or resistance (blue).

HLA LOCUS	DRB1	DRB1	DRB1	DRB1	DRB1	DRB1	DRB1	DRB1	DRB1	DRB1	DRB1
Location	60	67	67	67	70	70	70	71	71	74	74
EPITOPE	H	L	I	F	Q	D	R	K	R	R	E
PATIENT (N=170)	0	158	45	54	158	84	2	91	132	106	8
CONTROL (N=192)	31	142	84	104	97	157	52	33	185	24	46
Pcorr. Value	0.0003	0.0001	0.08	0.002	3.6E-18	7.4E-9	8.2E-12	3.0E-11	5.12E-6	4.2E-12	1.4E-5
OR	0.16	4.5	0.5	0.4	12.42	0.22	0.04	5.48	0.14	6.6	0.17
Associated alleles	14:01	03:01, 04:05, 04:01, 04:04, 04:08, 01:02, 04:07, 01:01, 04:03, 16:02, 10:01, 14:01	04:02, 16:05, 13:02, 11:02, 15:06, 12:01, 15:02, 13:03, 15:01, 13:01, 07:01	08:04, 13:05, 16:01, 11:03, 11:01, 11:04	03:01, 04:05, 04:01, 04:04, 04:08, 01:02, 04:07, 15:06, 01:01, 15:02, 04:03, 15:01	04:02, 08:04, 13:05, 16:05, 13:02, 16:01, 11:02, 11:03, 12:01, 13:03, 13:01, 07:01, 11:01, 16:02, 11:04	10:01, 14:01	03:01, 04:01, 13:03	04:05, 04:04, 08:04, 04:08, 13:05, 16:05, 01:02, 16:01, 04:07, 01:01, 12:01, 04:03, 07:01, 11:01, 16:02, 10:01, 11:04, 14:01	03:01	04:07, 04:03, 14:01

Table 23: The HLA-DRB1 non- pocket amino acids that are significant ($p<0.05$) and are either associated with susceptibility to T1D (red) or resistance (blue).

HLA LOCUS	DRB1	DRB1	DRB1	DRB1	DRB1	DRB1	DRB1	DRB1	DRB1	DRB1	DRB1
Location	10	31	33	40	58	73	73	77	77	96	96
EPITOPE	E	V	H	Y	E	G	A	N	T	Y	Q
PATIENT (N=170)	3	3	110	3	14	94	151	106	152	110	49
CONTROL (N=192)	24	24	37	24	67	50	189	24	192	37	99
Pcorr. Value	0.003	0.003	8.8E-17	0.003	6.2E-8	2.0E-6	0.01	4.2E-12	8.9E-5	8.8E-17	0.001
OR	0.07	0.07	7.6	0.07	0.17	3.5	0.14	6.6	0.02	7.6	0.38
Associated alleles	10:01	10:01	04:05, 04:02, 04:01, 04:04, 04:08, 04:07, 04:03	10:01	11:02, 11:03, 11:01, 11:04	03:01, 07:01	04:05, 04:02, 04:01, 04:04, 08:04, 04:08, 13:05, 16:05, 13:02, 01:02, 16:01, 04:07, 11:02, 15:06, 01:01, 11:03, 12:01, 15:02, 04:03, 13:03, 15:01, 13:01,11:01, 16:02, 10:01, 11:04, 14:01	03:01	04:05, 04:02, 04:01, 04:04, 08:04, 04:08, 13:05, 16:05, 13:02, 01:02, 16:01, 04:07, 11:02, 15:06, 01:01, 11:03, 12:01, 15:02, 04:03, 13:03, 15:01, 13:01, 07:01, 11:01, 16:02, 10:01, 11:04, 14:01	04:05, 04:02, 04:01, 04:04, 04:08, 04:07, 04:03	16:01, 15:02, 15:01, 16:02, 10:01

Table 23(continue): The HLA-DRB1 non- pocket amino acids that are significant ($p<0.05$) and are either associated with susceptibility to T1D (red) or resistance (blue).

HLA LOCUS	DRB1	DRB1	DRB1	DRB1	DRB1	DRB1	DRB1	DRB1	DRB1
Location	98	98	104	104	112	120	166	180	181
EPITOPE	K	E	S	A	Y	N	Q	L	M
PATIENT (N=170)	147	114	147	114	0	110	3	110	14
CONTROL (N=192)	187	65	187	65	31	61	24	37	51
Pcorr. Value	0.01	4.3E-8	0.01	4.3E-8	0.0003	4.4E-8	0.003	8.8E-17	0.0005
OR	0.18	3.95	0.18	3.9	0.16	3.9	0.07	7.6	0.26
Associated alleles	03:01, 08:04, 13:05, 13:02, 01:02, 16:01, 11:02, 01:01, 11:03, 12:01, 15:02, 13:03, 15:01, 13:01, 11:01, 16:02, 10:01, 11:04, 14:01	04:05, 04:02, 04:01, 04:04, 04:08, 04:07, 04:03, 07:01	03:01, 08:04, 13:05, 13:02, 01:02, 16:01, 11:02, 01:01, 11:03, 12:01, 15:02, 13:03, 15:01, 13:01, 11:01, 16:02, 10:01, 11:04, 14:01	04:05, 04:02, 04:01, 04:04, 04:08, 04:07, 04:03, 07:01	14:01	04:05, 04:02, 04:01, 04:04, 04:08, 04:07, 04:03, 10:01	10:01	04:05, 04:02, 04:01, 04:04, 04:08, 04:07, 04:03	07:01, 10:01

HLA-DQA1 locus

Table 24: Allele frequency analysis for HLA-DQA1 in the Cypriot population. The HLA-DQA1 alleles of the two populations. The table includes presence in the population and frequency, allele number and frequency, delta difference between the T1D and CTL population frequencies, a corrected P-value and the Odds Ratio (OR).

HLA-DQA1 locus											
Allele	Pop (T1D)	Freq (T1D)	Pop (CTL)	Freq (CTL)	Allele (T1D)	Freq (T1D)	Allele (CTL)	Freq (CTL)	Delta	p^corr	OR
03:01	111	65.29%	41	21.35%	126	37.06%	45	11.72%	43.94%	1.76E-16	6.84
05:01	94	55.29%	95	49.48%	114	33.53%	107	27.86%	5.81%	1	1.26
04:01	1	0.59%	0	0.00%	1	0.29%	0	0.00%	0.59%	1	3.41
01:10	0	0.00%	1	0.52%	0	0.00%	1	0.26%	-0.52%	1	0.37
02:01	12	7.06%	26	13.54%	12	3.53%	27	7.03%	-6.48%	0.46382	0.5
01:03	1	0.59%	17	8.85%	1	0.29%	17	4.43%	-8.26%	0.00124	0.09
01:02	54	31.76%	83	43.23%	55	16.18%	98	25.52%	-11.47%	0.23858	0.61
01:01	31	18.24%	81	42.19%	31	9.12%	89	23.18%	-23.95%	6.53E-06	0.31

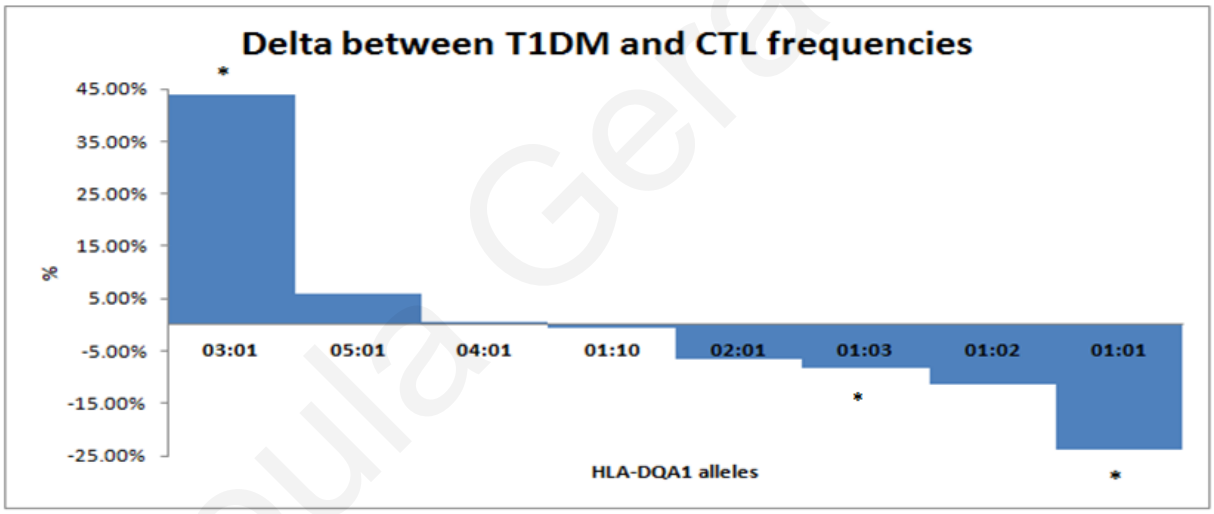


Figure 13: The difference (delta) between T1DM and CTL population frequencies for the HLA-DQA1 alleles. Each HLA-DQA1 allele is plotted against its delta difference between the two populations. Positive % denotes higher presence in the T1DM group, while negative % shows association to the control group. Significance is denoted with * ($p<0.05$).

The HLA-DQA1 allele profile of T1DM cohort and random control population is depicted above in Table 24. Opposing the other HLA class II alleles, HLA-DQA1 showed one allele to be strongly significantly present in the diabetic cohort and one allele to be strongly significantly present in the control population (Figure 13). HLA-DQA1*03:01 was most frequent in the diabetic cohort, 65.29%, as opposed to 21.35% in the control group

(pcorr=$1.76x10^{-16}$, OR=6.84). The allele with the highest frequency in the control group was the DQA1*01:01 with a significant pcorr. of $6.35x10^{-6}$ and OR of 0.31.

The HLA-DQA1 pocket amino acid profile of T1DM cohort and random control population is depicted in Table 25 and the non-pocket amino acids are depicted in Table 26. Figure 20 portrays the amino acids that had great difference between the two groups. For this HLA class II molecule, there are six pocket amino acids that show susceptibility to T1DM and six pocket amino acids that are mostly found in the control group suggesting a protective effect. The susceptibility amino acids that show significance of $p<0.05$ and OR range from 6.8 to 10.9 are amino acid at position R56, V76, Y11, R55, I66 and L69. The same pocket amino acid positions of the HLA-DQA1 molecule are the ones included in the resistance to T1DM amino acids list that include: G56, M76, C11, G55, M66 and A69 ($p<0.05$, OR=0.24) (Figure 18). The HLA-DQA1 non-pocket amino acids that have a high OR, a significant p-value, and therefore strong presence in the diabetic group, include: S26, Q47, T187, L50, R53, E175, R52, S18, V45, L48, F61, T64, S80, and H129. The non-pocket amino acids of the HLA-DQA1 molecule that are significantly associated with resistance are: F25, Q129, F18, A45, R47, W48, E50, S52, K53, G61, R64, Y80 and Q175. Summarizing tables 29 and 30, show the HLA-DQA1 alleles and their shared amino acid, at each significant pocket and non-pocket position.

Table 25: The HLA-DQA1 pocket Amino Acid typing of the two populations. The HLA-DQA1 pocket amino acid typing of the two populations. The table includes presence in the population and frequency, AA number and

frequency, delta difference between the T1D and CTL population frequencies, a corrected P-value and the Odds Ratio (OR).

DQA1							
AA at Position	**Population (T1D)**	**Freq (T1D)**	**Population (CTL)**	**Freq (CTL)**	**Delta**	**p^corr**	**OR**
R56	133	78.24%	45	23.44%	54.80%	1.61E-15	6.841
V76	133	78.24%	45	23.44%	54.80%	1.61E-15	6.841
Y11	165	97.06%	141	73.44%	23.62%	4.15E-09	10.952
R55	165	97.06%	141	73.44%	23.62%	4.15E-09	10.952
I66	165	97.06%	141	73.44%	23.62%	4.15E-09	10.952
L69	165	97.06%	141	73.44%	23.62%	4.15E-09	10.952
S75	119	70.00%	107	55.73%	14.27%	2.14E+01	1.261
E34	130	76.47%	133	69.27%	7.20%	1.14E+01	1.436
L76	105	61.76%	113	58.85%	2.91%	4.32E+01	1.128
T69	1	0.59%	0	0.00%	0.59%	3.43E+01	3.407
I75	150	88.24%	180	93.75%	-5.51%	6.84E+00	0.508
Q34	131	77.06%	163	84.90%	-7.84%	4.41E+00	0.601
C11	82	48.24%	153	79.69%	-31.45%	3.04E-08	0.24
G55	82	48.24%	153	79.69%	-31.45%	3.04E-08	0.24
G56	82	48.24%	153	79.69%	-31.45%	3.04E-08	0.24
M66	82	48.24%	153	79.69%	-31.45%	3.04E-08	0.24
A69	82	48.24%	153	79.69%	-31.45%	3.04E-08	0.24
M76	82	48.24%	153	79.69%	-31.45%	3.04E-08	0.24

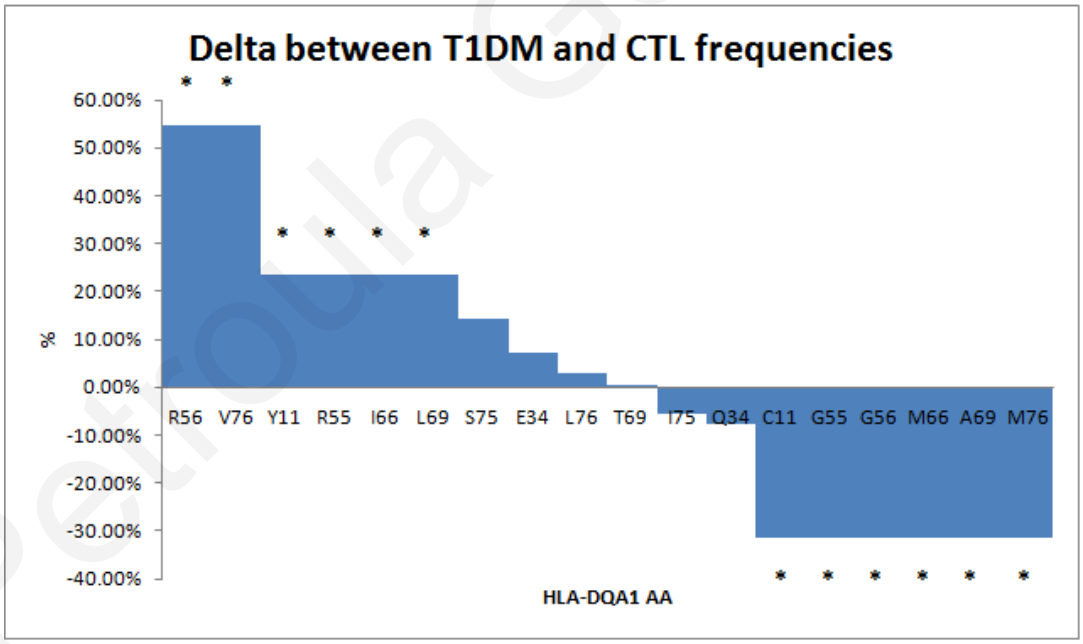


Figure 14: The difference (delta) between T1DM and CTL population frequencies for the HLA-DQA1 pocket amino acids. Each HLA-DQA1 amino acid is plotted against its delta difference between the two populations. Positive % denotes higher presence in the T1DM group, while negative % shows association to the control group. Significance is denoted with * ($p<0.05$).

Table 26: The HLA-DQA1 non-pocket Amino Acid typing of the two populations. The HLA-DQA1 non-pocket amino acid typing of the two populations. The table includes presence in the population and frequency, AA number and frequency, delta difference between the T1D and CTL population frequencies, a corrected P-value and the Odds Ratio (OR).

DQA1							
AA at Position	**Population (T1D)**	**Freq (T1D)**	**Population (CTL)**	**Freq (CTL)**	**Delta**	**p^corr**	**OR**
S26	133	78.24%	45	23.44%	54.80%	1.61E-15	6.841
Q47	133	78.24%	45	23.44%	54.80%	1.61E-15	6.841
T187	133	78.24%	45	23.44%	54.80%	1.61E-15	6.841
L50	115	67.65%	67	34.90%	32.75%	3.19E-08	3.869
R53	115	67.65%	67	34.90%	32.75%	3.19E-08	3.869
E175	115	67.65%	67	34.90%	32.75%	3.19E-08	3.869
R52	163	95.88%	123	64.06%	31.82%	3.53E-13	12.268
S18	165	97.06%	141	73.44%	23.62%	4.15E-09	10.952
V45	165	97.06%	141	73.44%	23.62%	4.15E-09	10.952
L48	165	97.06%	141	73.44%	23.62%	4.15E-09	10.952
F61	165	97.06%	141	73.44%	23.62%	4.15E-09	10.952
T64	165	97.06%	141	73.44%	23.62%	4.15E-09	10.952
S80	165	97.06%	141	73.44%	23.62%	4.15E-09	10.952
H129	165	97.06%	147	76.56%	20.50%	2.58E-07	9.282
I107	119	70.00%	107	55.73%	14.27%	2.14E+01	1.261
L156	119	70.00%	107	55.73%	14.27%	2.14E+01	1.261
E161	119	70.00%	107	55.73%	14.27%	2.14E+01	1.261
S163	119	70.00%	107	55.73%	14.27%	2.14E+01	1.261
K175	119	70.00%	107	55.73%	14.27%	2.14E+01	1.261
G40	95	55.88%	95	49.48%	6.40%	1.80E+01	1.291
C47	95	55.88%	95	49.48%	6.40%	1.80E+01	1.291
V50	95	55.88%	95	49.48%	6.40%	1.80E+01	1.291
L51	95	55.88%	95	49.48%	6.40%	1.80E+01	1.291
Q53	95	55.88%	95	49.48%	6.40%	1.80E+01	1.291
Y25	170	100.00%	189	98.44%	1.56%	1.83E+01	6.298
F54	170	100.00%	190	98.96%	1.04%	3.65E+01	4.475
K47-	14	8.24%	14	7.29%	0.94%	7.30E+01	0.374
H52-	14	8.24%	14	7.29%	0.94%	7.30E+01	0.374
L54-	14	8.24%	14	7.29%	0.94%	7.30E+01	0.374
R41	170	100.00%	192	100.00%	0.00%	7.30E+01	0.886
S130	170	100.00%	192	100.00%	0.00%	7.30E+01	0.886
E40-	150	88.24%	180	93.75%	-5.51%	6.84E+00	0.508
F51	150	88.24%	180	93.75%	-5.51%	6.84E+00	0.508
T107	150	88.24%	180	93.75%	-5.51%	6.84E+00	0.508
F156	150	88.24%	180	93.75%	-5.51%	6.84E+00	0.508
D161	150	88.24%	180	93.75%	-5.51%	6.84E+00	0.508
I163	150	88.24%	180	93.75%	-5.51%	6.84E+00	0.508
T26-	155	91.18%	188	97.92%	-6.74%	3.24E-01	0.239
A187	155	91.18%	188	97.92%	-6.74%	3.24E-01	0.239
K41-	1	0.59%	17	8.85%	-8.27%	4.23E+00	0.496
A130-	1	0.59%	17	8.85%	-8.27%	4.23E+00	0.496
F25-	13	7.65%	42	21.88%	-14.23%	1.45E-02	0.304
Q129-	81	47.65%	142	73.96%	-26.31%	2.32E-05	0.323
F18	82	48.24%	153	79.69%	-31.45%	3.04E-08	0.24
A45	82	48.24%	153	79.69%	-31.45%	3.04E-08	0.24
R47	82	48.24%	153	79.69%	-31.45%	3.04E-08	0.24
W48	82	48.24%	153	79.69%	-31.45%	3.04E-08	0.24
E50	82	48.24%	153	79.69%	-31.45%	3.04E-08	0.24
S52	82	48.24%	153	79.69%	-31.45%	3.04E-08	0.24
K53	82	48.24%	153	79.69%	-31.45%	3.04E-08	0.24
G61	82	48.24%	153	79.69%	-31.45%	3.04E-08	0.24
R64	82	48.24%	153	79.69%	-31.45%	3.04E-08	0.24
Y80	82	48.24%	153	79.69%	-31.45%	3.04E-08	0.24
Q175	82	48.24%	153	79.69%	-31.45%	3.04E-08	0.24

Table 27: The HLA-DQA1 pocket amino acid zygosity analysis. Homozygous and heterozygous inheritance of shared HLA-DQA1 epitopes associated with T1DM. Association with T1DM is designated with red and resistance with blue.

Locus	HLA-DQA1
Location	69
Epitope	L
P corr value (Homozygous)	$1.07x10^{-12}$
OR (Homozygous)	22.86
P corr value (Heterozygous)	$2.69x10^{-8}$
OR (Heterozygous)	8.82

Table 28: The HLA-DQA1 non-pocket amino acid zygosity analysis. Homozygous and heterozygous inheritance of shared HLA-DQA1 non-pocket epitopes associated with T1DM. Association with T1DM is designated with red and resistance with blue

Locus	HLA-DQA1	HLA-DQA1	HLA-DQA1	HLA-DQA1	HLA-DQA1
Location	25	52	129	129	175
Epitope	F	R	H	Q	E
P corr value (Homozygous)	NA	1.7E-15	2.7E-9	1.7E-5	1.2E-4
OR (Homozygous)	NA	29.9	13.9	0.11	42.96
P corr value (Heterozygous)	6.2E-4	1.1E-11	2.6E-7	2.6E-5	2.0E-8
OR (Heterozygous)	0.31	10.1	7.99	0.36	3.57

Zygosity analysis of the HLA-DQA1 pocket amino acids showed a strong association of Leucine (L) at position 69 in both homozygous or heterozygous state. Non-pocket amino acid that also portrayed a strong homozygosity included DQA R^{52} ($p= 1.7x10^{-15}$, OR 29.9), H^{129} ($p= 2.7x10^{-9}$, OR 13.9), E^{175} ($p= 1.2x10^{-4}$, OR 42.9).

Table 29: The HLA-DQA1 pocket amino acids that are significant ($p<0.05$) and are either associated with susceptibility to T1D (red) or resistance (blue).

HLA LOCUS	DQA1	DQA1	DQA1	DQA1	DQA1	DQA1	DQA1	DQA1	DQA1	DQA1	DQA1	DQA1
Location	11	11	55	55	56	56	66	66	69	69	76	76
EPITOPE	Y	C	R	G	R	G	I	M	L	A	V	M
PATIENT (N=170)	165	82	165	82	133	82	165	82	165	82	133	82
CONTROL (N=192)	141	153	141	153	45	153	141	153	141	153	45	153
Pcorr. Value	4.2E-9	3.0E-8	4.2E-9	3.0E-8	1.6E-15	3.0E-8	4.2E-9	3.0E-8	4.2E-9	3.0E-8	1.6E-15	3.0E-8
OR	11.0	0.21	11.0	0.24	6.8	0.24	11.0	0.24	11.0	0.24	6.8	0.24
Associated alleles	03:01, 05:01, 04:01, 02:01	01:03, 01:02, 01:01	03:01, 05:01, 04:01, 02:01	01:03, 01:02, 01:01	03:01	01:03, 01:02, 01:01	03:01, 05:01, 04:01, 02:01	01:03, 01:02, 01:01	03:01, 05:01, 02:01	01:03, 01:02, 01:01	03:01	01:03, 01:02, 01:01

Table 30: The HLA-DQA1 non- pocket amino acids that are significant (p<0.05) and are either associated with susceptibility to T1D (red) or resistance (blue).

HLA LOCUS	DQA1	DQA1	DQA1	DQA1	DQA1	DQA1	DQA1	DQA1	DQA1	DQA1	DQA1	DQA1	DQA1	DQA1
Location	18	18	25	26	45	45	47	47	48	48	50	50	52	52
EPITOPE	S	F	F	S	V	A	Q	R	L	W	L	E	R	S
PATIENT (N=170)	165	82	13	133	165	82	133	82	165	82	115	82	163	82
CONTROL (N=192)	141	153	42	45	141	153	45	153	141	153	67	153	123	153
Pcorr. Value	4.2E-9	3.0E-8	1.5E-2	1.6E-15	4.2E-9	3.0E-8	1.6E-15	3.0E-8	4.2E-9	3.0E-8	3.2R-8	3.0E-8	3.5E-13	3.0E-8
OR	10.9	0.24	0.30	6.8	10.9	0.24	6.8	0.24	10.9	0.24	3.9	0.24	12.3	0.24
Associated alleles	03:01, 05:01, 04:01, 02:01	01:03, 01:02, 01:01	02:01, 01:03	03:01	03:01, 05:01, 04:01, 02:01	01:03, 01:02, 01:01	03:01	01:03, 01:02, 01:01	03:01, 05:01, 04:01, 02:01	01:03, 01:02, 01:01	03:01, 02:01	01:03, 01:02, 01:01	03:01, 05:01, 04:01	01:03, 01:02, 01:01

Table 30 (continue): The HLA-DQA1 non- pocket amino acids that are significant (p<0.05) and are either associated with susceptibility to T1D (red) or resistance (blue).

HLA LOCUS	DQA1	DQA1	DQA1	DQA1	DQA1	DQA1	DQA1	DQA1	DQA1	DQA1	DQA1	DQA1	DQA1
Location	53	53	61	61	64	64	80	80	129	129	175	175	187
EPITOPE	R	K	F	G	T	R	S	Y	H	Q	E	Q	T
PATIENT (N=170)	115	82	165	82	165	82	165	82	165	81	115	82	133
CONTROL (N=192)	67	153	141	153	141	153	141	153	147	142	67	153	45
Pcorr. Value	3.2E-8	3.0E-8	4.2E-9	3.0E-8	4.2E-9	3.0E-8	4.2E-9	300E-8	2.6E-7	2.3E-5	3.2E-8	3.0E-8	1.6E-15
OR	3.9	0.24	10.9	0.24	10.9	0.24	10.9	0.24	9.2	0.32	3.9	0.24	6.8
Associated alleles	03:01, 02:01	01:03, 01:02, 01:01	03:01, 05:01, 04:01, 02:01	01:03, 01:02, 01:01	03:01, 05:01, 04:01, 02:01	01:03, 01:02, 01:01	03:01, 05:01, 04:01, 02:01	01:03, 01:02, 01:01	03:01, 05:01, 04:01,	01:02, 01:01	03:01, 04:01, 02:01	01:03, 01:02, 01:01	03:01

									02:01, 01:03				

HLA-DQB1 locus

Table 31: Allele frequency analysis for HLA-DQB1 in the Cypriot population. The HLA-DQB1 alleles of the two populations. The table includes presence in the population and frequency, allele number and frequency, delta difference between the T1D and CTL population frequencies, a corrected P-value and the Odds Ratio (OR).

HLA-DQB1 locus											
Allele	Pop (T1D)	Freq (T1D)	Pop (CTL)	Freq (CTL)	Allele (T1D)	Freq (T1D)	Allele (CTL)	Freq (CTL)	Delta	p^corr	OR
02:01	121	71.18%	53	27.60%	151	44.41%	56	14.58%	43.58%	1.05E-15	6.4
03:02	79	46.47%	22	11.46%	84	24.71%	23	5.99%	35.01%	1.19E-12	6.58
03:03	1	0.59%	0	0.00%	1	0.29%	0	0.00%	0.59%	1	3.41
03:04	1	0.59%	0	0.00%	1	0.29%	0	0.00%	0.59%	1	3.41
04:02	1	0.59%	1	0.52%	1	0.29%	1	0.26%	0.07%	1	1.13
06:04	5	2.94%	6	3.13%	5	1.47%	6	1.56%	-0.19%	1	0.95
02:03	0	0.00%	1	0.52%	0	0.00%	1	0.26%	-0.52%	1	0.37
06:01	1	0.59%	6	3.13%	1	0.29%	6	1.56%	-2.54%	1	0.25
03:05	0	0.00%	7	3.65%	0	0.00%	7	1.82%	-3.65%	0.23877	0.07
06:02	0	0.00%	7	3.65%	0	0.00%	7	1.82%	-3.65%	1	0.07
06:03	0	0.00%	13	6.77%	0	0.00%	13	3.39%	-6.77%	0.00387	0.04
05:02	50	29.41%	70	36.46%	50	14.71%	83	21.61%	-7.05%	1	0.73
05:01	31	18.24%	54	28.13%	31	9.12%	59	15.36%	-9.89%	0.51537	0.57
05:03	0	0.00%	31	16.15%	0	0.00%	31	8.07%	-16.15%	2.14E-08	0.02
03:01	15	8.82%	84	43.75%	15	4.41%	91	23.70%	-34.93%	2.85E-13	0.13

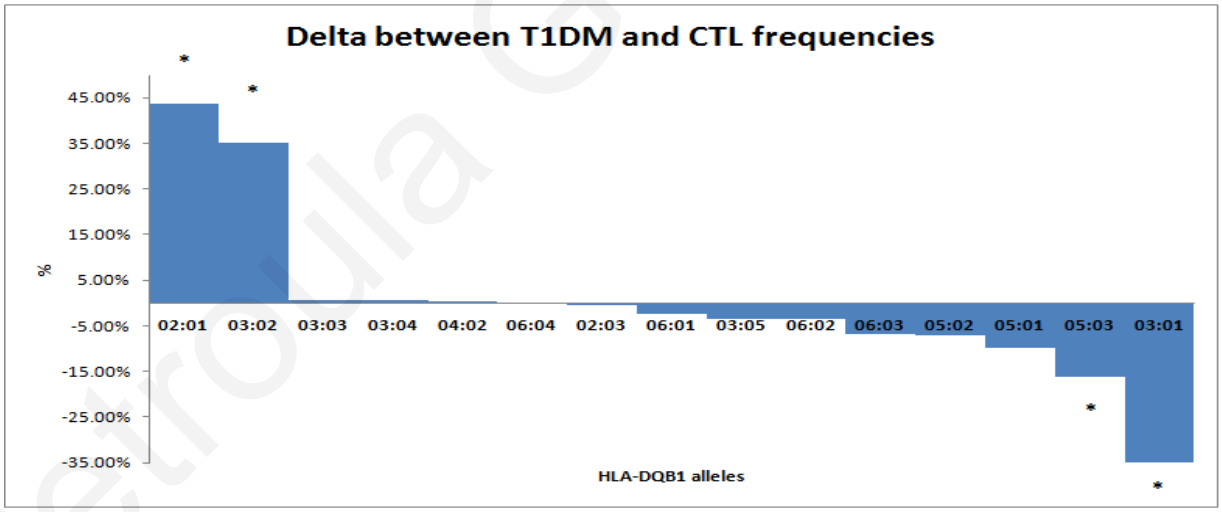


Figure 15: The difference (delta) between T1DM and CTL population frequencies for the HLA-DQB1 alleles. Each HLA-DQB1 allele is plotted against its delta difference between the two populations. Positive % denotes higher presence in the T1DM group, while negative % shows association to the control group. Significance is denoted with * ($p<0.05$).

The HLA-DQB1 allele profile of T1DM cohort and random control population is depicted above in Table 31. The two most dominantly present in the diabetic cohort HLA-DQB1 alleles are DQB1*02:01 (pcorr.= $1.05x10^{-15}$, OR = 6.4) and *03:02 (pcorr.=$1.19x10^{-12}$ and OR= 6.58). In contrast, the HLA-DQB1*05:03 (pcorr. = $2.14x10^{-8}$, OR= 0.13) and DQB1*03:01 ($2.85x10^{-13}$, OR=0.02) were most commonly found in the control population (Figure 15). There is no significant difference in the other allele frequencies between the two groups.

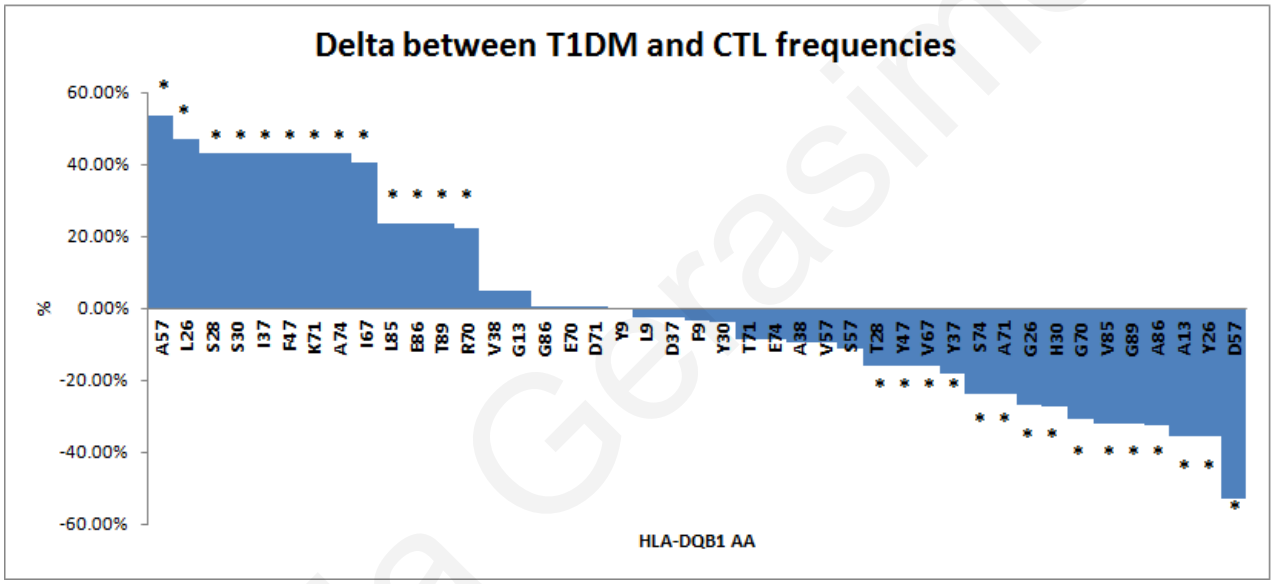


Figure 16: The difference (delta) between T1DM and CTL population frequencies for the HLA-DQB1 pocket amino acids. Each HLA-DQB1 amino acid is plotted against its delta difference between the two populations. Positive % denotes higher presence in the T1DM group, while negative % shows association to the control group. Significance is denoted with * ($p<0.05$).

Table 32: The HLA-DQB1 pocket Amino Acid typing of the two populations. The HLA-DQB1 pocket amino acid typing of the two populations. The table includes presence in the population and frequency, AA number and frequency, delta difference between the T1D and CTL population frequencies, a corrected P-value and the Odds Ratio (OR).

DQB1							
AA at Position	**Population (T1D)**	**Freq (T1D)**	**Population (CTL)**	**Freq (CTL)**	**Delta**	**p^corr**	**OR**
A57	160	94.12%	78	40.63%	53.49%	1.3271E-27	22.296
L26	161	94.71%	92	47.92%	46.79%	1.38E-22	18.47
S28	121	71.18%	54	28.13%	43.05%	1.61451E-14	6.238
S30	121	71.18%	54	28.13%	43.05%	1.61451E-14	6.238
I37	121	71.18%	54	28.13%	43.05%	1.61451E-14	6.238
F47	121	71.18%	54	28.13%	43.05%	1.61451E-14	6.238
K71	121	71.18%	54	28.13%	43.05%	1.61451E-14	6.238
A74	121	71.18%	54	28.13%	43.05%	1.61451E-14	6.238
I67	122	71.76%	60	31.25%	40.51%	7.69065E-13	5.532
L85	165	97.06%	141	73.44%	23.62%	5.22404E-09	10.952
E86	165	97.06%	141	73.44%	23.62%	5.22404E-09	10.952
T89	165	97.06%	141	73.44%	23.62%	5.22404E-09	10.952
R70	166	97.65%	145	75.52%	22.13%	1.55728E-08	12.079
V38	155	91.18%	166	86.46%	4.72%	17.003992	1.597
G13	170	100.00%	183	95.31%	4.69%	0.3703	17.654
G86	6	3.53%	6	3.13%	0.40%	92	0.954
E70	1	0.59%	1	0.52%	0.07%	92	1.13
D71	1	0.59%	1	0.52%	0.07%	92	1.13
Y9	170	100.00%	192	100.00%	0.00%	92	0.886
L9	1	0.59%	6	3.13%	-2.54%	11.6127	0.254
D37	1	0.59%	6	3.13%	-2.54%	11.6127	0.254
F9	1	0.59%	8	4.17%	-3.58%	3.6639	0.192
Y30	90	52.94%	109	56.77%	-3.83%	48.345448	0.857
T71	93	54.71%	122	63.54%	-8.84%	9.901684	0.694
E74	93	54.71%	122	63.54%	-8.84%	9.901684	0.694
A38	92	54.12%	122	63.54%	-9.42%	7.950548	0.678
V57	36	21.18%	59	30.73%	-9.55%	3.902548	0.609
S57	54	31.76%	83	43.23%	-11.46%	16.526328	0.728
T28	140	82.35%	189	98.44%	-16.08%	3.98829E-06	0.085
Y47	140	82.35%	189	98.44%	-16.08%	3.98829E-06	0.085
V67	139	81.76%	188	97.92%	-16.15%	1.10621E-05	0.106
Y37	136	80.00%	189	98.44%	-18.44%	1.87938E-06	0.082
S74	78	45.88%	134	69.79%	-23.91%	0.0003933	0.369
A71	77	45.29%	133	69.27%	-23.98%	0.000399666	0.369
G26	78	45.88%	140	72.92%	-27.03%	1.85509E-05	0.317
H30	81	47.65%	144	75.00%	-27.35%	8.39693E-06	0.306
G70	77	45.29%	146	76.04%	-30.75%	1.83779E-07	0.263
V85	82	48.24%	154	80.21%	-31.97%	1.71368E-08	0.232
G89	82	48.24%	154	80.21%	-31.97%	1.71368E-08	0.232
A86	78	45.88%	151	78.65%	-32.76%	1.29876E-08	0.232
A13	17	10.00%	88	45.83%	-35.83%	1.09692E-12	0.135
Y26	17	10.00%	88	45.83%	-35.83%	1.09692E-12	0.135
D57	17	10.00%	121	63.02%	-53.02%	4.50184E-25	0.067

The HLA-DQB1 pocket amino acid profile of T1DM cohort and random control population is depicted in Table 32 and the non-pocket amino acids are depicted in Table 33. Figure 16 portrays the amino acids that had great difference between the two groups. As in the HLA-DQA1 amino acid analysis, it is observed that the pocket amino acids of HLA-DQB1 that are associated with either resistance or susceptibility to T1DM belong to the same position but with different amino acid. Such pairs include A57 ($p=1.3x10^{-27}$, OR=22.3) versus D57 ($p=4.5x10^{-25}$, OR=0.07), L26 ($p=1.4x10^{-22}$, OR=18.5) versus Y26 ($p=1.1x10^{-12}$, OR=0.14), S28 ($p=1.6x10^{-14}$, OR= 6.2) versus T28 ($p=3.9x10^{-6}$, OR=0.09), S30 ($p=1.6x10^{-14}$, OR= 6.2) versus H30 ($p=8.4x10^{-6}$, OR0.31), I37 ($p=1.6x10^{-14}$, OR= 6.2) versus Y37 ($p=1.8x10^{-6}$, OR=0.08), F47 ($p=1.6x10^{-14}$, OR= 6.2) versus Y47 ($p=3.9x10^{-6}$, OR=0.09), K71 ($p=1.6x10^{-14}$, OR= 6.2) versus A71 (p=0.0004, OR=0.37), A74 (p=1.6x10-14, OR= 6.2) versus S74 (p=0.0004, OR=0.37), I67 (p=7.7x10-13, OR=5.5) versus V67 (p=1.1x10-5, OR=0.11), L85 (p=5.2x10-9, OR=10.9) versus V85 (1.7x10-8, OR=2.3), E86 (p=5.2x10-9, OR=10.9) versus A86 (p=1.3x10-8, OR=0.23) and T89 (p=5.2x10-9, OR=10.9) versus G89 (p=1.7x10-8, OR=0.23). Summarizing tables 36 and 37, show the HLA-DQB1 alleles and their shared amino acid, at each significant pocket and non-pocket position.

Table 33: The HLA-DQB1 non-pocket Amino Acid typing of the two populations. The HLA-DQB1 non-pocket amino acid typing of the two populations. The table includes presence in the population and frequency, AA number and frequency, delta difference between the T1D and CTL population frequencies, a corrected P-value and the Odds Ratio (OR).

DQB1							
AA at Position	**Population (T1D)**	**Freq (T1D)**	**Population (CTL)**	**Freq (CTL)**	**Delta**	**p^corr**	**OR**
E46	121	71.18%	54	28.13%	43.05%	1.61451E-14	6.238
L52	121	71.18%	54	28.13%	43.05%	1.61451E-14	6.238
L55	121	71.18%	54	28.13%	43.05%	1.61451E-14	6.238
D66	122	71.76%	60	31.25%	40.51%	7.69065E-13	5.532
I185	80	47.06%	28	14.58%	32.48%	1.12746E-09	5.134
S125	165	97.06%	133	69.27%	27.79%	0.000399666	0.369
A125	165	97.06%	140	72.92%	24.14%	2.58759E-09	11.244
L53	165	97.06%	141	73.44%	23.62%	5.22404E-09	10.952
Q84	165	97.06%	141	73.44%	23.62%	5.22404E-09	10.952
L87	165	97.06%	141	73.44%	23.62%	5.22404E-09	10.952
T90	165	97.06%	141	73.44%	23.62%	5.22404E-09	10.952
V116	166	97.65%	151	78.65%	19.00%	1.03638E-06	10.135
M14	166	97.65%	152	79.17%	18.48%	2.05749E-06	9.826
V75	156	91.76%	161	83.85%	7.91%	2.350048	2.105
R77	155	91.18%	161	83.85%	7.32%	3.72232	1.957
Q126	170	100.00%	179	93.23%	6.77%	0.02371208	25.646
R167	170	100.00%	183	95.31%	4.69%	0.3703	17.654
G45	170	100.00%	185	96.35%	3.65%	1.464456	13.787
A140	157	92.35%	175	91.15%	1.21%	65.03388	1.163
S182	157	92.35%	175	91.15%	1.21%	65.03388	1.163
S3	170	100.00%	191	99.48%	0.52%	92	2.671
Q130	6	3.53%	6	3.13%	0.40%	92	0.954
L56	1	0.59%	1	0.52%	0.07%	92	1.13
P56	170	100.00%	192	100.00%	0.00%	92	0.886
R130	170	100.00%	192	100.00%	0.00%	92	0.886
S197	170	100.00%	192	100.00%	0.00%	92	0.886
P55	89	52.35%	104	54.17%	-1.81%	69.209392	0.93
T185	164	96.47%	189	98.44%	-1.97%	29.012752	0.467
T140	89	52.35%	105	54.69%	-2.33%	61.998432	0.911
N182	89	52.35%	105	54.69%	-2.33%	61.998432	0.911
P3	1	0.59%	6	3.13%	-2.54%	11.6127	0.254
N197	1	0.59%	6	3.13%	-2.54%	11.6127	0.254
L75	93	54.71%	122	63.54%	-8.84%	9.901684	0.694
T77	93	54.71%	123	64.06%	-9.36%	7.890748	0.679
H126	54	31.76%	83	43.23%	-11.46%	16.526328	0.728
F87	1	0.59%	26	13.54%	-12.95%	6.34699E-05	0.056
G125	6	3.53%	32	16.67%	-13.14%	0.004328876	0.195
V46	140	82.35%	189	98.44%	-16.08%	3.98829E-06	0.085
P52	140	82.35%	189	98.44%	-16.08%	3.98829E-06	0.085
E66	139	81.76%	188	97.92%	-16.15%	1.10621E-05	0.106
Y87	81	47.65%	136	70.83%	-23.19%	0.000880274	0.377
L14	77	45.29%	133	69.27%	-23.98%	0.000399666	0.369
I116	77	45.29%	133	69.27%	-23.98%	0.000399666	0.369
R55	83	48.82%	155	80.73%	-31.91%	1.50402E-08	0.23
Q53	82	48.24%	154	80.21%	-31.97%	1.71368E-08	0.232
E84	82	48.24%	154	80.21%	-31.97%	1.71368E-08	0.232
I90	82	48.24%	154	80.21%	-31.97%	1.71368E-08	0.232
E45	16	9.41%	84	43.75%	-34.34%	6.18746E-12	0.137
H167	17	10.00%	88	45.83%	-35.83%	1.09692E-12	0.135

Table 34: The HLA-DQB1 pocket amino acid zygosity analysis. Homozygous and heterozygous inheritance of shared HLA-DQB1 pocket epitopes associated with T1DM. Association with T1DM is designated with red and resistance with blue.

Locus	HLA-DQB1	HLA-DQB1	HLA-DQB1	HLA-DQB1	HLA-DQB1	HLA-DQB1	HLA-DQB1	HLA-DQB1
Location	26	30	37	57	70	70	74	86
Epitope	L	H	Y	A	R	G	S	A
P corr value (Homozygous)	4.3E-21	2.1E-7	5.6E-11	1.3E-24	1.3E-10	2.3E-8	6.6E-5	3.9E-9
OR (Homozygous)	45.8	0.1	0.05	100.6	17.6	0.08	0.13	0.07
P corr value (Heterozygous)	3.9E-19	3.4E-5	5.0E-5	5.6E-23	2.8E-8	1.3E-6	3.5E-4	8.7E-8
OR (Heterozygous)	14.9	0.4	0.13	17.5	10.4	0.31	0.42	0.28

Table 35: The HLA-DQB1 non-pocket amino acid zygosity analysis. Homozygous and heterozygous inheritance of shared HLA-DQB1 non-pocket epitopes associated with T1DM. Association with T1DM is designated with red and resistance with blue.

Locus	HLA-DQB1	HLA-DQB1	HLA-DQB1	HLA-DQB1	HLA-DQB1	HLA-DQB1	HLA-DQB1	HLA-DQB1	HLA-DQB1
Location	14	45	55	87	87	116	125	125	185
Epitope	M	E	R	Y	F	V	A	G	I
P corr value (Homozygous)	2.E-7	NA	1.0E-9	3.3E-5	NA	1.8E-7	1.34E-11	NA	NA
OR (Homozygous)	10.9	NA	0.07	0.14	NA	11.1	18.57	NA	NA
P corr value (Heterozygous)	2.5E-7	2.0E-13	1.8E-7	7.5E-4	2.1E-6	1.3E-7	9.4E-9	1.4E-4	1.8E-11
OR (Heterozygous)	9.34	0.14	0.27	0.43	0.06	9.7	9.5	0.2	5.5

Homozygosity of susceptibility epitope DQB1 A^{57} had the strongest association with T1DM ($p= 1.25x10^{-24}$, OR 100.6), whilst inheritance of two copies of the resistance epitope at the same position, DQB1 D^{57}, had the strongest negative association ($p= 3.94x10^{-9}$, OR 0.03). A gene dose effect was apparent since inheritance of one copy of the A^{57} epitope decreased the probability of disease as shown by the lower OR ($p= 5.61x10^{-23}$, OR 17.5), in the same way inheritance of one copy of the susceptibility epitope D^{57} ($p= 4.8x10^{-22}$, OR 0.08) was not as protective as inheritance of two copies but still lowered probability of disease as compared to inheritance of one copy of A^{57}. However, resistance appears to be dominant since inheritance of only one copy of D^{57} still conferred a lower probability of diabetes (OR 0.08). A similar effect was observed for DQB1 L^{26}, inheritance of two copies is strongly associated with T1DM ($p= 4.3x10^{-21}$, OR 45.8), inheritance of one copy greatly decreased the probability of disease ($p= 3.91x10^{-19}$, OR 14.9) and two copies of G^{26} is protective ($p= 8.68x10^{-6}$, OR 0.11). Non-pocket amino acid that also portrayed a strong homozygosity included DQB1 M^{14} ($p= 2.0x10^{-7}$, OR 10.9), V^{116} ($p= 1.8x10^{-7}$, OR 11.1), A^{125} ($p= 1.34x10^{-11}$, OR 18.6).

Table 36: The HLA-DQB1 pocket amino acids that are significant ($p<0.05$) and are either associated with susceptibility to T1D (red) or resistance (blue).

HLA LOCUS	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1
Location	13	26	26	26	28	28	30	30	37	37	47	47	57	57	67	67
EPITOPE	A	L	Y	G	S	T	S	H	I	Y	F	Y	A	D	I	V
PATIENT (N=170)	17	161	17	78	121	140	121	81	121	136	121	140	160	17	122	139
CONTROL (N=192)	88	92	88	140	54	189	54	144	54	189	54	189	78	121	60	188
Pcorr. Value	1.1E-12	1.4E-22	1.1E-12	1.9E-5	1.6E-14	4.0E-6	1.6E-14	8.4E-6	1.6E-14	1.9E-6	1.6E-14	4.0E-6	1.3E-27	4.5E-25	7.7E-13	1.1E-5
OR	0.14	18.47	0.14	0.32	6.24	0.09	6.2	0.31	6.2	0.08	6.2	0.09	22.3	0.07	5.5	0.11
Associated alleles	03:04, 06:01, 03:01	02:01, 03:02, 03:03, 06:04, 02:03, 06:02, 06:03	03:04, 06:01, 03:01	04:02, 03:05, 05:02, 05:01, 05:03	02:01, 02:03	03:02, 03:03, 03:04, 04:02, 06:04, 06:01, 03:05, 06:02, 06:03, 05:02, 05:01, 05:03, 03:01	02:01, 02:03	06:04, 06:03, 05:02, 05:01, 05:03	02:01, 02:03	03:02, 03:03, 03:04, 04:02, 06:04, 03:05, 06:02, 06:03, 05:02, 05:01, 05:03, 03:01	02:01, 02:03	03:02, 03:03, 03:04, 04:02, 06:04, 06:01, 03:05, 06:02, 06:03, 05:02, 05:01, 05:03, 03:01	02:01, 03:02, 03:04, 03:05	03:03, 04:02, 02:03, 06:01, 06:02, 06:03, 05:03, 03:01	02:01, 04:02, 02:03, 06:01	03:02, 03:03, 03:04, 06:04, 03:05, 06:02, 06:03, 05:02, 05:01, 05:03, 03:01

Table 36 (continue): The HLA-DQB1 pocket amino acids that are significant ($p<0.05$) and are either associated with susceptibility to T1D (red) or resistance (blue)

HLA LOCUS	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1
Location	70	70	71	71	74	74	85	85	86	86	89	89
EPITOPE	R	G	K	A	A	S	L	V	E	A	T	G
PATIENT (N=170)	166	77	121	77	121	78	165	82	165	78	165	82
CONTROL (N=192)	145	146	54	133	54	134	141	154	141	151	141	154
Pcorr. Value	1.6E-8	1.8E-7	1.6E-14	0.0004	1.6E-14	0.0004	5.2E-9	1.7E-8	5.2E-9	1.3E-8	5.2E-9	1.7E-8
OR	12.1	0.26	6.2	0.37	6.2	0.37	11.0	0.23	11.0	0.23	11.0	0.23
Associated alleles	02:01, 03:02, 03;03, 03:04, 06:04, 02:03, 06:01, 03:05, 03:01	06:02, 06:03, 05:02, 05:01, 05:03	02:01, 02:03	05:02, 05:01, 05:03	02:01, 02:03	04:02, 05:02, 05:01, 05:03	02:01, 03:02, 03:03, 03:04, 04:02, 02:03, 03:05, 03:01	06:04, 06:01, 06:02, 06:03, 05:02, 05:01, 05:03	02:01, 03:02, 03:03, 03:04, 04:02, 02:03, 03:05, 03:01	06:01, 06:02, 06:03, 05:02, 05:01, 05:03	02:01, 03:02, 03:03, 03:04, 04:02, 02:03, 03:05, 03:01	06:04, 06:01, 06:02, 06:03, 05:02, 05:01, 05:03

Table 37: The HLA-DQB1 non- pocket amino acids that are significant ($p<0.05$) and are either associated with susceptibility to T1D (red) or resistance (blue).

HLA LOCUS	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1
Location	14	14	45	46	52	52	53	53	55	55	66	66	84	84
EPITOPE	M	L	E	E	L	P	L	Q	L	R	D	E	Q	E
PATIENT (N=170)	166	77	16	121	121	140	165	82	121	83	122	139	165	82
CONTROL (N=192)	152	133	84	54	54	189	141	154	54	155	60	188	141	154
Pcorr. Value	2.1E-8	0.0004	6.2E-12	1.6E-14	1.6E-14	4.0E-6	5.2E-9	1.7E-8	1.6E-14	1.5E-8	7.7E-13	1.1E-5	5.2E-9	1.7E-8
OR	9.8	0.37	0.14	6.2	6.2	0.09	10.9	0.23	6.2	0.23	5.5	0.11	10.9	0.23
Associated alleles	02:01, 03:02, 03:03, 03:04, 04:02, 06:04, 02:03, 06:01, 03:05, 06:02, 06:03, 03:01	05:02, 05:01, 05:03	03:04, 03:01	02:01, 02:03	02:01, 02:03	03:02, 03:03, 03:04, 04:02, 06:04, 06:01, 03:05, 06:02, 06:03, 05:02, 05:01, 05:03, 03:01	02:01, 03:02, 03:03, 03:04, 04:02, 02:03, 03:05, 03:01	06:04, 06:01, 06:02, 06:03, 05:02, 05:01, 05:03	02:01, 02:03	04:02, 06:04, 06:01, 06:02, 06:03, 05:02, 05:01, 05:03	02:01, 04:02, 02:03, 06:01	03:02, 03:03, 03:04, 06:04, 03:05, 06:02, 06:03, 05:02, 05:01, 05:03, 03:01	02:01, 03:02, 03:03, 03:04, 04:02, 02:03, 03:05, 03:01	06:04, 06:01, 06:02, 06:03, 05:02, 05:01, 05:03

Table 37 (continue): The HLA-DQB1 non- pocket amino acids that are significant ($p<0.05$) and are either associated with susceptibility to T1D (red) or resistance (blue).

HLA LOCUS	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1	DQB1
Location	87	87	87	90	90	116	116	125	125	125	167	185
EPITOPE	L	Y	F	T	I	V	I	A	G	S	H	I
PATIENT (N=170)	165	81	1	165	82	166	77	165	6	165	17	80
CONTROL (N=192)	141	136	26	141	154	151	133	140	32	133	88	28
Pcorr. Value	5.2E-9	0.0009	6.3E-5	5.2E-9	1.7E-8	1.0E-6	0.0004	2.6E-9	0.004	0.0004	1.1E-12	1.1E-9
OR	10.9	0.38	0.06	10.9	0.23	10.1	0.37	11.2	0.20	0.37	0.14	5.1
Associated alleles	02:01, 03:02, 03:03, 03:04, 04:02, 02:03, 03:05, 03:01	06:04, 05:02, 05:01, 05:03	06:01, 06:02, 06:03	02:01, 03:02, 03:03, 03:04, 04:02, 02:03, 03:05, 03:01	06:04, 06:01, 06:02, 06:03, 05:02, 05:01, 05:03	02:01, 03:02, 03:03, 03:04, 04:02, 06:04, 06:01, 03:05, 06:02, 06:03, 03:01	05:02, 05:01, 05:03	02:01, 03:02, 03:03, 03:04, 04:02, 03:05, 03:01	06:04, 06:01, 06:02, 06:03	05:02, 05:01, 05:03	03:04, 06:01, 03:01	03:02, 03:03, 04:02, 03:05

HLA-DPB1 locus

Table 38: Allele frequency analysis for HLA-DPB1min the Cypriot population. The HLA-DPB1 typing of the two populations. The table includes presence in the population and frequency, allele number and frequency, delta difference between the T1D and CTL population frequencies, a corrected P-value and the Odds Ratio (OR).

HLA-DPB1 locus											
Allele	Pop (T1D)	Freq (T1D)	Pop (CTL)	Freq (CTL)	Allele (T1D)	Freq (T1D)	Allele (CTL)	Freq (CTL)	Delta	p^corr	OR
03:01	51	30.00%	25	13.30%	56	16.47%	26	6.91%	16.70%	0.00306	2.76
02:01	90	52.94%	74	39.36%	105	30.88%	82	21.81%	13.58%	0.21908	1.73
30:01	5	2.94%	1	0.53%	5	1.47%	2	0.53%	2.41%	1	4.15
06:01	4	2.35%	0	0.00%	4	1.18%	0	0.00%	2.35%	0.99807	10.19
01:01	3	1.76%	0	0.00%	3	0.88%	0	0.00%	1.76%	1	7.88
17:01	12	7.06%	10	5.32%	12	3.53%	11	2.93%	1.74%	1	1.34
19:01	1	0.59%	0	0.00%	1	0.29%	0	0.00%	0.59%	1	3.34
51:01	1	0.59%	0	0.00%	1	0.29%	0	0.00%	0.59%	1	3.34
11:01	1	0.59%	1	0.53%	1	0.29%	1	0.27%	0.06%	1	1.11
15:01	0	0.00%	1	0.53%	0	0.00%	1	0.27%	-0.53%	1	0.37
71:01	0	0.00%	1	0.53%	0	0.00%	1	0.27%	-0.53%	1	0.37
14:01	17	10.00%	20	10.64%	18	5.29%	21	5.59%	-0.64%	1	0.94
46:01	0	0.00%	2	1.06%	0	0.00%	2	0.53%	-1.06%	1	0.22
04:01	92	54.12%	104	55.32%	112	32.94%	126	33.51%	-1.20%	1	0.95
05:01	1	0.59%	4	2.13%	1	0.29%	4	1.06%	-1.54%	1	0.36
09:01	2	1.18%	6	3.19%	2	0.59%	6	1.60%	-2.01%	1	0.42
23:01	1	0.59%	5	2.66%	1	0.29%	5	1.33%	-2.07%	1	0.3
13:01	8	4.71%	13	6.91%	8	2.35%	14	3.72%	-2.20%	1	0.68
10:01	5	2.94%	16	8.51%	5	1.47%	16	4.26%	-5.57%	0.80695	0.35
04:02	5	2.94%	58	30.85%	5	1.47%	62	16.49%	-27.91%	4.62E-12	0.07

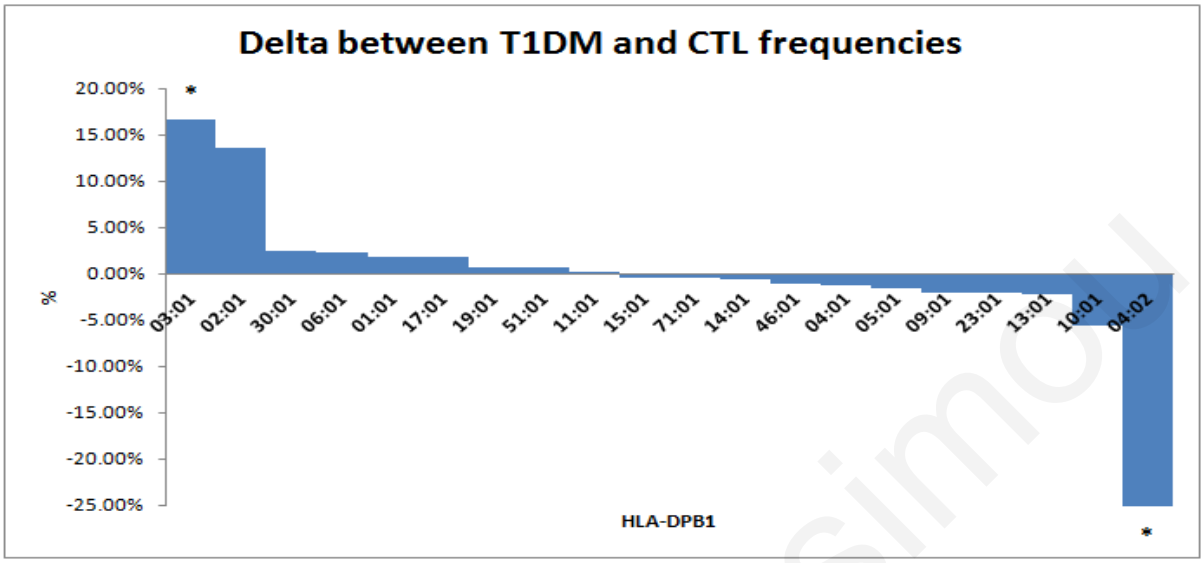


Figure 17: The difference (delta) between T1DM and CTL population frequencies for the HLA-DPB1 alleles. Each HLA-DPB1 allele is plotted against its delta difference between the two populations. Positive % denotes higher presence in the T1DM group, while negative % shows association to the control group. Significance is denoted with * ($p<0.05$).

The HLA-DPB1 allele profile of T1DM cohort and random control population is depicted above in Table 38. The HLA-DPB1 showed one allele to be strongly significantly present in the diabetic cohort and one allele to be strongly significantly present in the control population (Figure 17). HLA-DPB1*03:01 was most frequent in the diabetic cohort, 30%, as opposed to 13.3% in the control group (pcorr=0.003, OR=2.76). The allele with the highest frequency in the control group was the DPB1*04:02 with a significant pcorr. of 4.2×10^{-12} and OR of 0.07.

Table 39: The HLA-DPB1 pocket Amino Acid typing of the two populations. The HLA-DPB1 pocket amino acid typing of the two populations. The table includes presence in the population and frequency, AA number and

frequency, delta difference between the T1D and CTL population frequencies, a corrected P-value and the Odds Ratio (OR).

DPB1							
AA at Position	**Population (T1D)**	**Freq (T1D)**	**Population (CTL)**	**Freq (CTL)**	**Delta**	**p^corr**	**OR**
Y9	64	37.65%	38	19.79%	17.86%	0.01292232	2.367
L65	70	41.18%	45	23.44%	17.74%	0.02967874	2.212
L11	93	54.71%	82	42.71%	12.00%	2.032234	1.557
E69	116	68.24%	108	56.25%	11.99%	1.755774	1.586
V76	74	43.53%	61	31.77%	11.76%	1.74317	1.601
D84	95	55.88%	85	44.27%	11.61%	2.081776	1.531
V87	95	55.88%	85	44.27%	11.61%	2.081776	1.531
D55	144	84.71%	153	79.69%	5.02%	22.165008	1.261
V36	145	85.29%	158	82.29%	3.00%	35.460894	1.098
F35	169	99.41%	187	97.40%	2.02%	46	0.904
A36	100	58.82%	110	57.29%	1.53%	46	1.013
E55	7	4.12%	5	2.60%	1.51%	25.783874	1.53
A55	100	58.82%	113	58.85%	-0.03%	38.171766	0.948
R69	1	0.59%	2	1.04%	-0.45%	46	0.66
V84	0	0.00%	1	0.52%	-0.52%	46	0.367
Y35	11	6.47%	14	7.29%	-0.82%	38.46957	0.868
M76	156	91.76%	178	92.71%	-0.94%	13.62612	0.635
I65	161	94.71%	184	95.83%	-1.13%	7.208108	0.415
K69	143	84.12%	164	85.42%	-1.30%	20.706394	0.777
I76	9	5.29%	13	6.77%	-1.48%	30.384104	0.765
L35	1	0.59%	4	2.08%	-1.50%	17.23505	0.363
G11	152	89.41%	176	91.67%	-2.25%	8.384742	0.584
H9	38	22.35%	48	25.00%	-2.65%	24.666074	0.842
F9	151	88.82%	176	91.67%	-2.84%	6.091734	0.55
G84	149	87.65%	175	91.15%	-3.50%	4.764864	0.535
M87	149	87.65%	175	91.15%	-3.50%	46	0.535

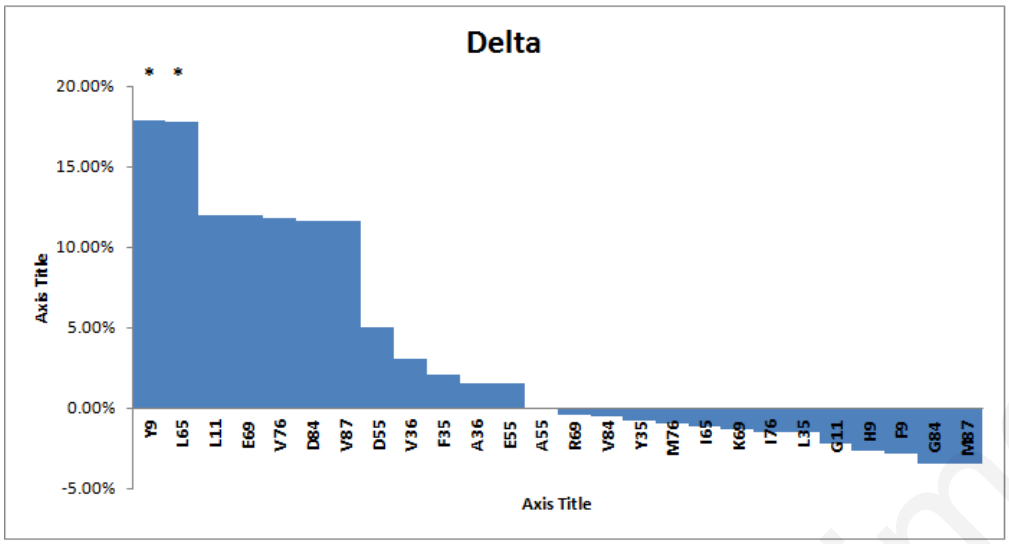


Figure 18: The difference (delta) between T1DM and CTL population frequencies for the HLA-DPB1 pocket amino acids. Each HLA-DPB1 amino acid is plotted against its delta difference between the two populations. Positive % denotes higher presence in the T1DM group, while negative % shows association to the control group. Significance is denoted with * ($p<0.05$).

The HLA-DPB1 pocket amino acid profile of T1DM cohort and random control population is depicted in Table 39 and the non-pocket amino acids are depicted in Table 40. Figure 18 portrays the amino acids that had great difference between the two groups. Only two pocket amino acids were associated with susceptibility to diabetes: Y9 ($p=0.01$, $OR=2.4$) and L65 ($p=0.03$, $OR=2.2$). No HLA-DPB1 pocket amino acids were associated with resistance to diabetes. The only non-pocket amino acid that showed a significant p-value of 1.1x10-11 and OR of 0.07 was M178. Summarizing tables 40 and 41, show the HLA-DQB1 alleles and their shared amino acid, at each significant pocket and non-pocket position.

Table 40: The HLA-DPB1 non-pocket Amino Acid typing of the two populations. The HLA-DPB1 non-pocket amino acid typing of the two populations. The table includes presence in the population and frequency, AA number and frequency, delta difference between the T1D and CTL population frequencies, a corrected P-value and the Odds Ratio (OR).

DPB1							
AA at Position	**Population (T1D)**	**Freq (T1D)**	**Population (CTL)**	**Freq (CTL)**	**Delta**	**p^corr**	**OR**
D57	80	47.06%	59	30.73%	16.33%	0.151892	1.936
V8	94	55.29%	83	43.23%	12.06%	1.57021	1.598
E85	95	55.88%	85	44.27%	11.61%	2.081776	1.531
A86	95	55.88%	85	44.27%	11.61%	2.081776	1.531
K96	76	44.71%	65	33.85%	10.85%	2.40097	1.526
I170	76	44.71%	65	33.85%	10.85%	2.40097	1.526
E56	144	84.71%	153	79.69%	5.02%	22.165008	1.261
L178	168	98.82%	181	94.27%	4.55%	8.242096	2.785
E33	170	100.00%	188	97.92%	2.08%	46	0.905
A56	106	62.35%	116	60.42%	1.94%	42.024404	1.028
Q194	2	1.18%	0	0.00%	1.18%	4.879772	7.878
R194	168	98.82%	188	97.92%	0.91%	10.340524	0.179
Q33	1	0.59%	2	1.04%	-0.45%	46	0.66
E57	158	92.94%	182	94.79%	-1.85%	6.645482	0.452
L8	151	88.82%	176	91.67%	-2.84%	6.091734	0.55
R96	148	87.06%	173	90.10%	-3.05%	7.530982	0.59
T170	148	87.06%	173	90.10%	-3.05%	7.530982	0.59
G85	149	87.65%	175	91.15%	-3.50%	46	0.535
P86	149	87.65%	175	91.15%	-3.50%	46	0.535
M178	5	2.94%	62	32.29%	-29.35%	1.06154E-11	0.074

Table 41: The HLA-DPB1 pocket amino acids that are significant ($p<0.05$) and are either associated with susceptibility to T1D (red) or resistance (blue).

HLA LOCUS	DPB1
Location	9
EPITOPE	Y
PATIENT (N=170)	64
CONTROL (N=192)	38
Pcorr. Value	0.01
OR	2.3
Associated alleles	03:01, 06:01, 01:01, 11:01, 15:01, 13:01

Table 42: The HLA-DPB1 non- pocket amino acids that are significant ($p<0.05$) and are either associated with susceptibility to T1D (red) or resistance (blue).

HLA LOCUS	DPB1
Location	178
EPITOPE	M
PATIENT (N=170)	5
CONTROL (N=192)	62
Pcorr. Value	1.1E-11
OR	0.07
Associated alleles	04:02

3.2.3 The HLA Risk Haplotype distribution of T1DM patients.

HLA gene variants in T1DM have been established as primary susceptibility determinants that confer as much as 50-60% of the overall disease risk (Noble and Erlich, 2012). Class II haplotypes, HLA-DR3/DQ2 and HLA-DR4/DQ8, carry the strongest association to disease with up to 50% of patient carrying both haplotypes (van Lummel et al., 2012). Genotypic combinations that phenotypically portray greater risk include DRB1*03:01-DQA1*05:01-DQB1*02:01 (**DR3**) and DRB1*04:xx-DQA1*03:01-DQB1*03:02 (**DR4**) (Erlich et al., 2008). Therefore, through the course of this study we have divided our patient cohort into groups according to the disease associated risk haplotype with focus on the two risk conferring alleles; DRB1*03 (DR3) and DRB1*04 (DR4). The six main genotype groups are as follows: DRB1*03:01-DQA1*05:01-DQB1*02:01 homozygous (DR3 homozygous), DRB1*04:xx-DQA1*03:01-DQB1*03:02 homozygous (DR4 homozygous), DRB1*03:01-DQA1*05:01-DQB1*02:01/DRB1*04:xx-DQA1*03:01-DQB1*03:02 heterozygoys (DR3/DR4 heterozygous), DRB1*03:01-DQA1*05:01-DQB1*02:01 heterozygous (DR3 heterozygous), DRB1*04:xx-DQA1*03:01-DQB1*03:02 (DR4 heterozygous) and Risk Haplotype absence being any other DRB1 group other than the risk conferring ones.

Table 43: Frequency analysis of T1DM Risk Haplotypes. The risk haplotype number (frequency %) in the T1DM and control (CTL) group. Significance is denoted with p<0.05. DR3 - DQ2.5 = DRB1*03:01-DQA1*05:01-DQB1*02:01, DR4 - DQ8 = DRB1*04:XX-DQA1*03:01-DQB1*03:02, DR4 - DQ2.3 = DRB1*04:XX-DQA1*03:01-DQB1*02:01.

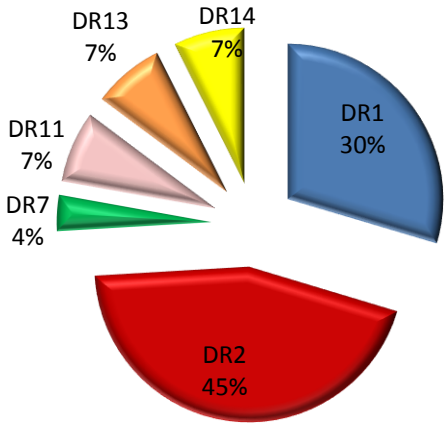
HLA class II Genotype	Patients (%) (N=170)	Control subjects (%) (N=192)	P value	OR
DR3 - DQ2.5 / DRX	30 (17.65)	22 (11.46)	0.1	1.66
DR3 - DQ2.5 / DR3 - DQ2.5	18 (10.59)	0	<0.0001	46.7
DR3 - DQ2.5 / DR4 - DQ2.3	5 (2.94)	1 (0.52)	0.1	5.79
DR4 - DQ8 / DRX	43 (25.29)	18 (9.38)	<0.0001	3.27
DR4 - DQ8 / DR4 - DQ8	5 (2.94)	1 (0.52)	0.1	5.79
DR4 - DQ8 / DR4 - DQ2.3	4 (2.35)	0	0.047	10.41
DR3 - DQ2.5 / DR4 - DQ8	30 (17.65)	1 (0.52)	<0.0001	40.93
DR4 - DQ2.3 / DRX	22 (12.94)	8 (4.17)	0.004	3.42
DRX / DRX (non-risk)	13 (7.65)	141 (73.44)	<0.0001	0.03

Table 44: Frequency analysis of T1DM Risk Haplotypes in the three age of onset groups. The risk haplotypes in the three age of onset groups. Significance is denoted with p<0.05.

HAPLOTYPE / ALLELE	Age Group 1	Age group 2	Age group 3	P-VALUE
non-Risk	11	11	13	
DR3/X	14	11	11	
DR3/DR3	3	8	7	
DR3/DR4	13	11	5	
DR4/X	18	17	12	
DR4/DR4	0	2	3	0.431
DR3 Presence	30	30	23	0.813
DR4 Presence	31	30	20	0.341

We aimed to further dissect our T1DM patient cohort with regards to their HLA genotypes in order to potentially explain disease susceptibility due to the presence of shared susceptibility epitopes. More specifically, among our T1DM patients the majority (135 of 170 or 79%) carried at least one or both of the susceptibility alleles DR3 - DQ2.5 and DR4 - DQ8 (Table 43). However, a significant number of patients (35 of 170 or 21%) could not be explained by the presence of the risk alleles as they did not carry any copies of either DR3 - DQ2.5 or DR4 - DQ8. We observed that a new haplotype DRB1*04:XX-DQA1*03:01-DQB1*02:01, that we are henceforth naming DR4 - DQ2.3, was dominant within this subgroup; 22 out of the 35 patients (63%) carrying non-risk haplotypes and 13% of all T1DM patients carried one copy of the DR4 - DQ2.3 haplotype. In contrast, only 9 of 192 control (4.7%) subjects carried the DR4 - DQ2.3 haplotype. Interestingly, no DR4 - DQ2.3 homozygous individuals were identified. The stratification of the different Risk Haplotype combinations among the three age of onset groups revealed no significant difference between them (Table 44).

a)



b)

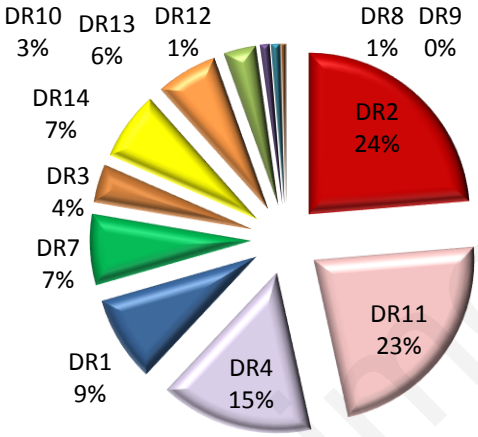
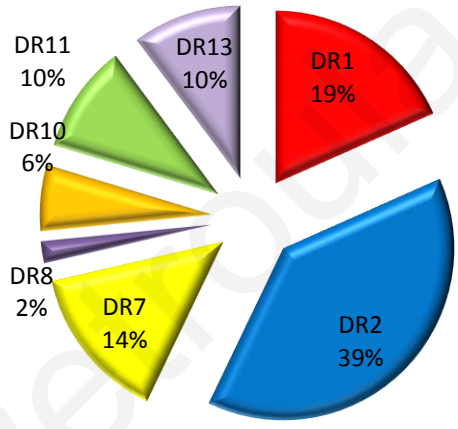


Figure 19: DRB1 partner allele to the risk associated DRB1*03.a) The DRB1 'X' partner allocation of the DRB1*03/ DRB1*X T1DM patients. b) Distribution of DRB1 'X' partner allocation of the DRB1*03/ DRB1*X in the general Cypriot population (n=123242).

a)



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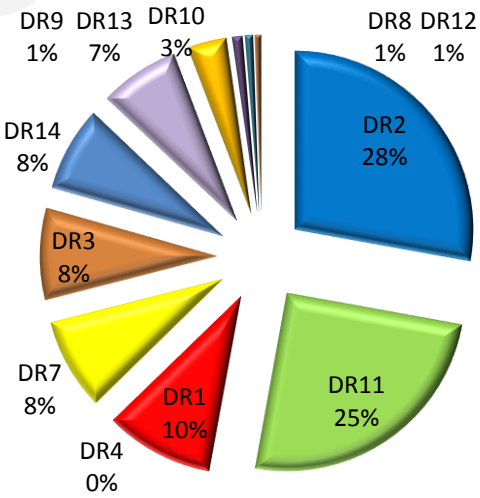

Figure 20: DRB1 partner allele to the risk associated DRB1*04 alleles The DRB1 'X' partner allocation of the DRB1*04/ DRB1*X T1DM patients (n=182). Distribution of DRB1 'X' partner allocation of the DRB1*04/ DRB1*X in general Cypriot population (n=123242).

The DRB1 'X' partner allocation of the DRB1*03/ DRB1*X T1DM patients is shown in Figure 19 along with the distribution of DRB1 'X' partner allocation of the DRB1*03/

DRB1*X in general Cypriot population (n=123242). As seen in the figure, there is a DRB1*02 (split antigens DRB1*15 and *16) prevalence followed by the DRB1*01 group. A similar depiction is observed in Figure 20, where the DRB1 'X' partner allocation of the DRB1*04/ DRB1*X T1DM patients shows a DRB1*02 (split antigens DRB1*15 and *16) prevalence followed by the DRB1*01 group.

Using the Cypriot general population allele frequencies, we also calculated the expected frequencies of the most significant class I alleles previously described to ensure that our randomly selected population was in fact representative of the general population. It is obvious in Figure 21 that the random control population is in fact representative of the general population and none of the alleles were over-represented. Furthermore, the frequencies of the most significant class I alleles were also calculated in the presence of the risk allele to decipher any linkage disequilibrium (LD) between the two and reveal any false significance observed in the diabetic group. Figure 22 shows that HLA alleles A*24:02, B*08:01 and B*41:01 frequencies observed are as expected from the general population. Therefore, it is concluded that these alleles are in LD with the risk haplotype. Nevertheless, HLA alleles A*25:01, B*50:01 and C*14:03 frequencies are observed significantly more in the diabetic population than expected from the general population, showing a selection bias in diabetes. For detailed haplotype frequencies please refer to Appendix I.

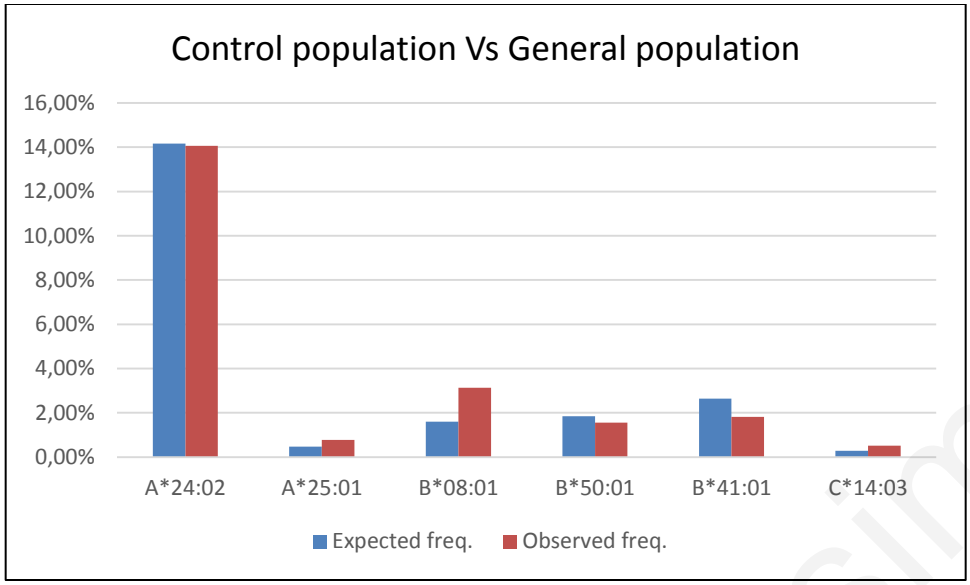

Figure 21: T1DM associated HLA-class I alleles frequency. Expected frequencies of the T1DM associated significant alleles from the general population against the observed frequencies in the randomly selected control population used in this study.

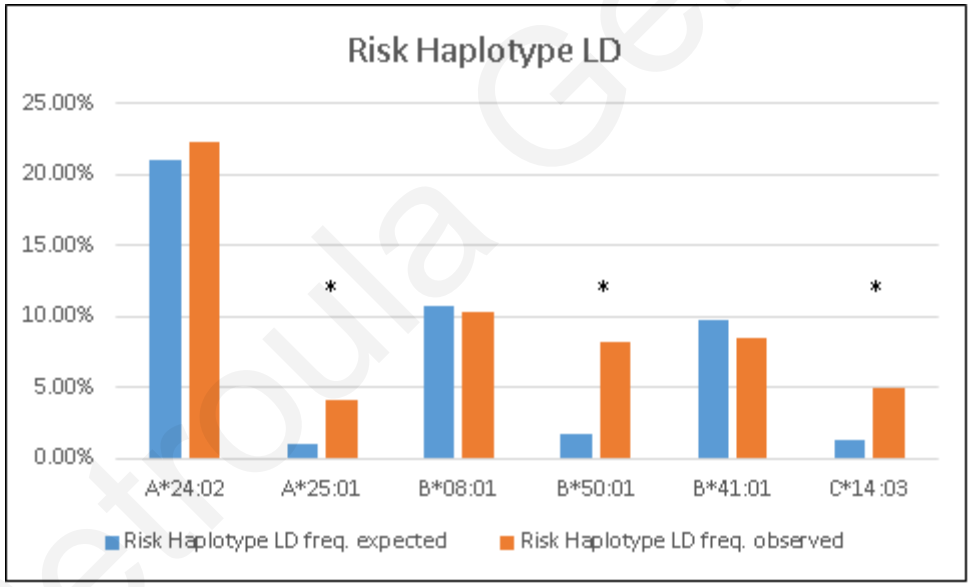

Figure 22: T1DM associated HLA-class I alleles frequency in LD with risk haplotype. Comparison of the expected frequencies versus the observed frequencies of the most significant alleles if in linkage disequilibrium with the Risk Haplotype.

Alignment of susceptibility and resistance alleles

The sequences of risk and protective HLA class II alleles identified by the allele frequency analysis were retrieved and aligned using the IMGT/HLA database of the European Bioinformatics Institute. Within these sequences we noted all the susceptibility and resistance epitopes, both pocket and non-pocket epitopes, which were identified in this study to investigate what differentiates risk and protective alleles. The risk associated alleles HLA-DRB1*04:02 and *04:05, as well as the protective alleles HLA-DRB1*10:01, *11:04, *13:01, *14:01 and *16:02 are shown in Figure 23. The risk associated HLA-DQB1*02:01 and *03:02 and protective alleles HLA-DQB1*05:03 and *03:01 are shown in Figure 24 and the risk associated HLA-DQA1*03:01 and protective alleles HLA-DQA1*01:01 and *01:03 are shown in Figure 25. As expected, the highest polymorphism for all alleles was observed within the 100 first amino acids which contain the antigen binding pockets. Overall, all risk associated HLA alleles contain more susceptibility epitopes and protective alleles contain more protective alleles. Some epitopes, however, might not be as critical as others. For example, HLA-DRB1 susceptibility epitopes E^{9} and L^{67} and resistance epitopes A^{73} and T^{77} are found in both risk and protective HLA-DRB1 alleles (Figure 23). In contrast, all risk HLA-DRB1 alleles contain the susceptibility epitope Q^{70}, which is absent from the protective alleles. The only notable exception is the risk allele HLA-DRB1*04:02 that contains the R^{70} resistance epitope instead. The sequence of this risk associated allele is very similar to the sequence of protective allele HLA-DRB1*13:01, except for the presence of susceptibility epitopes V^{11} and H^{13} in *04:02, but also the presence of a number of non-pocket susceptibility epitopes H^{33}, Y^{96}, E^{98}, A^{104}, N^{120} and L^{180}. This might suggest that non-pocket epitopes could play a role in altering the structure of the molecule, and thus affecting the binding pockets. Epitopes also alter the susceptibility of closely related alleles. For example, HLA-DQB1*03:01 and *03:02 have very similar amino acid sequences but differ in critical position 57, risk associated HLA-DQB1*03:02 contains alanine (A), whereas protective HLA-DQB1*03:01 contains aspartic acid (D) in the same position (Figure 24). In addition, risk associated HLA-DQB1*03:02 also contains the susceptibility epitope L^{26}.

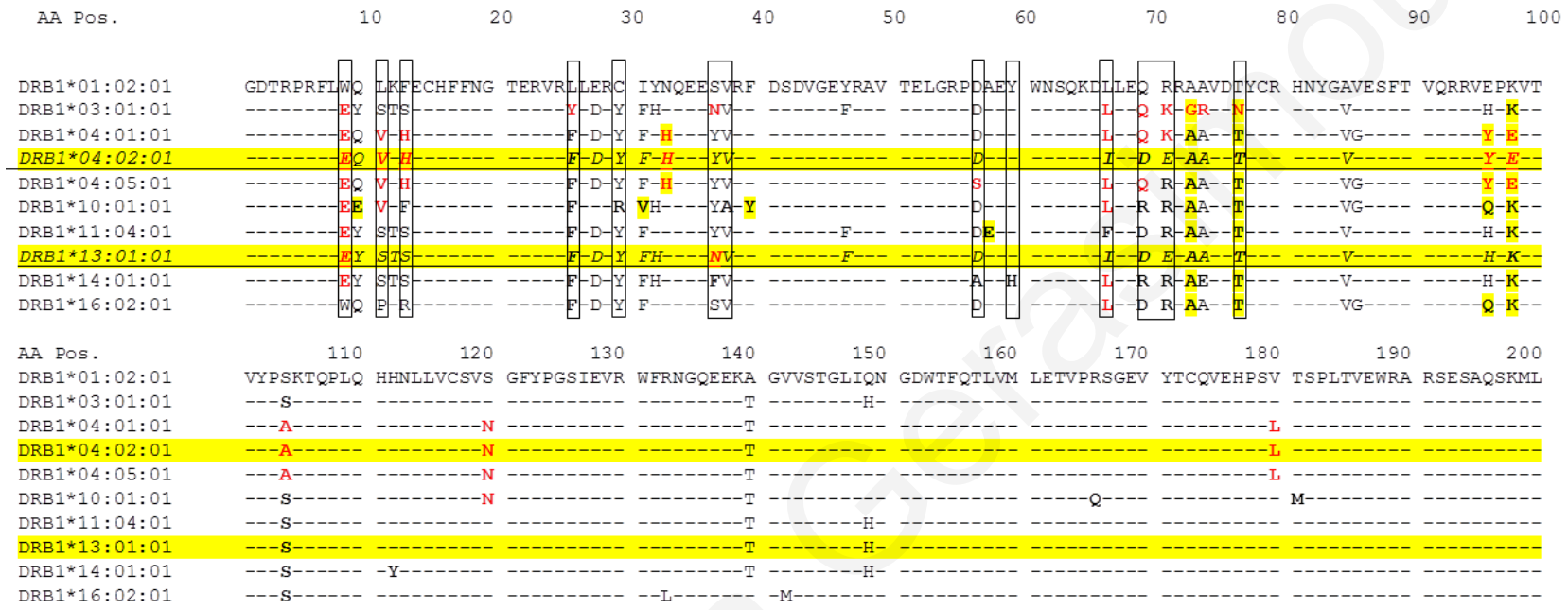

Figure 23: HLA-DRB1 allele alignment. The sequences of risk alleles HLA-DRB1*04:02 and *04:05 and protective alleles HLA-DRB1*10:01, *11:04, *13:01, *14:01 and *16:02 were retrieved and aligned using the IMGT/HLA database of the European Bioinformatics Institute. Susceptibility and resistance pocket and non-pocket epitopes identified in this study are presented. Resistance epitopes are shown in bold, susceptibility epitopes are shown in red, pocket epitopes are shown in boxes, non-pocket epitopes are highlighted. The sequence of susceptibility allele HLA-DRB1*04:02 and resistance allele HLA-DRB1*13:01 are also highlighted from comparison purposes.

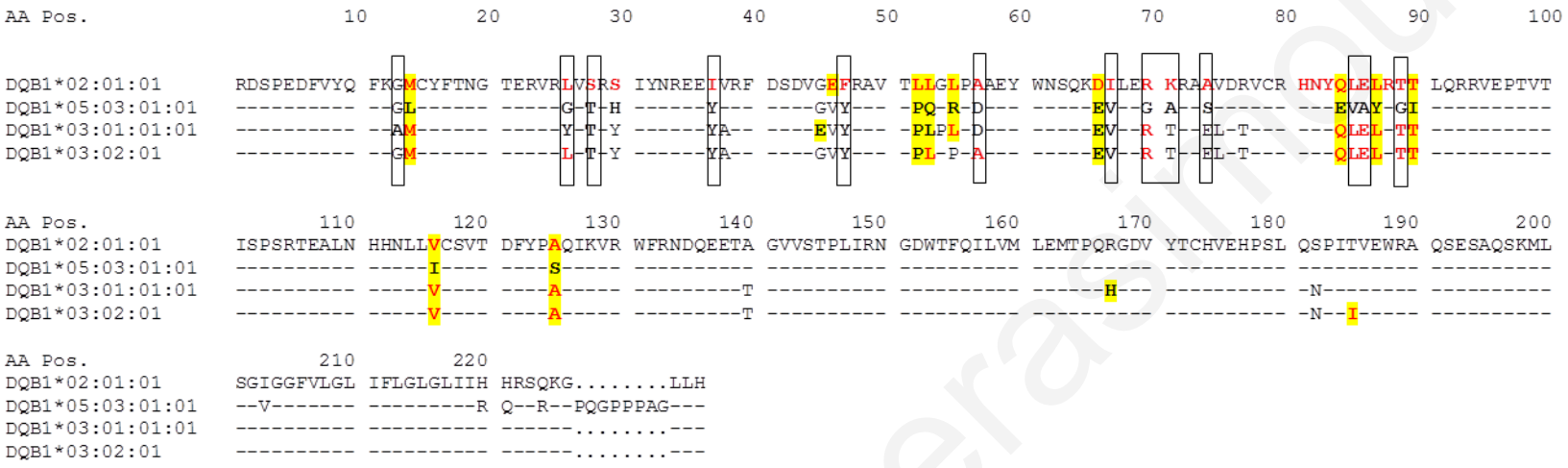

Figure 24: HLA-DQB1 allele alignment. The sequences of risk alleles HLA-DQB1*02:01 and *03:02 and protective alleles HLA-DQB1*05:03 and *03:01 were retrieved and aligned using the IMGT/HLA database of the European Bioinformatics Institute. Susceptibility and resistance pocket and non-pocket epitopes identified in this study are presented. Resistance epitopes are shown in bold, susceptibility epitopes are shown in red, pocket epitopes are shown in boxes, non-pocket epitopes are highlighted.

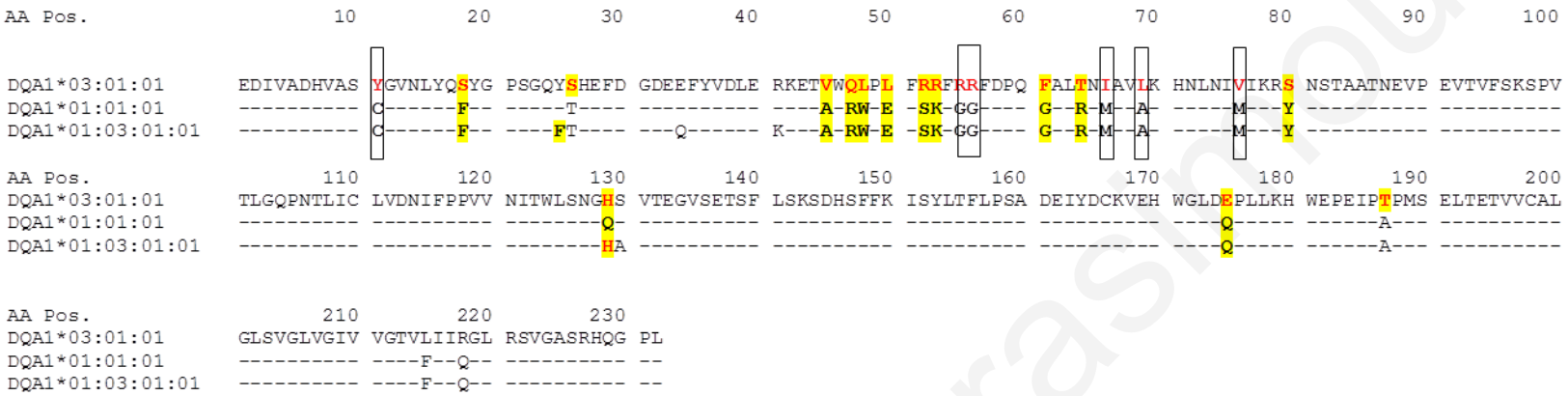

Figure 25: HLA-DQA1 allele alignment. The sequences of risk allele HLA-DQA1*03:01 and protective alleles HLA-DQA1*01:01 and *01:03 were retrieved and aligned using the IMGT/HLA database of the European Bioinformatics Institute. Susceptibility and resistance pocket and non-pocket epitopes identified in this study are presented. Resistance epitopes are shown in bold, susceptibility epitopes are shown in red, pocket epitopes are shown in boxes, non-pocket epitopes are highlighted.

Table 45: Linkage disequilibrium of DR/DQ susceptibility epitopes in T1DM patients and control subjects

HLA class II susceptibility epitopes	Patients (%) (N=170)	Control subjects (%) (N=192)	LD in patients P value (OR)	LD in controls P value (OR)
DRβ Q^{70+}/DQβ A^{57+}	153 (90)	57 (29.7)	0.001 (20.5)	$1.80x10^{-6}$ (4.9)
DRβ Q^{70+}/DQβ A^{57-}	5 (2.9)	40 (20.8)		
DRβ Q^{70-}/DQβ A^{57+}	7 (4.1)	21 (10.9)		
DRβ Q^{70-}/DQβ A^{57-}	5 (2.9)	74 (38.5)		

The prevalence of having both the highest susceptibility epitopes DRβ Q^{70} and the DQβ A^{57} was compared between T1DM patients and control subjects. The vast majority of patients (90%) had both epitopes compared with only 29.7% of the control subjects ($p \leq 0.0001$, OR 21.3). A very small percentage of patients had only the DRβ Q^{70} epitope (2.9%) or only the DQβ A^{57} epitope (4.1%) or none of the two epitopes (2.9%). Finally, among control subjects the highest percentage (38.5%) had none of these susceptibility epitopes ($p \leq 0.0001$, OR 0.05). The above analysis was also performed to test LD in DQα $Y^{11}R^{52}R^{55}F^{61}T^{64}I^{66}L^{69}V/L^{76}H^{129}E/K^{175}$, DRβ Q^{70} and DQβ A^{57}. The prevalence of both DQ and DR susceptibility epitopes was higher in patients than in control subjects and was not exclusively a result of linkage disequilibrium, suggesting that multiple HLA epitopes may work together to increase the risk of developing diabetes. Lastly, the SKDM software performed a combined action analysis and suggested that all the above parameters have a combined action.

HLA epitopes associated with susceptibility and resistance and their potential function

Further dissection of the most important identified amino acids that either renders susceptibility or resistance to T1DM according to their potential function is summarized in tables 37 to 42 according to loci. The DQA, DQB and DRB domains show a considerable number of polymorphisms that are mainly involved in antigen binding by the anchoring pockets, the heterodimer formation, T-cell receptor (TCR) binding, CD4 co-receptor binding and in the formation of the dimer of heterodimers.

The DRB1 amino acids that are part of the pockets and therefore the binding groove formation and are associated with diabetes are 9E, 11V, 13H, 26Y, 37N and 74R. The residue at position 57 is involved in the pocket 9 but also is responsible for the hydrogen bond formation to the peptide. A serine in that position was highly associated to T1DM while an alanine was

associated to susceptibility. The amino acids at positions 67, 70 and 71 were also part of the pocket formation but are also sites for TCR contact and had two residues that showed significance either with susceptibility or resistance. Lastly, amino acid at position 112 that was significant has a potential function in the homodimer of heterodimers and the amino acid at position 140 is a potential contact side for the CD4 co-receptor.

DQB1 included several amino acids that were significant in susceptibility or resistance to T1DM. Amino acids involved in the peptide pocket formation include positions 13, 26, 28, 30, 37, 47, 57, 67, 70, 71, 74, 85, 86, 89 and 90. Amino acids at position 30 and 57 are also involved in the formation of a hydrogen bond to the peptide while residues at position 67, 70 and 71 are also a potential TCR contact site. Amino acids 52, 53 and 55 act as homodimerization patch in the molecules dimer formation. Proposed amino acid functions are denoted above as previously described.(Schafer et al., 1995, Brown et al., 1993, Bondinas et al., 2007)

Pocket amino acids of the DQA1 molecule include residues at positions 11, 52, 66, 69 and 76. Amino acids at positions 69 and 76 also form a hydrogen bond to the peptide. Important amino acids from position 55 to 64 are potential TCR contact sites while amino acid at position 129 is a potential CD4 contact site. The position 175 residue upholds a function in the formation of the homodimer of heterodimers.

Table 46: Single-letter code of T1DM associated amino acids of polymorphic positions of DQA1 alleles.

	Amino Acid position									
									CD4	
			TCR				**HB**			**HH**
	P6	**P1**				**P6**	**P6/9**	**P9**		
DQA1 allele	**11**	**52**	**55**	**61**	**64**	**66**	**69**	**76**	129	**175**
Positively Associated alleles										
03:01	**Y**	**R**	**R**	**F**	**T**	**I**	**L**	**V**	H	**E**
05:01	**Y**	**R**	**R**	**F**	**T**	**I**	**L**	**L**	H	**K**
Negatively Associated alleles										
01:01	**C**	**S**	**G**	**G**	**R**	**M**	**A**	**M**	Q	**Q**
01:02	**C**	**S**	**G**	**G**	**R**	**M**	**A**	**M**	Q	**Q**
01:03	**C**	**S**	**G**	**G**	**R**	**M**	**A**	**M**	H	**Q**

Note: The position(s) identified in bold show amino acids that are exclusive to the T1DM positively or negatively associated alleles. Function of each amino acid is depicted on top. P; pocket number, HH; homodimer of heterodimer, HB; hydrogen bond to peptide, TCR; T-cell receptor contact site to HLA molecule.

Table 47: Single-letter code of the T1DM associated amino acids of polymorphic positions of DRB1 alleles

	Amino Acid position												
						HB						HH	
								TCR			TCR		CD4
	P9	P6	P4	P4	P9	P9	P7	P7	P4/7	P4			
DRB1 allele	9	11	13	26	37	57	67	**70**	71	74	77	112	140
Positively Associated alleles													
03:01	E	S	S	Y	N	D	L	**Q**	K	R	N	H	T
04:01	E	V	H	F	Y	D	L	**Q**	K	A	T	H	T
04:05	E	V	H	F	Y	S	L	**Q**	R	A	T	H	T
Negatively Associated alleles													
10:01	E	V	F	V	Y	D	L	**R**	R	A	T	H	T
11:04	E	S	S	F	Y	D	F	**D**	R	A	T	H	T
14:01	E	S	S	F	F	A	L	**R**	R	E	T	Y	T
16:02	W	P	R	F	S	D	L	**D**	R	A	T	H	A

Note: The position(s) identified in bold show amino acids that are exclusive to the T1DM positively or negatively associated alleles. Function of each amino acid is depicted on top. P; pocket number, HH; homodimer of heterodimer, HB; hydrogen bond to peptide, TCR; T-cell receptor contact site. CD4; CD4 contact site to HLA molecule.

Table 48: Single-letter code of the T1DM associated amino acid sequences of polymorphic position of DQB1 alleles

	Amino Acid position																	
							HH				TCR							
				HB						SB								
	P4	P4	P4/7	P6	P9	P7				P9		P7	P4	P4/7	P4	P1	P1	P1	P1
DQB1 allele	13	**26**	28	30	37	47	52	53	55	**57**	66	67	70	71	74	85	86	89	90
Positively Associated alleles																			
02:01	G	**L**	S	S	I	F	L	L	L	**A**	D	I	R	K	A	L	E	T	T
03:02	G	**L**	T	Y	Y	Y	P	L	P	**A**	E	V	R	T	E	L	E	T	T
Negatively Associated alleles																			
03:01	A	**Y**	T	Y	Y	Y	P	L	L	**D**	E	V	R	T	E	L	E	T	T
05:03	G	**G**	T	H	Y	Y	P	Q	P	**D**	E	V	G	A	S	V	A	G	I

Note: The position(s) identified in bold show amino acids that are exclusive to the T1DM positively or negatively associated alleles. Function of each amino acid is depicted on top. P; pocket number, HH; homodimer of heterodimer, SB; salt bridge, TCR; T-cell receptor contact site to HLA molecule, HB; hydrogen bond to peptide.

Re-evaluation of the HLA alleles according to their amino acid composition suggests contribution of the HLA molecules DR/DQ to T1DM pathogenesis. Amino acids DQβ L^{26} and

A^{57} that are part of the binding pocket but also at position that contributes in the stability of the heterodimer, are mainly found in the risk DQB1 alleles *02:01 and *03:02 (Table 46 and 47). Similarly, all the significant amino acids on the risk DQA1 *03:01 and *05:01 alleles are unique from all the other alleles (Table 48). Therefore, re-calculating the risk of our T1DM cohort it was shown that 97% of our patients were carriers of the DQα **$Y^{11}R^{52}R^{55}F^{61}T^{64}I^{66}L^{69}V/L^{76}H^{129}E/K^{175}$** with or without the DRβ **Q^{70}** and/or DQβ **$L^{26}A^{57}$**, while the remaining 3% patients carried the DRβ **Q^{70}** and/or DQβ **$L^{26}A^{57}$** only.

3.3 HLA-G GENOTYPING

HLA-G has also been recently investigated in its involvement in inflammatory and autoimmune disease. The HLA-G receptor functions towards immune inhibition and tolerance through direct immune-inhibitory functions by effectors cells blocking, regulatory cell generation through indirect immune-inhibitory functions and other HLA-G functions that result in immune-inhibition. Studies on HLA involvement in T1DM predisposition also include independent associations with HLA class I alleles (Noble et al., 2002), suggesting a particular role in the age of onset of T1DM (Noble et al., 2002, Noble et al., 2006, Noble et al., 2010, Nejentsev et al., 2007, Howson et al., 2009). A study by Baschal, et al. (2011) suggested that risk for type I diabetes lies in polymorphisms of the telomeric MHC locus, a region closest to classical and non-classical HLA class I alleles (Baschal et al., 2011). For these reasons, this study investigated the involvement of this allele in the context of diabetes and possible association with other HLA alleles.

3.3.1 The HLA-G 3'UTR 14bp polymorphism

Patients and controls were characterized in the context of the 3'-UTR 14bp *DEL/INS (rs371194629)*, with frequencies not revealing any significant difference between the two groups in either genotype or allele presence (P>0.05). In fact, the distribution of the two alleles was in Hardy-Weinberg equilibrium in both groups.

Table 49: Comparison of the HLA-G 3'UTR 14bp polymorphism between the T1DM patients and the control population. HLA-G 3'UTR 14bp polymorphism allele and genotype presence or absence between the T1DM cohort and control population. No significance was observed.

	POPULATIONS				
	DIABETIC		CONTROL		
HLA-G	**PRESENCE**	**ABSENCE**	**PRESENCE**	**ABSENCE**	**p-value**
DELETION allele	142 (84%)	28 (16%)	153 (80%)	39 (20%)	> 0.05
INSERTION allele	114 (67%)	56 (33%)	137 (71%)	55 (29%)	
I/I genotype	28 (16%)	142 (84%)	39 (20%)	153 (80%)	> 0.05
I/D genotype	86 (51%)	84 (49%)	98 (51%)	94 (49%)	
D/D genotype	56 (33%)	114 (67%)	55 (29%)	138 (71%)	

Table 50: Comparison of the HLA-G 3'UTR 14bp polymorphism between T1DM patients that are carriers or non-carriers of the disease associated HLA-DR/DQ risk haplotypes. HLA-G 3'UTR 14bp polymorphism allele and genotype presence in comparison with the frequency of disease associated HLA-DR/DQ risk haplotype in the T1DM cohort. (DR3; DRB1*03:01-DQA1*05:01-DQB1*02:01, DR4; DRB1*04:xx-DQA1*03:01-DQB1*03:02). No significance was observed.

	DIABETIC		
HLA-G	**Risk Haplotype CARRIERS**	**Risk Haplotype ABSENCE**	**p-value**
DELETION allele	113 (84%)	29 (83%)	> 0.002
INSERTION allele	88 (65%)	26 (74%)	
I/I genotype	22 (16%)	6 (17%)	> 0.002
I/D genotype	66 (49%)	20 (57%)	
D/D genotype	47 (35%)	9 (26%)	

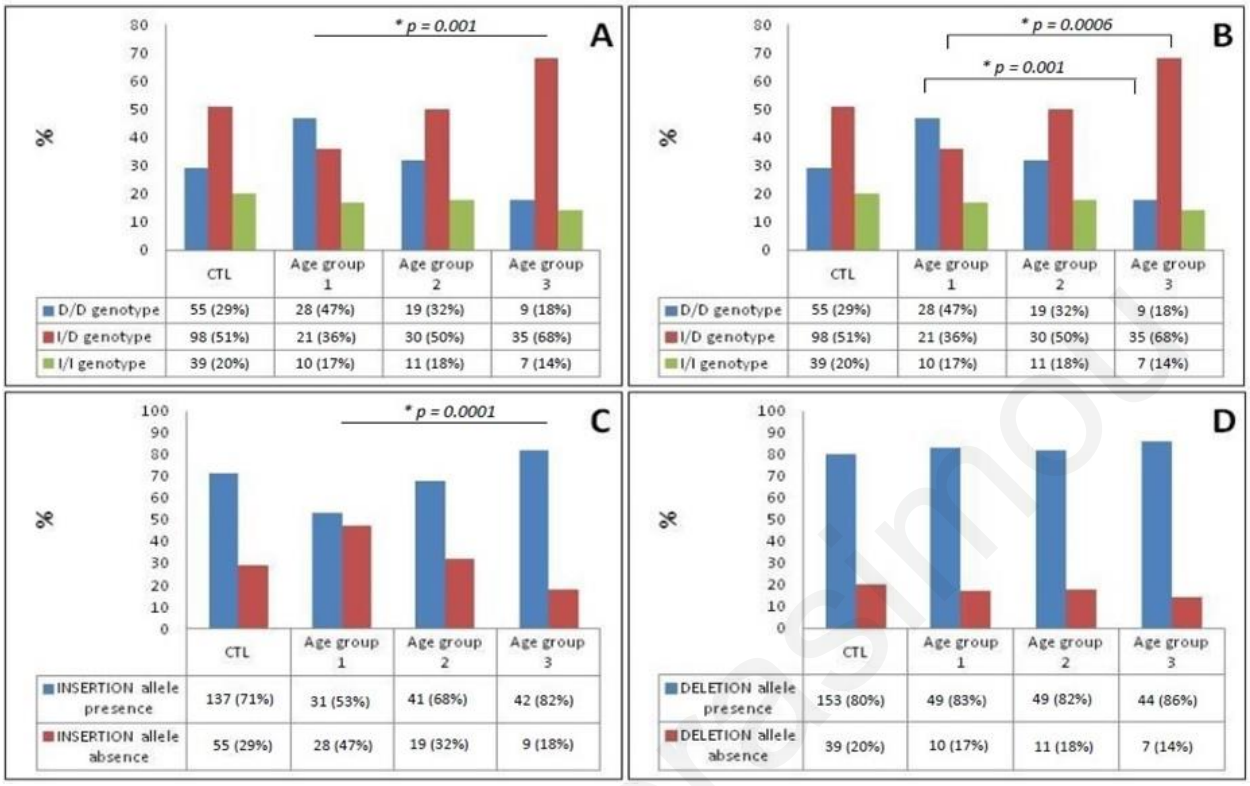

Figure 26: HLA-G 14bp genotype frequencies (%) and age of onset A. The Freeman-Halton extension of the Fisher exact probability test for a 2x3 contingency table was performed to determine significance between HLA-G 14bp genotype frequencies (%) and age of onset 0 to 8 years old (Age group 1), age of onset 9-13 years old (Age group 2), age of onset 14-39 years old (Age group 3) and control group. **B.** The 2x2 Fisher exact statistical test was performed to determine significant difference between the three age-of-onset groups in terms of individual HLA-G 14bp genotype frequencies (%). **C. and D**. The 2x2 Fisher exact statistical test was performed to determine significance between the presence and absence of the Insertion or Deletion allele at the different age groups and control population. * Significance was denoted with a 'P' value less than 0.002, corrected using the Bonferoni multiple testing correction system (n=29).

Stratification of patients into age of onset groups revealed genotypic significant differences among groups, with the most significant being the difference between age group 1 and age group 3, with a P value of 0.001. Further analysis to decipher if the presence or absence of an HLA-G 14bp genotype was responsible for the significant difference among these two groups, revealed that the presence of the *D/D* genotype was significantly higher in the Age group 1 than in Age group 3 (p=0.001), while the opposite was observed with the *I/D* genotype, for which the numbers are higher in the Age group 3 (p=0.0006). No significant difference was observed for the *I/I* genotype among age groups (Figure 26A and 1B). Our cohort was further dissected according to *INS* or *DEL* allele presence/absence, in order to investigate the contribution of either the *INS* or *DEL* allele in the aforementioned significance. This stratification revealed that statistical significant difference lies among Age group 1 and Age group 3, but only in the presence or absence of the *INS* allele (p=0.0001) and not in the presence

or absence of the *DEL* allele (p>0.002) (Figure 26C and 26D). The statistical comparison of the control population and the age groups presented no significant difference in either the two alleles or the genotypes, while the frequencies followed a similar pattern in both groups.

In order to investigate the independent association of the *HLA-G INS/DEL* polymorphism from the diabetes associated risk haplotype presence, patients were also stratified according to *DRB1*03:01-DQA1*05:01-DQB1*02:01 (DR3) and DRB1*04:xx-DQA1*03:01-DQB1*03:02 (DR4)* haplotypes. 79% of T1DM patients were carriers for the disease associated risk haplotype, while only 20% of the control population were carriers. Comparison of the *HLA-G* 3'UTR 14bp polymorphism between the T1DM patients that are carriers and non-carriers of the disease associated *HLA-DR/DQ* risk haplotype revealed no significant skewness of a particular genotype or allele presence towards one group. *HLA-G INS/DEL* polymorphism allocation among *HLA-DR3, HLA-DR4* and absence of risk haplotypes portrayed a similar pattern showing no significant presence of a particular *HLA-G* genotype at a specific *HLA-DR/DQ* (Figure 27).

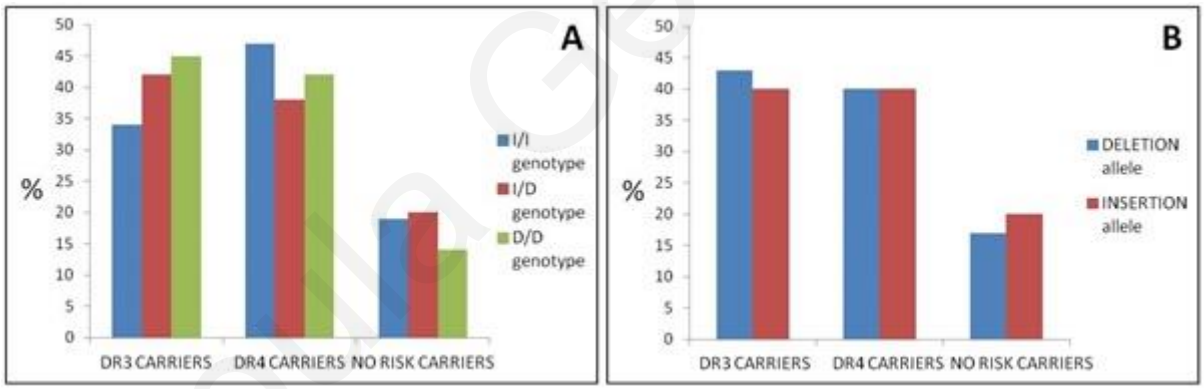

Figure 27: HLA-G 3'UTR 14bp polymorphism allele and genotype according to risk-associated class II haplotype. HLA-G 3'UTR 14bp polymorphism allele and genotype presence or absence allocated according to patients' DRB1*03:01-DQA1*05:01-DQB1*02:01 (DR3) and DRB1*04:xx-DQA1*03:01-DQB1*03:02 (DR4) haplotypes. No significant difference was observed among the different groups.

3.3.2 The HLA-G promoter, genotype and 3'UTR haplotypes

The HLA-G promoter haplotypes were analysed by our laboratory and Dr. Castelli's Laboratory in Sao Paolo for confirmation and probable identification of new alleles. Our laboratory performed a Fisher's excact and a Logit analysis to determine significance between T1DM patients and controls where as Dr. Castelli's Laboratory performed a GWASpi analysis. Patient and control numbers and frequencies as well as delta differences between the groups are shown in the following tables.

Promoter Haplotype	T1D n=182	Frequency %	CTL n=192	Frequency %	Delta	p-value	Logit p-value
Promo 0104a	67	36.81%	56	29.17%	7.65%	0.124	0.500
Promo 010101f	11	6.04%	7	3.65%	2.40%	0.337	0.282
Promo 0103e	7	3.85%	5	2.60%	1.24%	0.566	0.302
Promo 010101a	95	52.20%	98	51.04%	1.16%	0.409	0.751
Promo-recom-unkown	5	2.75%	4	2.08%	0.66%	0.745	0.225
Promo 0103d	15	8.24%	16	8.33%	-0.09%	1.000	0.304
Promo 010102aLikeA	0	0.00%	1	0.52%	-0.52%	1.000	NA
Promo 010101c	4	2.20%	6	3.13%	-0.93%	0.751	0.947
Promo 010101b	30	16.48%	38	19.79%	-3.31%	0.424	0.381
Promo 010102a	95	52.20%	119	61.98%	-9.78%	0.060	0.010

Table 51: The HLA-G promoter haplotypes. The combination of a set of promoter SNPs are deciphered in extended promoter-haplotypes previously described (Castelli et al., 2014b). The table summarizes the HLA-promoter haplotype frequencies, frequency difference (delta) and p-value with Fisher's Exact and Logit.

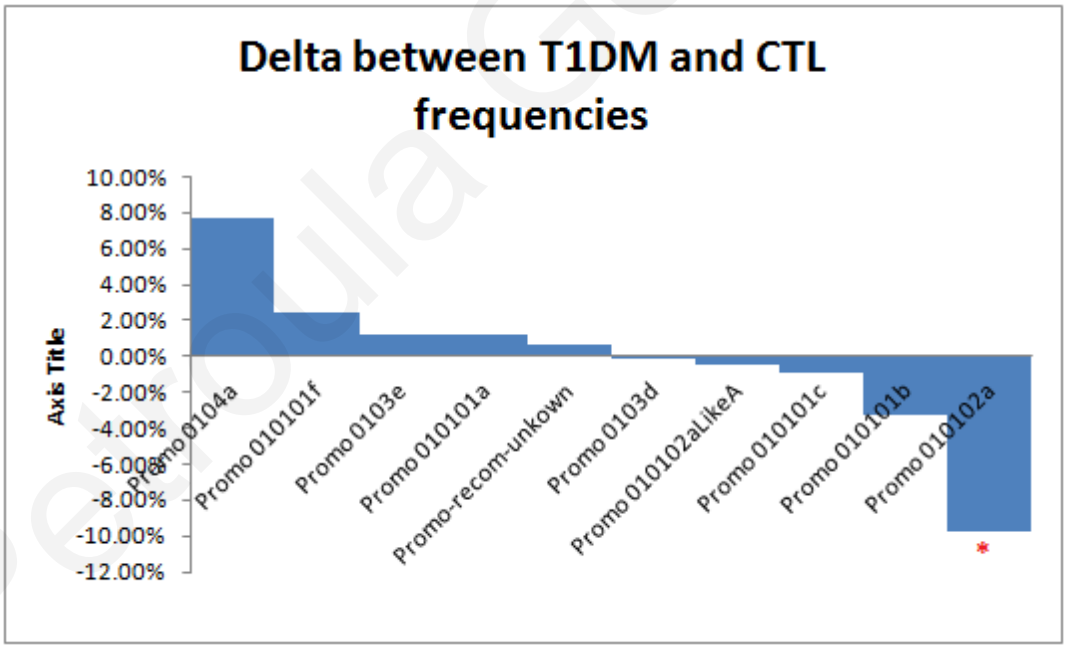

Figure 28: The difference between the T1DM and CTLs' HLA-G promoter haplotypes. Each HLA-G promoter haplotype is plotted against its delta difference between the two populations. Positive % denotes higher presence in the T1DM group while negative % show association to the control group. Significance is denoted with black * (Fisher's exact $p<0.05$). Red * denoted significance with Logit analysis.

Table 52: The HLA-G genotypes. Summary of the HLA-promoter haplotype frequencies, frequency difference (delta) and p-value with Fisher's Exact and Logit

HLA-G Genotypes	T1D n=182	Frequency %	CTL n=192	Frequency %	Delta	p-value	Logit p-value
G010401	66	36.26%	53	27.60%	8.66%	0.077	0.046
G01010103	24	13.19%	16	8.33%	4.85%	0.136	0.018
G01010101	73	40.11%	68	35.42%	4.69%	0.393	0.003
G01010106	7	3.85%	1	0.52%	3.33%	0.033	0.012
G0105N	11	6.04%	8	4.17%	1.88%	0.483	0.061
G010404	7	3.85%	4	2.08%	1.76%	0.370	0.160
G01010104	22	12.09%	20	10.42%	1.67%	0.627	0.144
G010107	2	1.10%	1	0.52%	0.58%	0.614	0.147
G01030102	21	11.54%	22	11.46%	0.08%	1.000	0.578
G01010202	1	0.55%	1	0.52%	0.03%	1.000	0.457
G0106	23	12.64%	25	13.02%	-0.38%	1.000	0.159
G010120	2	1.10%	3	1.56%	-0.46%	1.000	0.211
G010403	1	0.55%	2	1.04%	-0.49%	1.000	0.942
G01010102	0	0.00%	1	0.52%	-0.52%	1.000	NA
G01010302	2	1.10%	5	2.60%	-1.51%	0.450	0.639
G010108	0	0.00%	4	2.08%	-2.08%	0.124	NA
G010112	9	4.95%	16	8.33%	-3.39%	0.218	0.797
G01010201	46	25.27%	56	29.17%	-3.89%	0.418	0.185
G01010303	16	8.79%	25	13.02%	-4.23%	0.246	0.080
G01010105	4	2.20%	13	6.77%	-4.57%	0.045	0.018

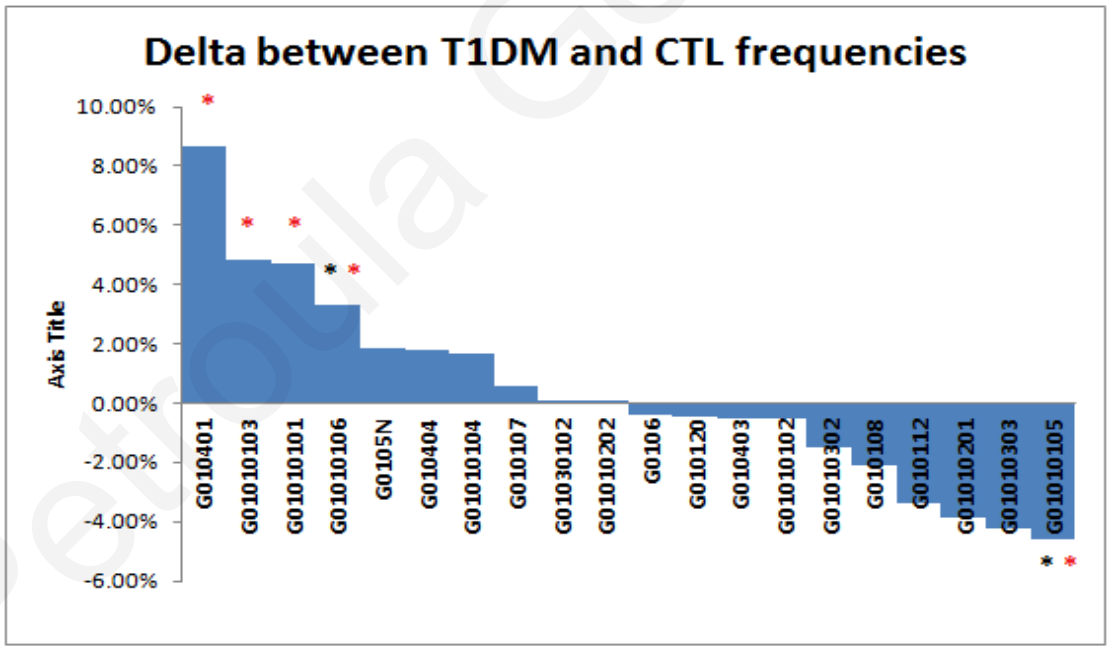

Figure 29: The difference between the T1DM and CTLs' HLA-G genotypes. Each HLA-G genotype is plotted against its delta difference between the two populations. Positive % denotes higher presence in the T1DM group, while negative % shows association to the control group. Significance is denoted with black * (Fisher's exact $p<0.05$). Red * denoted significance with Logit analysis.

Table 53: The HLA-G '3UTR haplotypes. The combination of a set of promoter SNPs are deciphered in extended promoter-haplotypes previously described (Castelli et al., 2010). Table summarizes the HLA-3'UTR haplotype frequencies, frequency difference (delta) and p-value with Fisher's Exact and Logit.

'3 UTR Haplotype	T1D n=182	Frequency %	CTL n=192	Frequency %	Delta	p-value	Logit p-value
UTR3	60	32.97%	53	27.60%	5.36%	0.263	0.016
UTR13	3	1.65%	0	0.00%	1.65%	0.114	NA
UTR18	8	4.40%	6	3.13%	1.27%	0.592	0.384
UTR1Like	2	1.10%	0	0.00%	1.10%	0.236	NA
UTR8Like	2	1.10%	0	0.00%	1.10%	0.236	NA
UTR6	17	9.34%	16	8.33%	1.01%	0.856	0.134
UTR4	33	18.13%	33	17.19%	0.94%	0.892	0.148
UTR3Like	2	1.10%	1	0.52%	0.58%	0.614	0.954
UTR8	2	1.10%	2	1.04%	0.06%	1.000	0.418
UTR9	1	0.55%	1	0.52%	0.03%	1.000	0.666
UTR14	0	0.00%	1	0.52%	-0.52%	1.000	NA
UTR7Like	0	0.00%	1	0.52%	-0.52%	1.000	NA
UTR2	82	45.05%	88	45.83%	-0.78%	0.917	0.296
UTR5	23	12.64%	27	14.06%	-1.43%	0.762	0.218
UTR10	1	0.55%	4	2.08%	-1.53%	0.373	0.136
UTR1	66	36.26%	76	39.58%	-3.32%	0.524	0.027
UTR7	18	9.89%	29	15.10%	-5.21%	0.160	0.712

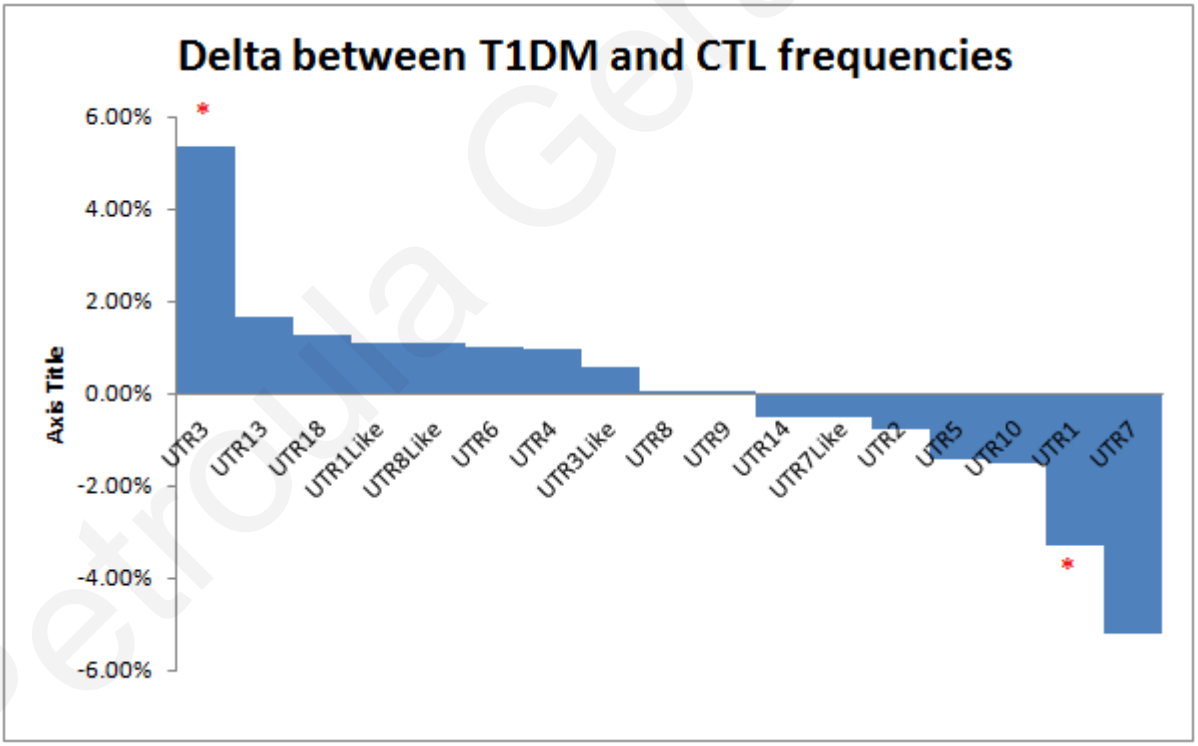

Figure 30: The difference between the T1DM and CTLs' HLA-G 3'UTR haplotypes. Each HLA-G 3'UTR haplotype is plotted against its delta difference between the two populations. Positive % denotes higher presence in the T1DM group, while negative % shows association to the control group. Significance is denoted with black * (Fisher's exact $p<0.05$). Red * denoted significance with Logit analysis.

Table 54: The HLA-G promoter haplotypes at the different age of onset. The combination of a set of promoter SNPs are deciphered in extended promoter-haplotypes previously described (Castelli et al., 2014b)

Promoter Haplotypes	Age group 1	Age group 2	Age group 3	P-VALUE
Promo010101a	33	29	29	0.602
Promo010101b	9	9	8	0.995
Promo010101c	2	1	1	0.805
Promo010101f	5	0	5	0.053
Promo010102a	24	34	31	0.077
Promo010102aLikeA	0	0	0	NA
Promo010104a	28	24	23	0.058
Promo0103d	4	5	5	0.847
Promo0103e	4	1	2	0.372
Promo-recom-unkown	0	3	2	0.240

Table 55: The HLA-G genotypes at the different age of onset. The HLA-G genotypes at the different age of onset. No significance was observed for HLA-G genotypes among the three age of onset patient groups.

HLA-G Genotypes	Age group 1	Age group 2	Age group 3	P-VALUE
G01010101	20	22	25	0.233
G01010102	0	0	0	NA
G01010103	12	9	3	0.092
G01010104	8	2	10	0.026
G01010105	3	1	0	0.195
G01010106	2	3	1	0.686
G01010201	10	20	12	0.114
G01010202	0	0	1	0.309
G01010302	0	2	0	0.156
G01010303	6	5	4	0.900
G010107	0	1	0	0.398
G010108	0	0	0	NA
G010112	1	4	4	0.300
G010120	0	1	1	0.578
G01030102	8	5	7	0.591
G010401	25	26	13	0.100
G010404	3	2	2	0.888
G010403	1	0	0	0.388
G0105N	3	3	5	0.512
G0106	8	6	7	0.789

Table 56: The HLA-G '3UTR haplotypes at the different age of onset. The combination of a set of 3'UTR SNPs are deciphered in extended promoter-haplotypes previously described (Castelli et al., 2010). No significance was observed for HLA-G genotypes among the three age of onset patient groups.

3UTR Genotypes	Age group 1	Age group 2	Age group 3	P-VALUE
UTR1	18	22	22	0.390
UTR2	20	28	28	0.081
UTR3	24	22	12	0.146
UTR4	16	8	7	0.091
UTR5	8	7	7	0.935
UTR6	7	2	6	0.176
UTR7	6	7	4	0.798
UTR8	0	1	1	0.578
UTR9	0	0	1	0.309
UTR10	1	0	0	0.388
UTR13	2	1	0	0.403
UTR14	0	0	0	NA
UTR18	4	1	2	0.372
UTR1Like	0	2	0	0.156
UTR3Like	1	1	0	0.648
UTR7Like	0	0	0	NA
UTR8Like	0	2	0	0.156

Table 57: The HLA-G promoter haplotypes. The combination of a set of promoter SNPs. Table summarizes the HLA-G promoter haplotype frequencies, frequency difference (delta) and p-value with GWASpi.

Promoter Haplotype		T1D (2n=360)	Frequency %	CTL (2n=384)	Frequency %	Delta	GWASpi p-value
0104A	h3	75	20.83%	59	15.36%	5.47%	0.056
0103E	h4	12	3.33%	6	1.56%	1.77%	
010101G	h7	11	3.06%	5	1.30%	1.75%	
010101F	h1	22	6.11%	18	4.69%	1.42%	
010101D	h8	6	1.67%	1	0.26%	1.41%	
010102F	h2	1	0.28%	0	0.00%	0.28%	
010101C	h5	12	3.33%	12	3.13%	0.21%	
010102D	h6	4	1.11%	4	1.04%	0.07%	
010101I	h9	0	0.00%	1	0.26%	-0.26%	
010101J	h10	0	0.00%	1	0.26%	-0.26%	
0103A	h11	0	0.00%	1	0.26%	-0.26%	
010102B	h13	0	0.00%	1	0.26%	-0.26%	
010102C	h15	0	0.00%	1	0.26%	-0.26%	
010102E	h17	0	0.00%	1	0.26%	-0.26%	
010101H	h19	0	0.00%	2	0.52%	-0.52%	
0103D	h16	11	3.06%	17	4.43%	-1.37%	
010101B	h18	14	3.89%	22	5.73%	-1.84%	
010101A	h14	74	20.56%	89	23.18%	-2.62%	
010102A	h12	118	32.78%	143	37.24%	-4.46%	

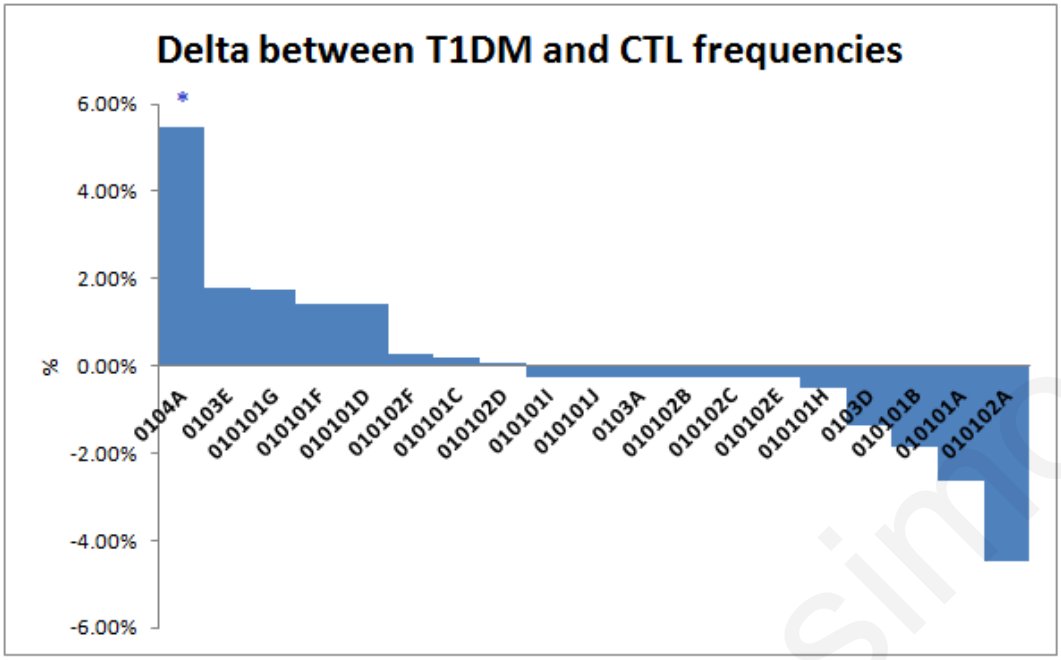

Figure 31: The difference between the T1DM and CTLs' HLA-G promoter haplotypes. Each HLA-G promoter haplotype is plotted against its delta difference between the two populations. Positive % denotes higher presence in the T1DM group, while negative % shows association to the control group. Significance is denoted with blue * (GWASpi $p<0.05$).

Table 58: The HLA-G genotypes. Table summarizes the HLA-G genotype frequencies, frequency difference (delta) and p-value with GWASpi.

Genotype		T1D (2n=360)	Frequency %	CTL (2n=384)	Frequency %	Delta	GWASpi p-value
G*01:04:01	h8	64	17.78%	51	13.28%	4.50%	
undef-h9	h9	7	1.94%	1	0.26%	1.68%	P=0.0329
G*01:01:01:04	h18	11	3.06%	6	1.56%	1.49%	
G*01:01:01:01-like-newD	h17	10	2.78%	5	1.30%	1.48%	
undef-h23	h23	6	1.67%	1	0.26%	1.41%	
G*01:04:04	h15	9	2.50%	5	1.30%	1.20%	
G*01:05N	h13	12	3.33%	9	2.34%	0.99%	
undef-7	h7	3	0.83%	1	0.26%	0.57%	
G*01:06-like-newC	h20	2	0.56%	0	0.00%	0.56%	
G*01:01:01:04-like-newE	h1	12	3.33%	11	2.86%	0.47%	
G*01:03:01:02-like-newB	h27	1	0.28%	0	0.00%	0.28%	
G*01:01:01:01-like-newE	h10	1	0.28%	0	0.00%	0.28%	
G*01:01:01:01-like-newF	h28	1	0.28%	0	0.00%	0.28%	
undef-h11	h11	1	0.28%	0	0.00%	0.28%	
undef-h14	h14	1	0.28%	0	0.00%	0.28%	
undef-h21	h21	1	0.28%	0	0.00%	0.28%	
G*01:01:03:01-LIKE-NEWI	h25	1	0.28%	0	0.00%	0.28%	
undef-h26	h26	1	0.28%	0	0.00%	0.28%	
undef-h30	h30	1	0.28%	0	0.00%	0.28%	
undef-31	h31	1	0.28%	0	0.00%	0.28%	
undef-32	h32	1	0.28%	0	0.00%	0.28%	
G*01:06	h6	24	6.67%	25	6.51%	0.16%	
undef-h19	h19	4	1.11%	4	1.04%	0.07%	
G*01:03:01:02-like-newA	h24	1	0.28%	1	0.26%	0.02%	
undef-h22	h22	1	0.28%	1	0.26%	0.02%	
G*01:03:01:02	h2	21	5.83%	23	5.99%	-0.16%	
G*01:01:12	h34	0	0.00%	1	0.26%	-0.26%	
G*01:01:01-like-newG	h33	0	0.00%	1	0.26%	-0.26%	
undef-h43	h43	0	0.00%	1	0.26%	-0.26%	
undef-h35	h35	0	0.00%	1	0.26%	-0.26%	
G*01:01:03:03-LIKE-NEWJ	h36	0	0.00%	1	0.26%	-0.26%	
undef-h37	h37	0	0.00%	1	0.26%	-0.26%	
undef-h38	h38	0	0.00%	1	0.26%	-0.26%	
undef-h40	h40	0	0.00%	1	0.26%	-0.26%	
undef-h41	h41	0	0.00%	1	0.26%	-0.26%	
undef-h42	h42	0	0.00%	1	0.26%	-0.26%	
undef-h44	h44	0	0.00%	1	0.26%	-0.26%	
undef-h45	h45	0	0.00%	1	0.26%	-0.26%	
undef-h46	h46	0	0.00%	1	0.26%	-0.26%	
G*01:01:02:01	h3	54	15.00%	59	15.36%	-0.36%	
G*01:01:14	h39	0	0.00%	3	0.78%	-0.78%	
G*01:01:01:01-like-newH	h29	1	0.28%	6	1.56%	-1.28%	
undef-h16	h16	8	2.22%	15	3.91%	-1.68%	
G*01:01:01:05	h5	25	6.94%	34	8.85%	-1.91%	
G*01:01:03:03	h12	17	4.72%	29	7.55%	-2.83%	
G*01:01:01:01	h4	57	15.83%	81	21.09%	-5.26%	

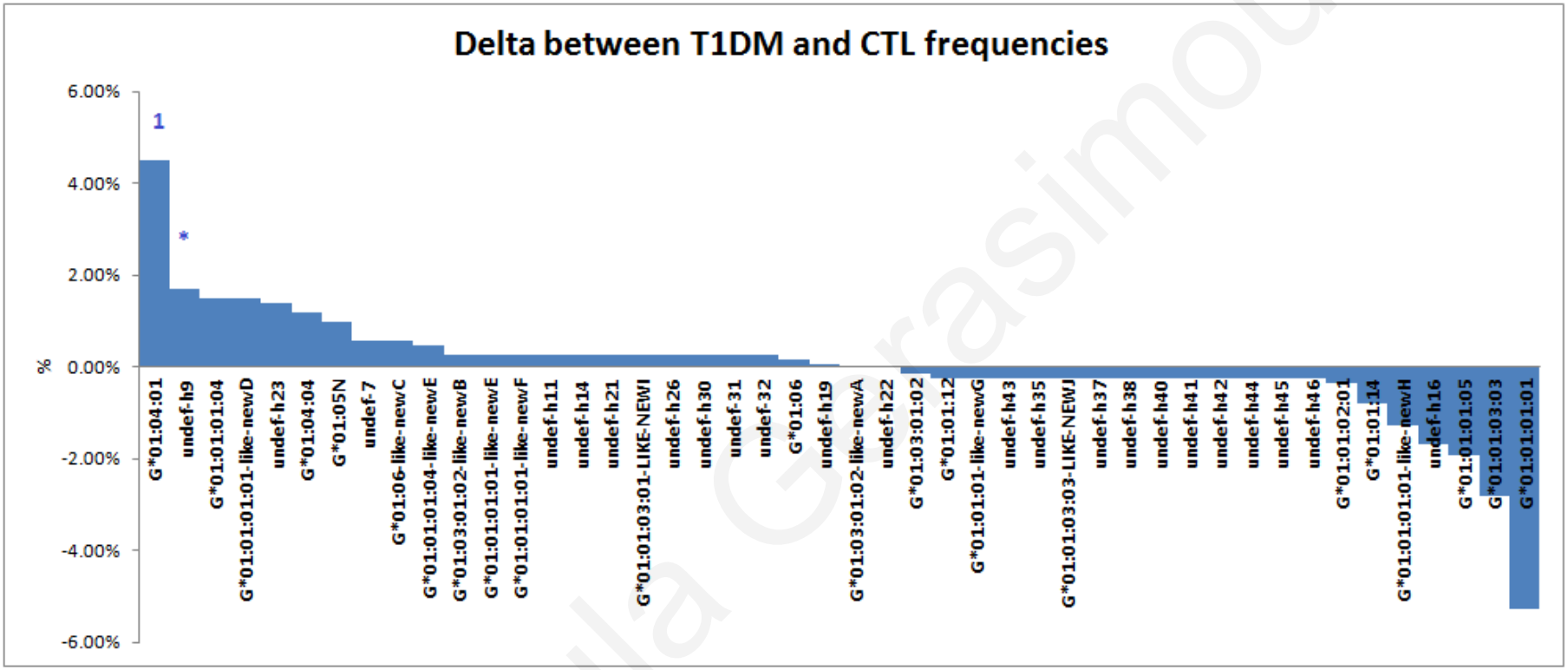

Figure 32: The difference between the T1DM and CTLs' HLA-G genotypes. The difference between the T1DM and CTLs' HLA-G genotypes. Significance is denoted by *. 1: * SNP +755 (non-synonymous mutation associated with G*01:04) detected by GWASpi as a susceptibility factor (P=0.0271, OR=1.51815). Most of the G*01:04 alleles are presented in higher frequency in Diabetes patients, but they were not associated individually.

Table 59: The HLA-G '3-UTR haplotypes.**Summary of the HLA-3'UTR haplotype frequencies, frequency difference (delta) and p-value with GWASpi.**

Genotype		T1D (2n=360)	Frequency %	CTL (2n=384)	Frequency %	Delta	GWASpi p-value
UTR-3	h6	71	19.72%	52	13.54%	6.18%	P=0.0295
new-A	h7	7	1.94%	1	0.26%	1.68%	P=0.0329
UTR-18	h12	10	2.78%	6	1.56%	1.22%	
UTR-6	h1	17	4.72%	15	3.91%	0.82%	
UTR-2	h3	92	25.56%	96	25.00%	0.56%	
UTR-5	h2	23	6.39%	23	5.99%	0.40%	
UTR-3	h13	1	0.28%	0	0.00%	0.28%	
UTR-1	h14	1	0.28%	0	0.00%	0.28%	
UTR-10	h16	1	0.28%	0	0.00%	0.28%	
new-C	h17	1	0.28%	0	0.00%	0.28%	
new-B	h11	4	1.11%	4	1.04%	0.07%	
UTR-8	h18	0	0.00%	1	0.26%	-0.26%	
UTR-5	h19	0	0.00%	1	0.26%	-0.26%	
UTR-4	h20	0	0.00%	1	0.26%	-0.26%	
new-D	h21	0	0.00%	1	0.26%	-0.26%	
UTR-2	h22	0	0.00%	1	0.26%	-0.26%	
UTR-4	h23	0	0.00%	1	0.26%	-0.26%	
UTR-13	h10	3	0.83%	6	1.56%	-0.73%	
UTR-4	h5	26	7.22%	32	8.33%	-1.11%	
UTR-1	h15	1	0.28%	6	1.56%	-1.28%	
UTR-1	h4	75	20.83%	89	23.18%	-2.34%	
UTR-2	h9	8	2.22%	18	4.69%	-2.47%	
UTR-7	h8	19	5.28%	30	7.81%	-2.53%	

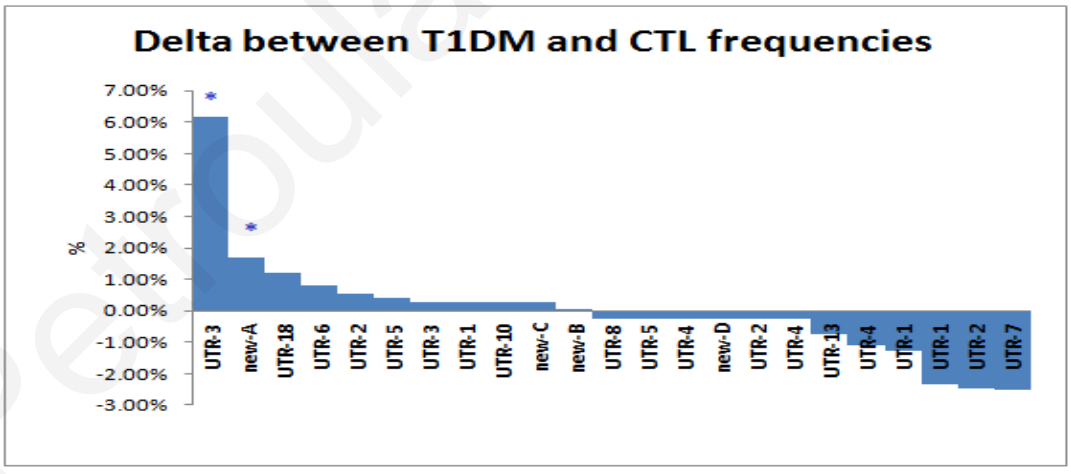

Figure 33: The difference between the T1DM and CTLs' HLA-G '3-UTR haplotypes. Each HLA-G 3'UTR haplotype is plotted against its delta difference between the two populations. Positive % denotes higher presence in the T1DM group, while negative % shows association to the control group. Significance is denoted with blue * with GWASpi $p<0.05$.

Table 60: HLA-G promoter haplotypes according to age of onset. Table summarizes the HLA-G promoter haplotype in each age of onset group. Only significant p-values are included in the table.

Promoter Haplotypes	Age group 1	Age group 2	Age group 3	P-VALUE
010101A	20	26	28	
010101B	5	6	3	
010101C	8	2	2	
010101D	2	1	3	
010101F	9	3	7	
010102A	31	46	39	
0103A	0	1	0	
0103D	3	3	2	
0103E	5	2	5	
0104A	31	28	13	0.030

Table 61: HLA-G genotypes according to age of onset. Table summarizes the HLA-G genotypes in each age of onset group. Only significant p-values are included.

HLA-G Genotypes	Age group 1	Age group 2	Age group 3	P-VALUE
G*01:01:01:01	20	24	29	
G*01:01:01:03	0	0	1	
G*01:01:01:04	9	4	7	
G*01:01:01:05	13	7	5	
G*01:01:01:06	2	3	1	
G*01:01:02:01	14	25	19	
G*01:01:02:02	0	0	1	
G*01:01:03:03	6	8	3	
G*01:03:01:02	8	6	7	
G*01:04:01	27	27	13	0.060
G*01:04:04	4	3	2	
G*01:05N	3	4	5	
G*01:06	8	7	9	

Table 62: HLA-G 3'UTR haplotypes according to age of onset. Table summarizes the HLA-G 3'UTR haplotypes in each age of onset group. Only significant p-values are included.

3UTR Genotypes	Age group 1	Age group 2	Age group 3	P-VALUE
UTR-1	17	24	27	
UTR-10	0	1	0	
UTR-13	2	1	0	
UTR-18	5	1	3	
UTR-2	25	35	34	
UTR-3	29	27	13	0.045
UTR-4	13	8	5	
UTR-5	8	6	7	
UTR-6	7	2	6	
UTR-7	6	8	4	
UTR-new-A	2	2	1	
UTR-new-B	0	1	0	
UTR-new-C	0	2	2	

The promoter haplotypes and their frequency between patients and controls analysed by our laboratory are portrayed in Table 42 and Figure 25, while GWASpi analysis is shown in Table 48 and Figure 28. It must be noted that two samples could not be included in the haplotype analysis by Dr.Castelli. The population in this study presents the same promoter haplotypes already described, with several new rare ones (010101H, 010101I, 010101J) mainly because there are variable sites here that were not previously described. Promo-G0104A presented a borderline P value when using the Fisher exact test. SNP -483 was detected as a susceptibility factor by using GWASpi and Logit tools, but the Fisher exact test did not confirm it. As seen from both GWASpi and Logit tools analysis, the most significant promoter haplotype identified to be associated with T1DM is the Promo-0104A (p=0.056) and the most significantly associated with the control group is the Promo-010102A (p=0.010).

The HLA-G genotypes and their frequency between patients and controls analysed by our laboratory are portrayed in Table 43 and Figure 26, while GWASpi analysis is shown in Table 49 and Figure 29. It was apparent from analysis that our population presented a number of new HLA-G alleles. The most common ones detected worldwide were also represented here, but there were a lot of new alleles due to the presence of crossing-over and new mutation. Such alleles were named as "undef" for undefined. Furthermore, the undefined allele (h9) was strongly associated with diabetes (p=0.03). On the SNP level, SNP +755 (non-synonymous

mutation) was associated with G*01:04, detected by GWASpi as a susceptibility factor (P=0.0271, OR=1.51815). This allele was in Linkage Disequilibrium with promo-0104a. None of the other alleles were associated with Diabetes by GWASpi analysis, but Logit analysis revealed significance for the following genotypes: G*010101, G*01010103, G*01010101, G*01010106 and G*01010105 (Figure 26). G*01010106 and G*01010105 also showed significance by Fisher's Exact test.

The HLA-G 3'UTR haplotypes and their frequency between patients and controls analysed by our laboratory are portrayed in Table 44 and Figure 27, while GWASpi analysis is shown in Table 50 and Figure 30. Our population presents the same 3'UTR haplotypes already described, with several new rare ones mainly because there are variable sites here that were not previously described. UTR-3 was associated with Diabetes by both Logit (p=0.016) and GWASpi (p=0.029) analysis. A new-A haplotype was also highly associated with diabetes (p=0.03). On the SNP analysis level, SNP +3092 was detected as a susceptibility marker for Diabetes by the GWAsip. This SNP was carried by the new-A haplotype. For more detailed tables, see Appendix I.

In summary, the commonly described extended haplotype Promo-G*0104a/G*01:04/UTR-3 was associated with Diabetes. A new 3'UTR haplotype named here new-A and the SNP associated with it (+3092) is associated with Diabetes. There were numerous new HLA-G coding alleles in our population.

Further stratification of patients into the age of onset groups and analysis against promoter-haplotypes, HLA-G genotypes and 3'UTR-haplotypes are depicted in Tables 45-47 and 51-53. Tables 51-53 revealed a significantly higher number in Age group 1 of the HLA-G promoter haplotype 0104A (p=0.03), the HLA-G genotype G*01:04:01 (p=0.06) and the HLA-G 3'UTR haplotype UTR-3 (p=0.045).

3.4 NON-HLA GENETIC ASSOCIATIONS TO T1DM

The genetic association between non-HLA loci and T1DM were also questioned for the course of this study. The effect of other immune associated elements is also investigated to further decipher genetic susceptibility to T1DM. These factors include cytokines TNF-alpha, IL10, IL6, IGN-gamma, TGF-beta and protein receptor cytotoxic T-lymphocyte-associated protein 4 (CTLA-4).

Table 63: Cytokine and non-HLA associated with T1DM factors. Table summarizes the cytokines (TNF-α, IFN-γ, TGF-β, IL6 and IL10) and non-HLA associated with T1DM factors (CTLA-4) frequencies, frequency difference (delta) and p-value with Fisher's Exact and Logit analysis ($p<0.05$).

CATEGORY	HAPLOTYPE / ALLELE	T1D n=182	T1D Frequency %	CTL n=192	CTL Frequency %	Delta	P-value	Logit P-value
TNFalpha	A/A	3	1.65%	0	0.00%	1.65%		NA
promoter -308	A/G	48	26.37%	37	19.27%	7.10%		0.038
	G/G	131	71.98%	155	80.73%	-8.75%	0.046*	0.023
	A	51	28.02%	37	19.27%	8.75%	0.050*	0.029
	G	179	98.35%	192	100.00%	-1.65%	0.114	NA
	HIGH	51	28.02%	37	19.27%	8.75%		0.038
	LOW	131	71.98%	155	80.73%	-8.75%	0.050*	0.023
IL10	ACC/ACC	24	13.19%	28	14.58%	-1.40%		0.681
promoter	ACC/ATA	38	20.88%	36	18.75%	2.13%		0.859
-1082, -819, -592	ATA/ATA	13	7.14%	20	10.42%	-3.27%		0.443
	GCC/ACC	43	23.63%	57	29.69%	-6.06%		0.373
	GCC/ATA	39	21.43%	24	12.50%	8.93%		0.269
	GCC/GCC	25	13.74%	27	14.06%	-0.33%	0.207	NA
	ACC	105	57.69%	121	63.02%	-5.33%	0.341	0.805
	ATA	90	49.45%	80	41.67%	7.78%	0.146	0.174
	GCC	107	58.79%	108	56.25%	2.54%	0.676	0.468
	HIGH	25	13.74%	27	14.06%	-0.33%		NA
	INTERMEDIATE	82	45.05%	81	42.19%	2.87%		0.846
	LOW	75	41.21%	84	43.75%	-2.54%	0.850	0.920
IL6	C/C	11	6.04%	5	2.60%	3.44%		0.069
promoter -174	G/C	56	30.77%	55	28.65%	2.12%		0.835
	G/G	115	63.19%	132	68.75%	-5.56%	0.206	0.835
	C	67	36.81%	60	31.25%	5.56%	0.276	0.835
	G	171	93.96%	187	97.40%	-3.44%	0.127	0.092
	HIGH	171	93.96%	187	97.40%	-3.44%		0.090
	LOW	11	6.04%	5	2.60%	3.44%	0.127	0.069
IFNG	A/A	32	17.58%	36	18.75%	-1.17%		0.751
intron 1 +874	T/A	104	57.14%	114	59.38%	-2.23%		0.683
	T/T	46	25.27%	42	21.88%	3.40%	0.738	0.686
	T	150	82.42%	156	81.25%	1.17%	0.790	0.968
	A	136	74.73%	150	78.13%	-3.40%	0.466	0.597
	HIGH	46	25.27%	42	21.88%	3.40%		0.686
	INTERMEDIATE	104	57.14%	114	59.38%	-2.23%		0.683
	LOW	32	17.58%	36	18.75%	-1.17%	0.738	0.751
TGFB	C/C	36	19.78%	51	26.56%	-6.78%		0.094
codon 10	T/C	87	47.80%	89	46.35%	1.45%		0.689
	T/T	59	32.42%	52	27.08%	5.33%	0.248	0.645
	T	146	80.22%	141	73.44%	6.78%	0.142	0.193
	C	123	67.58%	140	72.92%	-5.33%	0.308	0.645
TGFB	C/G	53	29.12%	72	37.50%	-8.38%		0.229
codon 25	G/G	129	70.88%	120	62.50%	8.38%	0.100	0.134
	C	53	29.12%	72	37.50%	-8.38%	0.100	0.229
	G	182	100.00%	192	100.00%	0.00%	NA	NA
TGFB	HIGH	105	57.69%	88	45.83%	11.86%		0.038
	INTERMEDIATE	65	35.71%	85	44.27%	-8.56%		0.050
	LOW	12	6.59%	19	9.90%	-3.30%	0.065	0.125
CTLA4	A/A	11	6.04%	12	6.25%	-0.21%		NA
exon 1 +49	A/G	79	43.41%	64	33.33%	10.07%		0.038
	G/G	92	50.55%	116	60.42%	-9.87%	0.127	0.023
	A	90	49.45%	76	39.58%	9.87%	0.050*	0.035
	G	171	93.96%	180	93.75%	0.21%	1.000	0.318

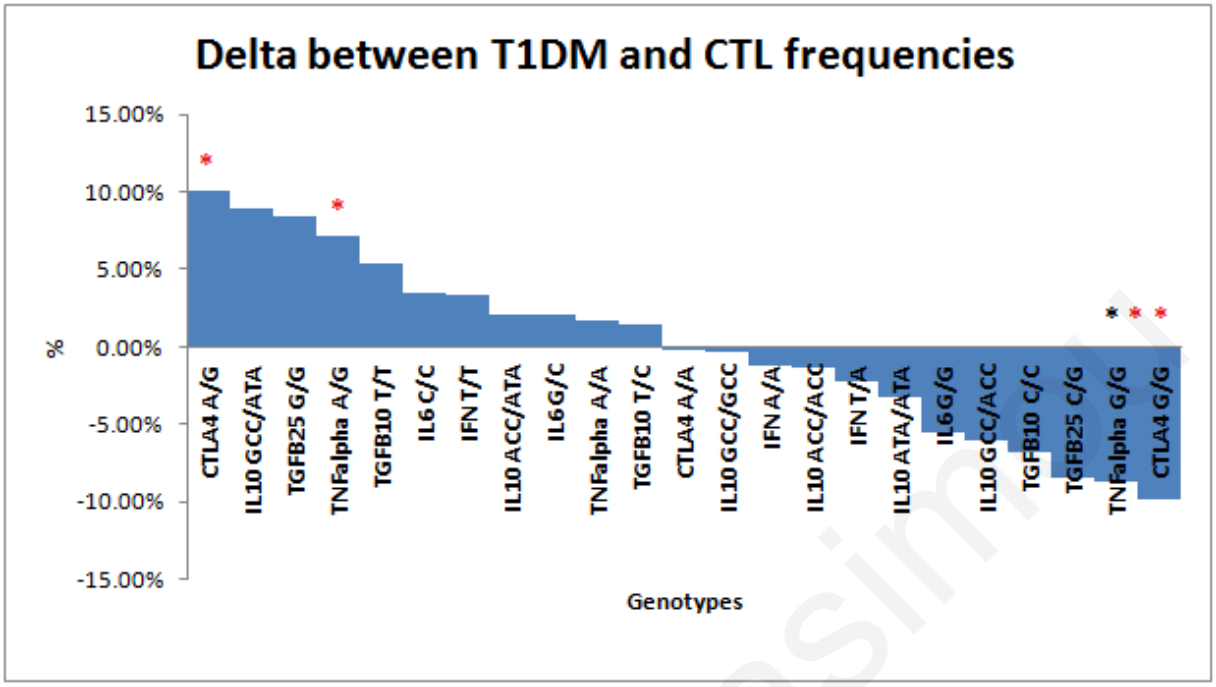

Figure 34: The genotype frequencies difference between the two populations. Each genotype is plotted against its delta difference between the two populations. Positive % denotes higher presence in the T1DM group while negative % show association to the control group. Significance is denoted with black * for Fisher's exact p<0.05 and red * for Logit p<0.05.

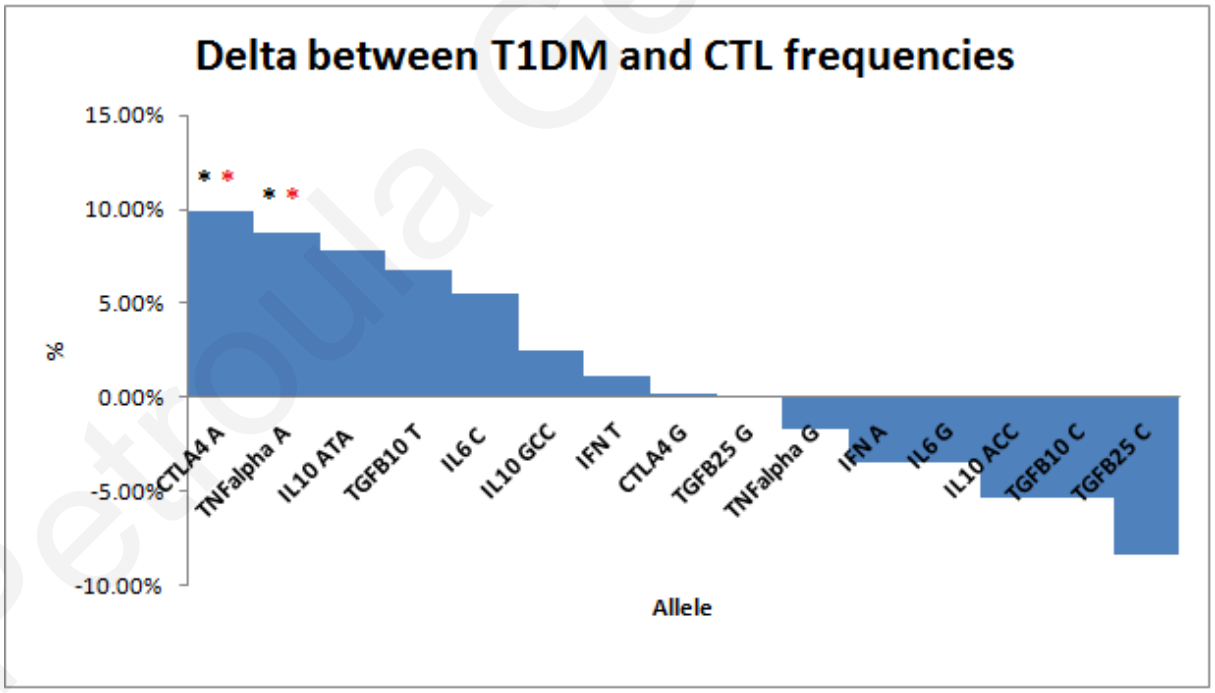

Figure 35: The allele frequencies difference between the two populations. Each allele is plotted against its delta difference between the two populations. Positive % denotes higher presence in the T1DM group, while negative % shows association to the control group. Significance is denoted with black * for Fisher's exact p<0.05 and red * for Logit p<0.05.

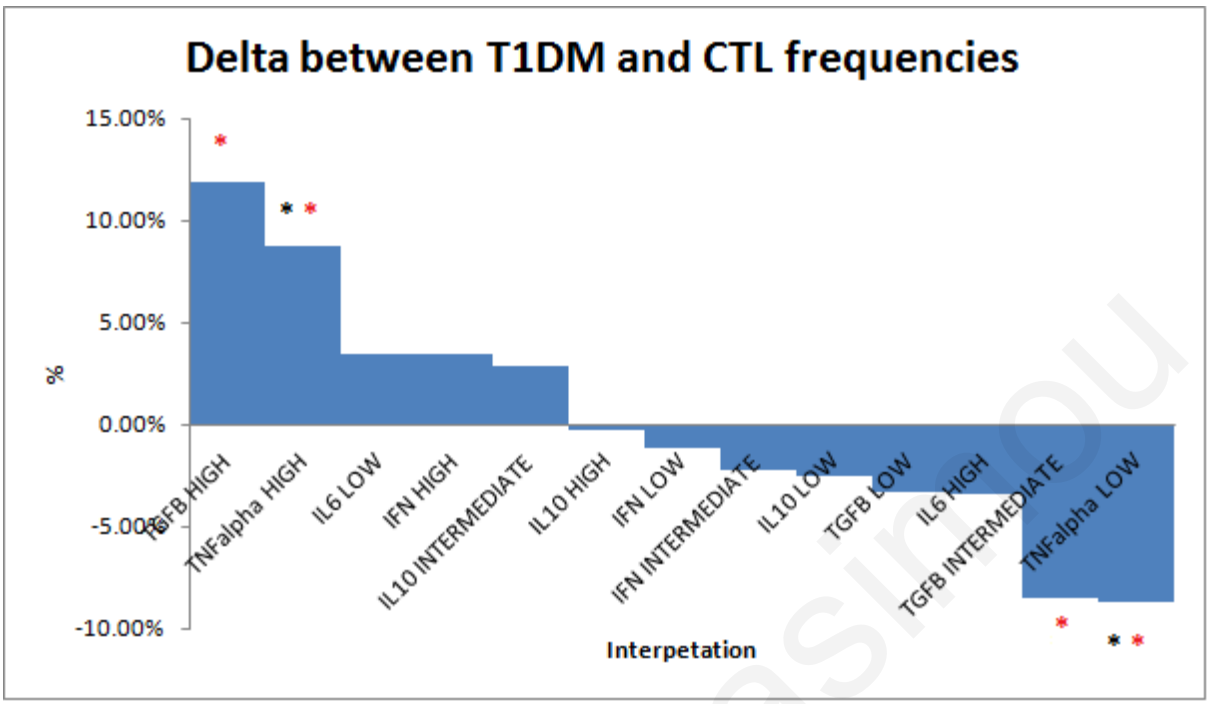

Figure 36: The interpreted cytokine difference between the two populations. Each allele interpretation is plotted against its delta difference between the two populations. Positive % denotes higher presence in the T1DM group, while negative % shows association to the control group. Significance is denoted with black * for Fisher's exact $p<0.05$ and red * for Logit $p<0.05$.

Table 64: Cytokine and non-HLA factors associated with T1DM distributed to the th

CATEGORY	HAPLOTYPE / ALLELE	Age Group 1	Age group 2	Age group 3	P-VALUE
TNFalpha	A/A	2	0	1	
promoter -308	A/G	15	14	15	
	G/G	42	46	35	0.621
	A	17	14	16	0.621
	G	57	60	60	0.37
	HIGH	17	14	16	
	LOW	42	46	35	0.621
IL10	ACC/ACC	8	8	6	
promoter	ACC/ATA	17	9	9	
-1082, -819, -592	ATA/ATA	4	4	4	
	GCC/ACC	11	16	13	
	GCC/ATA	10	12	15	
	GCC/GCC	9	11	4	0.584
	ACC	36	33	28	0.749
	ATA	31	25	28	0.319
	GCC	30	39	32	0.246
	HIGH	9	11	4	
	INTERMEDIATE	21	28	28	
	LOW	29	21	19	0.185
IL6	CC	2	6	2	
promoter -174	GC	17	14	21	
	GG	40	40	28	0.164
	C	19	20	23	0.308
	G	57	54	49	0.24
	HIGH	57	54	49	
	LOW	2	6	2	0.24
IFNG	AA	10	13	8	
intron 1 +874	TA	29	33	34	
	TT	20	14	9	0.273
	T	49	47	43	0.683
	A	39	46	42	0.135
	HIGH	20	14	9	
	INTERMEDIATE	29	33	34	
	LOW	10	13	8	0.273
TGFB	CC	9	11	12	
codon 10	TC	31	26	25	
	TT	19	23	14	0.637
	T	50	49	39	0.538
	C	40	37	37	0.472
TGFB	CG	19	16	16	
codon 25	GG	40	44	35	0.779
	C	19	16	16	0.779
	G				NA
	HIGH	36	35	27	
	INTERMEDIATE	18	23	20	
	LOW	5	2	4	0.649
CTLA4	AA	4	2	4	
exon 1 +49	AG	22	30	23	
	GG	33	28	24	0.589
	A	26	32	27	0.529
	G	55	58	47	0.564

ree age of onset groups. Table summarizes the cytokine and non-HLA associated with T1DM factors according to the three age of onset groups. No significance is observed.

A summary of actual number and frequency of each factor in each group, frequency difference (delta) between the two groups, Fisher's Exact p-value and Logit p-value is described in Table 54. For each factor previously associated with T1DM the frequency of the genotype, single allele and interpretation where possible was analysed for significant difference between diabetic and controls using both Fisher's exact and Logit analysis. The three main factors that showed significant association with either susceptibility or resistance to diabetes were cytokines TNF-alpha, TGF-beta and protein receptor cytotoxic T-lymphocyte-associated protein 4 (CTLA-4).

For each genotypes, alleles or interpretation analysis a figure was created to illustrate the frequency differences between the T1DM and control population. It is clear from these figures that factors associated with either group appear into clusters showing association to diabetes at the far left side of the figure and association to control at the far right side of the figure. Therefore, even though some factors missed statistical significance show clear 'preference' to one group.

Figure 31 shows the differences in frequencies between the different SNP genotypes. TNF-alpha G/G genotype was found to be significant with both Fisher's exact test (p=0.046) and Logit analysis (p=0.023) and was strongly associated with resistance to diabetes. On on the contrary, TNF-alpha A/G was associated to susceptibility to diabetes (Logit p=0.038). Through Logit analysis, CTLA-4 G/G was also associated with resistance (p=0.023), while CTLA-4 A/G was significantly present in the diabetic group (p=0.038). Factors that had a difference but missed significance include: IL10 GCC/ATA in T1D versus IL10 GCC/ACC in control, TGFBcodon25 G/G in T1D versus TGFBcodon25 C/G in control, TGFBcodon10 T/T in T1D versus TGFBcodon10 C/C in control and IL6 C/C in T1D versus IL6 G/G in control.

Figure 32 shows the differences in frequencies between the different allele SNPs. TNF-alpha A and CTLA-4 A SNPs were found to be significant with both Fisher's exact test (p=0.05 and p=0.05 respectively) and Logit analysis (p=0.029 and p=0.035 respectively), and were strongly associated with susceptibility to diabetes. Nevertheless, a cluster of factors that showed high frequency difference between the groups but were not statistically significant in the control group included IL10 ACC, TGFBcodon10 C and TGFB codon25 C.

Figure 33 shows the differences in frequencies between the different factor interpretations. TNF-alpha High and Low were found to be significant with both Fisher's exact test (p=0.05) and Logit analysis (p=0.038 and p=0.023 respectively). TNF-alpha High was

associated with diabetes susceptibility, while TNF-alpha Low was predominant in the control group. In addition, through the Logit analysis TGF-beta High was found significant in the T1D group (p=0.038), while TGF-beta Intermediate was significantly higher (p=0.05) in the control population. A factor that barely missed significance was the IL6 Low found in the diabetic cohort against IL6 High predominance in the control population.

Further dissection of patients into the three ages of onset groups revealed no significance with either statistical method showing no effect of these factors to the age of disease manifestation (Figure 48).

3.5 FUNCTIONAL STUDIES

3.5.1 HLA-G+ Treg induction

In the periphery, HLA-G positive Tregs of thymic origin exist in limited numbers and proliferating ability (Feger et al., 2007). Recently, it was demonstrated that hypomethylating agent azacitidine (Aza) induced FOXP3 negative HLA-G expressing immunomodulatory T cells and, therefore, acquiring the best of both worlds; the immune-modulatory effects of Tregs and HLA-G.

Table 65: The induction of HLA-G$^+$ Tregs following treatment with hypomethylating agent azacitidine (Aza). The percentage of T cell populations after healthy-donor PBMCs incubation for 72 hours with 2μM Aza. *Significant difference between the percentage of each population with and without Aza as determined by flow cytometry.

	CONTROL 72H %	AZA 72H %
HLA-G+	2.21	16.70*
CD4+	45.86	41.02
CD4+HLA-G+	0.49	2.58*
CD4+CD25+	2.56	3.13
CD4+CD35+HLA-G+	1.15	11.11*
CD8+	32.9	25.29
CD8+HLA-G+	4.2	4.98
CD8+CD25+	0.41	0.28
CD8+CD25+HLA-G+	NA	NA

Table 65 and Figure 37 depict the induction of HLA-G positive Tregs via the actions of the hypomethylating agent azacitidine (Aza). The percentage of total HLA-G positive T-cells is increased by 14.5% after 72 hours incubation with 2μM, while significant increase was observed only for the CD4+ T cells. CD4+/HLA-G+ T-cells increased by 2% while CD4+/CD25+/HLA-G+ T-cells increased by 10%. CD8+ T-cells showed no difference after 72 hours incubation with Aza.

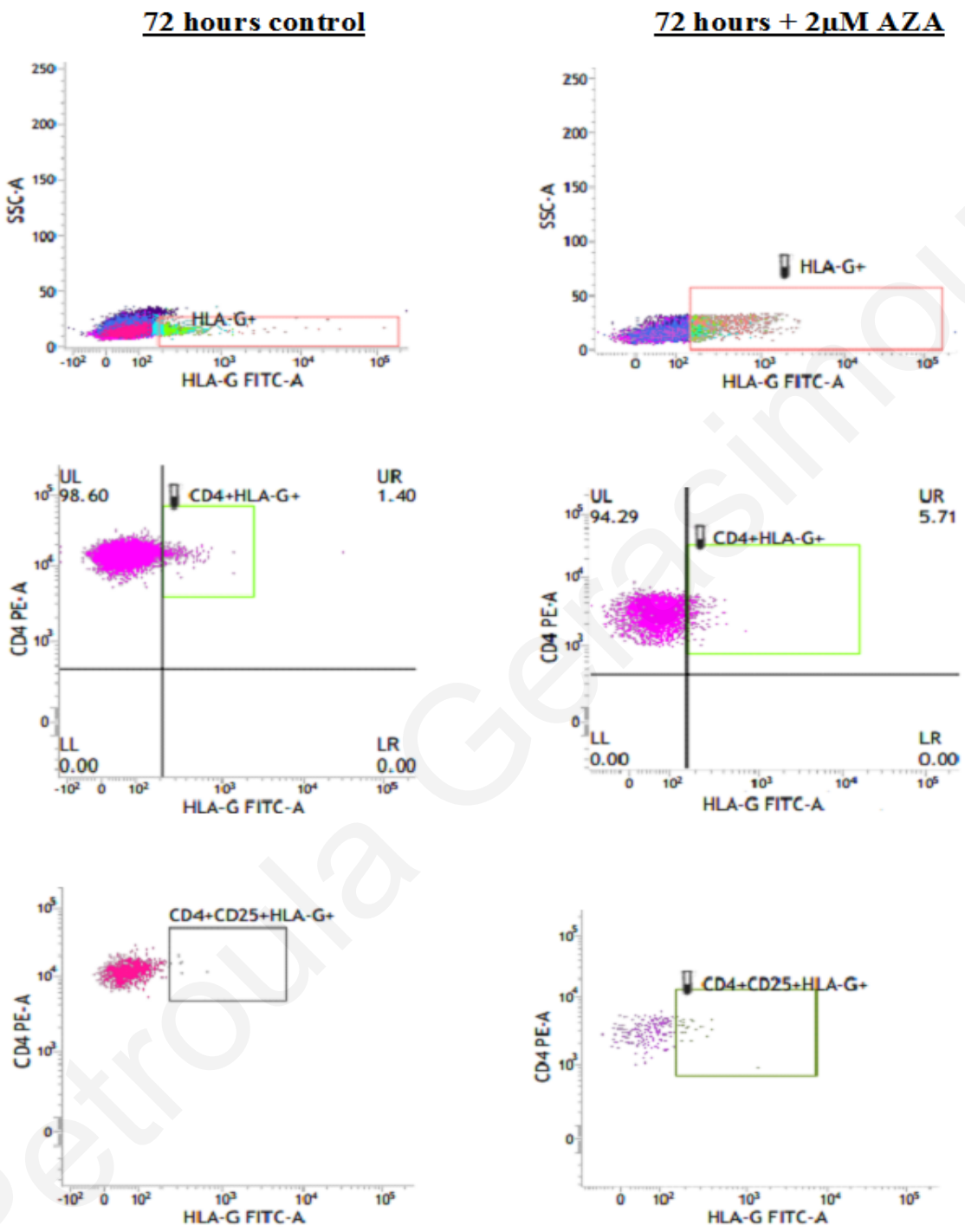

Figure 37: The induction of HLA-G positive Tregs via the actions of hypomethylating agent azacitidine (Aza). The induction of HLA-Gpos Tregs via the actions of hypomethylating agent azacitidine (Aza). Flow cytometry figures depict gating according to CD4, CD25 and HLA-G.

3.5.2 HLA-G gene expression

The HLA-G gene expression was also studied in the three age of onset groups to provide evidence that genotypic differences between the groups affect the mRNA expression. Using the TaqMan Gene expression assay and the ΔΔCt analytical method it became obvious that the three age of onset groups had different mean values in their HLA-G expression. A fold change calculation revealed a 2-fold higher HLA-G expression by the late age of onset group when compared to the early age of onset. The fold-change differences between the groups are portrayed in the following figure.

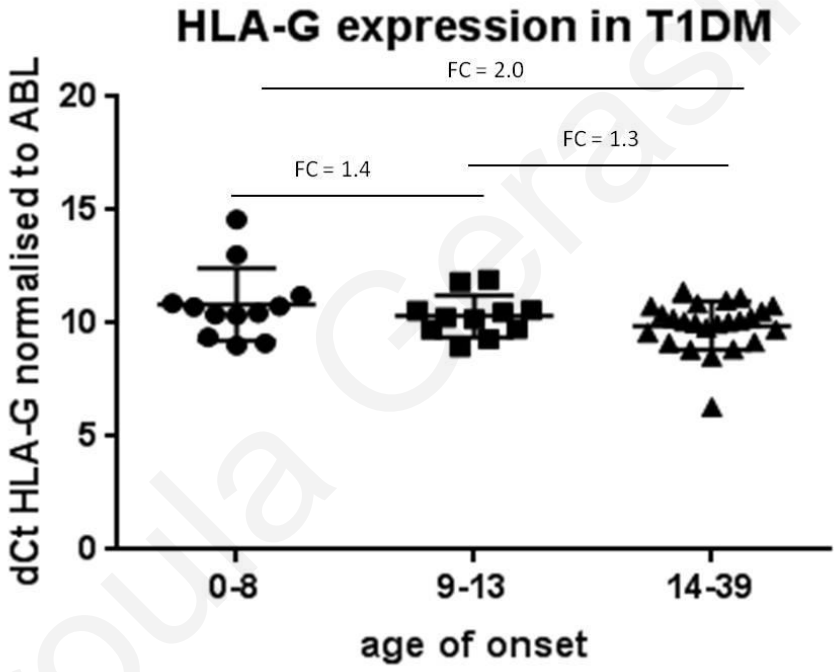

Figure 38: The HLA-G expression in T1DM. The HLA-G expression dCt as normalised from the expression of HLA-G and control gene ABL in the three age of onset groups. Real-time PCR Taqman analysis was performed on patient cDNA synthesised by patient RNA (from whole blood).The calculated fold change is also shown.

CHAPTER 4

DISCUSSION

4.1 HLA INVOLVEMENT IN T1DM

4.1.1 THE HLA

HLA class I and class II analysis

The genetic association of HLA components and autoimmune diseases is well documented and explored, even though the exact mechanism that confers the disease risk is yet to be fully understood. High linkage disequilibrium within the MHC itself makes precise mapping of specific loci to specific autoimmune disease complicated. Nevertheless, various studies have been conducted and concluded on specific HLA loci and T1DM susceptibility, due to high rate of occurrence within a population or among populations.

HLA gene variants in T1DM have been established as primary susceptibility determinants that confer as much as 50-60% of the overall disease risk (Noble and Erlich, 2012). Class II haplotypes, DRB1*03:01-DQA1*05:01-DQB1*02:01 (HLA-DR3/DQ2) and DRB1*03:01-DQA1*05:01-DQB1*02:01, DRB1*04:xx-DQA1*03:01-DQB1*03:02 (HLA-DR4/DQ8), carry the strongest association to disease with up to 50% of patients carrying both haplotypes (van Lummel et al., 2012, Erlich et al., 2008).

In our study population, 79% of patients carried either the risk haplotype in heterozygous or homozygous, or carried both haplotypes, while only 20% of the control population were carriers ($p<0.001$). As 21% of our diabetic cohort does not carry the risk associated allele, it can be concluded that other immunogenetic factors are involved in the overall pathogenesis. As a result, this study re-evaluated both HLA class I and class II in the context of diabetes. The HLA alleles that were most strongly associated with T1DM ($p<0.05$) were the A*24:02 and A*25:01, B*08:01, B*50:01and B*41:01, C*14:03, DRB1*03:01 and *04:05, DQB1*02:01 and *03:02, DQA1*03:01 and DPB1*03:01. On the contrary, the HLA alleles that were mostly associated with the control population include: A*32:01 and *11:01, B*35:01, *51:01 and *35:03, C*12:02, DRB1*14:01, *11:04, *10:01, *16:02 and *13:01, DQB1*05:03 and *03:01, DQA1*01:01, DPB1*04:02. A comparison among our diabetic significant class I alleles and the frequencies of these in the presence of the risk haplotype in the general population, discriminated among significance due to LD with the risk alleles and distinct contribution of some alleles to T1DM susceptibility. It was determined that the high frequency HLA alleles

A*24:02, B*08:01 and B*41: 01 in our diabetic cohort was due to LD with the disease associated DRB1 and DQB1 alleles rather than independent association. Nevertheless, HLA alleles A*25:01, B*50:01 and C*14:03 had significantly higher frequency in the diabetic group than expected as compared with frequencies in the general population, showing a selection bias in diabetes and hinting a possible role of HLA class I alleles to the susceptibility of diabetes.

The association of class II alleles with T1DM disease susceptibility has long been appreciated and several studies have investigated allele frequencies in patient and control cohorts in numerous, diverse populations. The allele frequencies of T1DM Cypriot patients have not been previously reported. In concordance with previous studies, we identified susceptibility alleles HLA-DRB1*03:01 and *04:05, HLA-DQB1*02:01 and *03:02, HLA-DQA1*03:01 as having the highest frequency in patients as compared with control subjects. The vast majority of our T1DM patients carried the established risk haplotypes DR3 - DQ2.5 and DR4 - DQ8, which are actually the first and second most common haplotypes respectively in European Caucasians. In contrast, in the Cypriot population these haplotypes rank 87th and 91st respectively. We thus believe that the Cypriot population represents an excellent sample to study that can allow further dissection of T1DM disease susceptibility. The genetic heterogeneity of the Cypriot population is very strikingly illustrated when calculating the cumulative incidence of the 50 most frequent HLA haplotypes, which accounts for about 30% of the Cypriot population as compared to other European populations who are more homogeneous and where the 50 most frequent HLA haplotypes cover over 50% of the population. Using our highly diverse, albeit small, cohort of Cypriot patients we were able to identify a new predisposing haplotype DR4 – DQ2.3 carried by 13% of our T1DM patients, but only 4.7% of control subjects. This haplotype failed to reach significance in a large T1D Genetics Consortium investigating HLA-DR-DQ haplotypes in T1DM families of mostly European descent (Erlich et al., 2008). Further verification of the significance of this haplotype in T1DM susceptibility should be pursued in a larger patient cohort.

HLA amino acid composition, function and T1DM susceptibility

In addition to conventional allele frequency studies, recent studies support the importance of epitope analysis as an important additional piece to the complex puzzle of deciphering autoimmune disease susceptibility (Roark et al., 2014, Freed et al., 2011). Therefore, in an attempt to explain the DRB1 'X' partner allocation of the DRB1*03/ DRB1*X and DRB1*04/ DRB1*X T1DM patients, and to further investigate the significance observed in the above mentioned HLA class I alleles, we performed an amino acid analysis to reveal possible associations with other HLA alleles that were not included in the risk haplotype, but that may share similar risk conferring amino acids in their binding domain.

Epitope analysis can uncover associations with alleles that are missed due to their low frequency in the population, or they are disparate alleles that share peptide-binding motifs, known as shared epitopes. HLA molecules function to present antigenic peptides to T cells, and thus have a central role in immune cell activation and autoimmune disease. The peptide-binding grooves of HLA molecules are made of amino acids arranged in pockets; these amino acids are highly polymorphic and create millions of possible epitopes either as single or groups of continuous or non-continuous residues. These epitopes determine the repertoire of peptides a given HLA allele can present. Class I molecules (HLA-A, -B and -C) have binding grooves made of six pockets. Class II molecules (HLA-DR, -DQ, -DP) are heterodimers comprised of α and β chains creating binding grooves with four major pockets. In the case of HLA-DR heterodimer, polymorphism lays only within the β chains, whereas both α and β chains of the HLA-DQ and HLA-DP heterodimers are polymorphic.

The polymorphic residues of the HLA class II molecules are important to peptide binding, T cell receptors, and CD4 interaction and dimerization of the heterodimer. Therefore, it cannot be excluded that amino acids that are located in one region of the DR or DQ molecule, may affect the physicochemical properties of an adjacent region by influencing side-chain interactions. Various studies have been performed in order to determine the role of individual amino acids or in combination to test their association with T1DM. In T1DM, positive and negative associations with specific residues of DQα (residue 52) (Khalil et al., 1990) and DQβ (residue 57) have been identified to be associated (Todd et al., 1987). Nevertheless, results have been conflicting over different populations. (Sanjeevi et al., 1995, Yamagata et al., 1989, Dorman et al., 1990) Further on, the polymorphic residue Asp (D) in the beta domain position

57 appears to form a salt bridge with a conserved Arg (R) at the alpha domain position 76. (Brown et al., 1993) Aspartic acid (D) at this position has been shown as protective to T1DM as HLA-DQ molecules with side chains of β 57 incapable of forming a salt bridge were linked to T1DM susceptibility (Todd et al., 1988). This residue has also been investigated due to its proximity with residues β 52 and 55 that are involved in the formation of the dimer of heterodimers. Another published amino acid that showed importance in conferring susceptibility or resistance to T1DM was the DQα 69 (Sanjeevi et al., 1995). A study by Sanjeevi et.al. 2002, implicated amino acid residues (DQβ 45 and 70) that are involved in TCR contact and proposed that susceptibility is not only due to peptide binding, but also through T cell recognition. These polymorphic residues outside the peptide binding groove may restrict TCR function. (Sanjeevi et al., 2002)

Epitope differences can account for closely related alleles differing in T1DM risk, and in addition, the sharing of epitopes by disparate alleles can reveal and explain allele associations not previously known. Therefore, through this study we hypothesis that even though the peptide binding groove is important in antigen presentation, the same HLA alleles are involved in several autoimmune diseases suggesting that true susceptibility does not lay on the auto-antigenic peptides but in the polymorphisms of amino acid residues as a whole. Amino acid substitution can change the conformation of the DR or DQ heterodimer or dimer of heterodimer, alter charge or hydrophobicity of the molecule that may result in enhancement in binding of a peptide that is capable of eliciting an immune reaction directed against islet beta cell auto-antigens or vary TCR and CD4 communication and thus T-cell activation patterns.

Using the SKDM HLA Tool, an independent tool different from the one used in previous studies by others, we were able to confirm the significance of a number of previously reported susceptibility and resistance epitopes in T1DM. Susceptibility epitope HLA-DQβ A^{57} and resistant epitope consisting of aspartic acid (D) in the same position were found to have the strongest association in agreement with previous reports (Sato et al., 1999, Roark et al., 2014). Similarly, we were able to identify previously reported susceptibility epitopes HLA-DRβ H^{13} and K^{71}, but not their corresponding resistance epitopes HLA-DRB1 R^{13} and A^{71}; in the place of alanine in position 71, we identified an epitope consisting of arginine (R) to be associated with resistance. The most significantly associated HLA-DRB1 susceptibility epitope identified in our study was Q^{70} not previously associated with T1DM. In addition to Q^{70}, we found epitopes HLA-DRβ V^{11}, H^{13} and L^{67} that were previously reported to have the highest

association with RA susceptibility, whereas D^{70} strongly correlated with resistance (Freed et al., 2011). The same study identified a two amino acid epitope $QA^{70,74}$ associated with RA susceptibility. In addition to resistance epitope HLA-DRβ D^{70}, we identified an alternative epitope in the same position of the HLA-DRB1 allele, R^{70}, which was actually more protective. It could be possible that the R^{70} epitope was not present in the RA cohort of Freed and colleagues; we were not able to identify R^{70+}/R^{70+} homozygous individuals.

A previous study showed that multiple susceptibility epitopes correlate with earlier age of onset whilst multiple resistance epitopes correlate with later age of onset (Roark et al., 2014). We attempted a similar analysis by counting the number of HLA-DRB1, HLA-DQB1 and HLA-DQA1 alleles that contained susceptibility and resistance epitopes for each patient or control subject but we were not able to verify this finding, even though there is an apparent trend, probably due to the small number of patients in each age group. Because the DR-DQ region is separated by fewer than 110kb, there is a high degree of linkage disequilibrium, which makes it difficult to analyze the independent effect of each locus. However, we showed that the concomitant presence of DR-DQ susceptibility epitopes in T1DM patients could not be just due to linkage disequilibrium, as 90% of patients had both the DRB1 Q^{70} and the DQB1 A^{57} susceptibility epitopes as compared to only 29.7% of the control subjects ($p \leq 0.0001$, OR 21.3). Our study reports for the first time the presence of a new haplotype, DR4 – DQ2.3 in T1DM patients. In addition, our study lends further support to the significant role of HLA epitopes in T1DM risk. Amino acid differences alter the binding characteristics of HLA molecules and thus determine immune cell activation and autoimmune disease susceptibility. This has been illustrated by studies showing increased binding of autoantigens by susceptibility epitopes as compared to resistance epitopes. For example, the HLA-DRB1 epitope $LA^{67,74}$ had increased binding to citrullinated vimentin compared to D^{70}, whilst the presence of the latter abrogated the binding this RA autoantigen (Freed et al., 2011). To definitively decipher the involvement of specific epitopes in T1DM, further studies are required to compare the binding of T1DM autoantigens to susceptibility and resistance epitopes.

T1DM risk conferring HLA alleles explained through amino acid composition

The importance of the epitope analysis becomes apparent when considering closely related haplotypes or alleles with different risk determined by the presence of certain epitopes. For example, the closely related haplotypes DRB1*04:01-DQA1*03:01-DQB1*03:02 and DRB1*04:04-DQA1*03:01-DQB*03:02 differ only at amino acid positions 71 (lysine vs. arginine) and 86 (glycine vs. valine) of DRB1; however, the former is highly predisposing whereas the latter haplotype is neutral (Erlich et al., 2008). Similarly, we compared the sequences of the HLA-DRB1*04:02 risk associated allele and that of the HLA-DRB1*13:01 protective allele that are very similar and both contain the resistance epitope R^{70}. The only differences are susceptibility epitopes V^{11} and H^{13} in *04:02, but also a number of position outside the binding pockets that were identified to confer susceptibility suggesting that these locations might also influence. In addition to explaining susceptibility difference in closely related alleles, sharing of epitopes by disparate alleles may explain some patients that do not have the high risk alleles. For example, we observed that whilst the majority of our T1DM patient cohort carried the established DR3 - DQ2.5 and DR4 - DQ8 risk haplotypes, 13% carried one copy of the DR4 - DQ2.3 haplotype and a small number of patients did not carry any of the known risk alleles. We were able to find that all patients however, even those that did not carry susceptibility haplotypes, carried susceptibility epitopes that we have identified in their DRB1 locus and one or more in the DQA1 and DQB1 loci.

Re-evaluation of the HLA alleles according to their amino acid composition suggests selected contribution of the HLA molecules DR/DQ to T1DM pathogenesis. Amino acids DQβ L^{26} and A^{57} that are part of the binding pocket but also at position that contributes in the stability of the heterodimer, are exclusively found in the risk DQB1 alleles *02:01 and *03:02. Similarly, all the significant amino acids on the risk DQA1 *03:01 and *05:01 alleles are uniquely different from all the other alleles. These ten amino acids (positions 11, 52, 55, 61, 64, 66, 69, 79, 129 and 175) have significant function either as pocket amino acids, TCR contact sites or part of the dimer of heterodimer formation suggesting that these DQA1 alleles, due to their distinctive amino acids composition, are contributors in the disease pathogenesis while other genetic factors may act as disease modifiers.

4.1.2 Non-classical HLA

Even though it managed to explain the HLA class II non-risk allele presence through the shared epitope hypothesis, the amino acid analysis failed to explain the significance observed in the above HLA class I alleles since only a few pocket amino acids in HLA-B class were found significant.

Studies on HLA involvement in T1DM predisposition have shown independent associations with HLA class I alleles (Noble and Erlich, 2012), suggesting a particular role in the age of onset of T1DM (Noble et al., 2010, Noble et al., 2002, Tait et al., 2003, Valdes et al., 2005, Nejentsev et al., 2007, Howson et al., 2009). A study by Baschal et al. suggested that risk for T1DM lay in polymorphisms of the telomeric MHC locus, a region closest to classical and non-classical HLA class I alleles (Baschal et al., 2011). This also became apparent from the strong associations of HLA class I molecules in our study population described above, that were not due to LD with the risk associated HLA-class II risk haplotypes.

HLA-G and T1DM

In this context, and due to its immunomodulatory characteristics, HLA-G has been identified as a potential key player in the pathogenesis and/or disease progression (Eike et al., 2009). Although HLA-G has been associated with autoimmune diseases, like multiple sclerosis (Wiendl et al., 2005), rheumatoid arthritis (Verbruggen et al., 2006), systemic lupus erythematosus (Rizzo et al., 2008) and psoriasis (Borghi et al., 2008, Aractingi et al., 2001), its involvement in T1DM is currently understudied.

Even though the HLA-G gene polymorphism is low, the association of the HLA-G 3'UTR 14bp polymorphism and the gene's expression, alternative splicing and concentration in sera has been widely explored (Hviid et al., 2003, O'Brien et al., 2001, Hviid et al., 2004, Hiby et al., 1999, Rousseau et al., 2003). In summary, the presence of the 14bp insertion generates an additional splice site, where 92 bases are removed from the start of exon 8 rendering the molecule resistance to mRNA degradations (Rousseau et al., 2003). It was concluded by many investigators that the 14bp *INS/INS* genotype is associated with lower serum and plasma levels of sHLA-G as compared to the *INS/DEL* and *DEL/DEL* genotypes (Chen et al., 2008, Hviid et al., 2004, Hviid et al., 2006). Therefore, it became evident that the HLA-G 3'UTR is involved in the post-transcriptional regulation of the HLA-G molecule (Castelli et al., 2011).

In this study, no significant observations could be drawn in either allele or genotype distribution of the 14bp *INS/DEL* between T1DM patients and controls (P>0.002). This is in agreement with a recent study published on rheumatoid arthritis (RA) that also failed to show overall significance of the 14bp *INS/DEL* (Mariaselvam et al., 2015). However, our findings are in contrast with the findings of this study, which show that the insertion allele is involved with early age of onset of RA. Instead, we demonstrate herein that the *DEL/DEL* genotype was significantly more frequent in the early age of onset group with T1DM, in contrast to RA. Moreover, our results are in complete agreement with a recent publication that implicated the HLA-G 14-bp INS/DEL polymorphisms in T1DM susceptibility and concluded that the homozygous deletion genotype was associated with earlier age of onset (Silva et al., 2015). Further on, in our study, age group 2, with mean age of onset of 11 years, had similar frequencies and distribution with the control group. Significance in frequencies was observed between the early and late age of onset groups, hinting a possible association between the 14bp *INS/DEL* and the disease age of onset.

This association became more apparent through the comparison between the different age of onset groups. Age group 1, that is the early onset patients, showed significant difference of the *INS/DEL* genotype when compared to age group 3 (P=0.0006). The *INS/DEL* genotype pattern showed an increasing trend with increasing age of onset. Opposing the increasing trend of the *INS/DEL* genotype, the *DEL/DEL* genotype showed a decreasing pattern with progressing age of onset. A significant decrease was also observed in the frequencies of this genotype between age group 1 and 3 (P=0.001). The presence of the *DEL/DEL* genotype in early onset T1DM was almost 3-fold higher as compared to late onset patients. This data strongly suggests that the *DEL/DEL* genotype is highly associated with earlier expression of autoimmune diabetes.

Further dissection of our cohort according to *INS* or *DEL* allele presence/absence revealed an increased presence of the *Insertion* allele in age group 3 (P=0.0001) when compared to age group 1, possibly due to the increasing numbers in *INS/DEL* genotype frequencies. This was not however observed from the statistical analysis of results from the presence/absence of the *Deletion* allele. As shown in Figure 1, significant difference between the early age of onset and the late age of onset groups was solely due to the presence or absence of the *Insertion* allele. Therefore, this reveals a probable dominant effect of the *Insertion* allele over the *Deletion* in

both homozygous and heterozygous that may offer a partial protective effect and prolong the age at which the disease presents.

In conclusion, even though there was no significant association between the *HLA-G* 14bp polymorphism and T1DM incidence, a strong association was observed between this polymorphism and disease age of onset. More specifically, the *DEL/DEL* genotype was significantly more frequent in patients with early onset of T1DM as compared to those with later disease onset, whereas the opposite was observed for the insertion allele, which was more prevalent in late onset patients. Therefore, the insertion allele may confer *HLA-G* mRNA stability, thereby possibly offering disease delay or protection, as opposed to the deletion allele. Although studies have associated the *INS/INS* genotype with lower sHLA-G values, no association was reported for the effect of this polymorphism on the levels of the other isoforms of the *HLA-G* molecule. In the case of localized autoimmune diseases, such as T1DM, cytoplasmic and membrane-bound *HLA-G* isoforms need to be further explored. We speculate that the insertion allele may influence the levels of the intracellular and membrane-bound *HLA-G*, thus affecting its tolerogenic role at the tissue level. Similarly, the increased incidence of the *DEL/DEL* genotype in patients with early onset T1DM may indicate a failure of *HLA-G* to confer sufficient immune-tolerance at the pancreatic level. The gene expression study performed to measure the HLA-G gene expression levels in the three age of onset groups confirm this theory as early age of onset patients portray 2-fold lower HLA-G expression than late onset and 1.4-fold less than age group 2.

The data presented herein is supportive of the involvement of HLA-G in the pathogenesis of T1DM in the context of age of onset independently from the HLA class II disease associated risk haplotypes, supporting the finding of the first study that investigated the HLA-G 14bp polymorphism in Brazilian T1DM patients (Silva et al., 2015). The above significant observations were drawn in two geographical distinct populations, further strengthening the notion of the HLA-G involvement in T1DM pathogenesis.

New HLA-G gene alleles

The HLA-G promoter haplotypes, genotypes and 3'UTR haplotypes, were analysed for the identification of new alleles. The populations in this study present the same promoter haplotypes already described, with several new rare ones (010101H, 010101I, 010101J) mainly because there are variable sites that were not previously described. Promoter haplotype Promo-G0104A presented a borderline P value when using the Fisher exact test. SNP -483 was detected as a susceptibility factor by using GWASpi and Logit tools, but the Fisher exact test did not confirm it. As seen from both GWASpi and Logit tools analysis, the most significant promoter haplotype identified to be associated with T1DM was the Promo-0104A (p=0.056) and the most significantly associated with the control group was the Promo-010102A (p=0.010).

In the case of HLA-G allele genotype, it was apparent by the analysis that our control population presented a number of new HLA-G alleles. The most common ones detected worldwide were also represented here, but there were a number of new alleles due to the presence of crossing-over and new mutation. Such alleles were named as "undef" for undefined. Furthermore, the undefined allele (h9) was strongly associated with diabetes (p=0.03). In addition, SNP +755, a non-synonymous mutation, was associated with G*01:04, detected by GWASpi as a susceptibility factor (P=0.0271, OR=1.51815). This allele was in LD with promo-0104a. None of the other alleles were associated with T1DM by GWASpi analysis, but Logit analysis revealed further significance for the following genotypes: G*010101, G*01010103, G*01010101, G*01010106 and G*01010105. G*01010106 and G*01010105 also showed significance through Fisher's Exact test.

Analysis of the HLA-G 3'UTR in our populations presents the same 3'UTR haplotypes already described, with several new rare ones mainly because there are variable sites that have not been previously reported. UTR-3 was associated with T1DM by both Logit (p=0.016) and GWASpi (p=0.029) analysis. A new-A haplotype that was also highly associated with diabetes (p=0.03). On the SNP analysis level, SNP +3092 was detected as a susceptibility marker for Diabetes by the GWASip. This SNP was carried by the new-A haplotype. In addition, further dissection into the three age of onset groups revealed that this HLA-G Promo-G*0104a, G*01:04:01 and UTR-3 were over-represented in Age group 1 in comparison to Age group 3, showing an overall involvement of the HLA-G in T1DM progression.

In summary, the previously described extended haplotype Promo-G*0104a/G*01:04/UTR-3 was associated with T1DM. Also, the new 3'UTR haplotype named here new-A and the HLA-G allele Undef-h9 was strongly associated with T1DM. Considering

these findings, an attempt was made to explain the distinct association of HLA-class I alleles with diabetes. Since as described above, HLA-A and –B alleles portrayed an association to diabetes which for some alleles was independent from the HLA-class II risk alleles, an analysis for LD was performed between HLA-A alleles and HLA-G 3'UTR, due to their proximal location on the MHC. Therefore, the new haplotype "new-A" that was associated with T1DM is associated with A*01:03 (in all cases), the UTR-3, which is also associated with diabetes, is usually associated with A*24:02:01, A*23:01:01 or A*33:03:01, the new haplotype "new-B" is associated with A*30:04:01 in all cases, the UTR-6 and UTR-18 are associated with A*29 alleles and the UTR-7 was in absolute LD with A*11:01:01. It is thus important to stress that even though it was suggested above that the HLA-A*24:02 was significantly higher in our population due to LD with the class II risk haplotype, it is now further questioned whether this high frequency is due to HLA-G 3'UTR selection. In addition, the population frequency of HLA-A*01:03 is low in the Cypriot general population (0.3%), while a frequency of 1.76% is observed in our diabetic cohort. The extended haplotype HLA-A*01:03-B*73:01-C*15:05-DRB1*04:05-DQB1*02:01 has a frequency of 0.3% (56 in 17398 typed at 5 loci) in our general population confirming that this haplotype is in complete LD, with relative risk 11.5 and $p<0.001$. This can now be explained due to selection bias of the significant HLA-G 3'UTR new-A haplotype in diabetics, further strengthening the notion that elements in the MHC other than HLA-class II play a significant role in diabetes susceptibility and probably pathogenesis.

HLA-G gene expression analysis

Our results show that, the UTR-3 haplotype (14bpDEL/+3001C/+3003T/+3010C/+3027C/+3035C/+3142G/+3187A/+3196C) is over-represented in diabetic patients. This 3'UTR haplotype is associated with low HLA-G mRNA and soluble HLA-G expression (de Albuquerque et al., 2016) and was also confirmed by our study through the gene expression assay. Therefore, the decreased expression of HLA-G at the pancreatic level in individuals genetically prone to produce less HLA-G could be damaging. In order to prove this hypothesis, we are currently investigating the HLA-G^+ Tregs of patients and controls with different HLA-G 3'UTR haplotype to reveal potential impairment in their HLA-G production. In addition to the 3'UTR influence on HLA-G expression levels, it has also been widely described that the SNP -725G variant in the promoter region is associated with higher

expression levels (Castelli et al., 2014b). In our patients and controls, the most dominant promoter haplotype that was also over-represented in the early age of onset was Promo-0104A (p=0.03). This promoter haplotype, carries the -725C variant, therefore is associated with lower HLA-G expression levels. As a result, the most common HLA-G extended haplotype (Promo-G*0104A/G*01:04/UTR-3) is not only associated with T1DM, but it is also involved in the disease progression as it is more frequent in the early age of onset group. A number of studies have previously described that this haplotype is related to lower HLA-G expression levels.(Castelli et al., 2014a)

4.2 NON- HLA INVOLVEMENT IN T1DM

The immune system is composed of Th1-mediated cellular immunity and Th2-mediated humoral immunity, a balance of which is essential to maintain health. Excessive activation of both Type 1 and Type 2 immunity may cause various immune diseases such as diabetes by Type 1, and allergy or tumour genesis by Type 2. Therefore, the regulation of the 'immune balance' between Type 1 and Type 2 immunity is critical for prevention and therapy of immune diseases.

Genetic association between non-HLA loci and T1DM were also questioned in the course of this study. These factors include cytokines TNF-α, IL10, IL6, IFN-gamma, TGF-β and protein receptor cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). Therefore, in addition to the HLA-G involvement in Type 1 diabetes, the effect of other immunomodulatory elements such as cytokines was investigated. The three main factors that showed significant association with either susceptibility or resistance to T1DM were cytokines TNF-α, TGF-β and CTLA-4.

TNF-α and T1DM

Tumor necrosis factor-α (TNF-α) is an important pro-inflammatory cytokine implicated in the pathogenesis of T1DM. TNF-α G/G genotype was found to be significant with both Fisher's exact test (p=0.046) and Logit analysis (p=0.023) and was associated with resistance to T1DM. On the contrary, TNF-alpha A/G was associated with susceptibility to T1DM (Logit p=0.038). These findings translate into a TNF-α high production in diabetics and TNF-α low production in the control group. The TNF gene locus is located within the MHC class III region

and its genetic polymorphisms have been reported to be associated with T1D (Nishimura et al., 2003). Nevertheless, it is unclear whether these associations are primary or because of LD with other predisposing genes within the MHC. The allele frequency of TNF-α SNP -308A, was determined to be significantly increased among patients compared to controls. This observed positive association of TNF-α SNP -308A was caused by its presence on HLA-B*50-DRB1*03-DQB1*02 (B50-DR3-DQ2) haplotype which is associated with T1DM in our patient population. This finding confirms the already reported combined effect of this B50-DR3-DQ2 and TNF-α A haplotype (Kumar et al., 2012). Therefore, these results indicate that TNF-alpha associations with T1D maybe caused by their linkage disequilibrium with specific HLA-DR3-DQ2 haplotypes in the Cypriot population. As it was shown above that the HLA-B*50 was significantly increased independently from the HLA class II risk haplotype, it is suggested that its significance derives solely due to LD with the TNF-α A allele. Since polymorphism in the promoter region regulates TNF expression levels, it retains crucial immunological significance in the development of T1D and its management.

CTLA-4 and T1DM

Another knownT1DM risk allele lies in the gene encoding CTLA-4 (Marron et al., 2000). CTLA-4 acts as vital modifier of the immune system and specifically the down-regulation of T-cell activation via binding with its ligands CD80 and CD86. Numerous research groups have investigated the different CTLA4 genotypes contribution in different autoimmune diseases. One of the most studied polymorphisms of this gene is in exon 1. The A49G polymorphism is the only polymorphism that leads to change to the primary amino acid sequence of CTLA-4. The G allele of an A-G transition at position 49, results in a Thr-Ala substitution in the leader peptide and is located 5.3 kb of the (AT)n locus. *In vitro* studies of A49G CTLA-4 have shown that this mutant form of CTLA-4 is aberrantly processed in the endoplasmic reticulum, leading to reduced surface expression (Anjos et al., 2002). Nevertheless, the exact mechanism for how this polymorphism affects CTLA-4 function remains unclear. In addition to effects in processing and intracellular trafficking, this polymorphism may affect oligomerization and surface retention (Teft et al., 2006). The predominant hypothesis in humans, however, is that the allelic variant lowers the mRNA levels of the soluble CTLA-4 splice variant (Ueda et al., 2003). Even though most studies implicate

the CTLA-4 49 G allele with susceptibility to autoimmune disease, current literature is not definitive on this matter (L.M. Gómez Osorio, 2005). It was observed that the A49 allele dominates in whites, whereas the G49 allele is more common in Asian populations (Yung et al., 2002). Ethnic and regional heterogeneity may, therefore, contribute weakly to explaining the differences between association studies. Through our analysis, CTLA-4 G/G was also associated with the control group (p=0.023), while CTLA-4 A/G was significantly over-represent in the diabetic group (p=0.038). CTLA-4 A SNP was also found to be significant and strongly associated with the diabetic group. Since the predisposing CTLA-4 allele is the 49G, one may conclude from our study that the negative regulation of immune responses and T-cell activation through CTLA-4 is not impaired in our diabetics.

Furthermore, CTLA-4 is expressed constitutively by T cells that suppress effector T-cell responses, referred to as T-regulatory cells (Tregs). 'Naturally occurring' $CD4^+$ T regulatory cells (nTreg) are derived centrally in the thymus and constitutively express CD25 and CTLA-4 (Workman et al., 2009). It has been shown that blockade of CTLA-4 *in vivo* inhibits nTreg cell–mediated suppression (Read et al., 2006). These cells generally appear to exert suppressive effects by direct cell contact rather than cytokine production, even though they can also function through the induction of inhibitory cytokines such as TGF-β. TGF-β induces cell-cycle arrest and apoptosis of targeted immune cells (Grossman et al., 2004). Such observations may conclude that the autoimmune diseases may develop as a result of a more fundamental failure of underlying immune regulation, rather than a simple skewing of immune response along a Th1/Th2 homeostasis as previously thought.

TGF-β and T1DM

TGF-β was also found to have significant difference between our study populations. TGF-β high expression was found significant in the T1D group (p=0.038), while TGF-β intermediate expression was significantly present (p=0.05) in the control population. This strengthens the notion that since CTLA-4 levels are presumably high and TGF-β levels are high in our diabetic cohort, T-cell activation mechanisms are not impaired and the Treg numbers are not deficient. Nonetheless, Treg mechanisms may be otherwise impaired (Buckner, 2010b, Liberal et al., 2015).

Similarly, induced Tregs that mature from $CD4^+$ precursors in peripheral sites, are influenced by TGF-β (Fu et al., 2004), and acquire the typical Treg markers CD25 and CTLA-4, show different characteristics from nTregs. These cells are named Th3 cells and their differentiation depends upon IL-2 and TGF-β (Sakaguchi et al., 1995). Due to the regulatory and tolerogenic ability of Tregs, their interactions with other immunomodulatory components, such as HLA-G, was greatly investigated (Huang et al., 2009b, Huang et al., 2009a, LeMaoult et al., 2007). The presence of $HLA\text{-}G^+$ T cells at sites of inflammation suggests an important function in modulating inflammatory responses in vivo.

$HLA\text{-}G^+$ T-regulatory cells and T1DM

It is yet to be determined though whether the function or differentiation of $HLA\text{-}G^+$ Treg cells is impaired in autoimmune diseases. As aforementioned, only the $FOXP3^-$ precursor cells give rise to Treg subsets. (Maynard et al., 2007) In the periphery, $HLA\text{-}G^+$ Tregs of thymic origin exist in limited numbers and with limited proliferating ability (Feger et al., 2007). Recently, it was demonstrated that hypomethylating agent azacitidine (Aza) induced $FOXP3^-$ HLA-G expressing immunomodulatory T cells (Spyridonidis et al.), and therefore acquiring the best of both worlds: the immune-modulatory effects of Tregs and HLA-G. This was also replicated in our laboratory, where $CD4^+/CD25^+/HLA\text{-}G^+$ T-cells in the periphery increased by 10%. In view of the fact that, CTLA-4 and TGF-β mechanisms do not seem impaired in our diabetic population but, as shown above, the HLA-G expression plays a vital role in the disease onset, this subtype of Tregs is a good candidate for explaining events in autoimmune disease.

In therapeutic context and due to its immunomodulatory role, studies and clinical trials are exploring the possibility of Treg administration to improve immune tolerance in the periphery of T1DM patients (Marek-Trzonkowska et al., 2012). Therefore, we suggest that $HLA\text{-}G^+$-Tregs may portray a greater effect in reversing T1DM phenotype than other proposed medical protocols.

Summary of Results

In summary, this study reports for the first time the HLA genotypes of the Cypriot diabetic population giving strong evidence of the independent involvement of HLA alleles to the overall genetic susceptibility. We report herein, a new susceptibility HLA haplotype, the DR4-DQ2.3 that shares the same T1DM risk conferring alleles as the widely recognised DR3-DQ2.5 and DR4-DQ8 risk conferring haplotypes and accounts for 92% of our diabetic cohort.

Further on, this study hypothesized the involvement of a 14bp INS/DEL polymorphism (*rs371194629*) at the 3' untranslated region (UTR) of HLA-G in the context of T1DM and age of onset. Findings that emerge from this study show a strong association between the HLA-G 14bp-polymorphism and T1DM with respect to the age of onset. Specifically, the deletion/deletion (*DEL/DEL*) genotype was found to be associated with an early age of onset, while the presence of the insertion allele was associated to a later age of onset, portraying a possible dominant effect over the deletion allele, a role in delaying disease onset and an overall involvement of HLA-G in the pathogenesis of Type I diabetes. In addition, this 14bp polymorphism is part of the most common HLA-G extended haplotype (Promo-G*0104a/G*01:04/UTR-3) that is not only associated with T1DM, but is also involved in the disease progression as it is more frequent in the early age of onset. Studies have previously described that this haplotype is related to lower HLA-G expression levels. Moreover, new HLA-G promoter haplotypes, genotypes and 3'UTR haplotypes have been recognised in the Cypriot population due to new SNPs combinations and the high diversity of our population.

The significant differences between T1DM patients and controls for genetic susceptibility in the CTLA-4 gene and the TGF-β hinted a possible involvement of Treg, as both factors are strongly required in their augmentation. Therefore, we believe that HLA-G localised at the pancreatic level, but also HLA-G^+ Tregs fail to render immune modulation during autoimmune response due to HLA-G genotypic differences and propose these cells as potential therapeutic targets.

Lastly, we also suggest an alternative approach to genetic susceptibility through the presence or absence of specific amino acids that compose a common epitope between already recognised susceptibility alleles and other non-risk associated ones. Through the same analysis, we report for the first time a single amino acid substitution in the DRB1 allele at position 70 from Arginine (R) to Aspartate (D) that alone discriminated between resistance and susceptibility to T1DM respectively. The amino acid composition of the HLA alleles, suggest exclusive contribution of the HLA molecules DR/DQ to T1DM pathogenesis. Amino acids

DQβ L^{26} and A^{57} that are part of the binding pocket but also at position that contributes in the stability of the heterodimer, are found in the risk DQB1 alleles *02:01 and *03:02. Similarly, all the significant amino acids (DQα **$Y^{11}R^{52}R^{55}F^{61}T^{64}I^{66}L^{69}V/L^{76}H^{129}E/K^{175}$**) on the risk DQA1 *03:01 and *05:01 alleles are entirely different from all the other alleles. These amino acids have significant function either as pocket amino acids, TCR contact sites or part of the dimer of heterodimer formation. 97% of our patients were carriers of the DQα **$Y^{11}R^{52}R^{55}F^{61}T^{64}I^{66}L^{69}V/L^{76}H^{129}E/K^{175}$** with or without the DRβ **Q^{70}** and/or DQβ **$L^{26}A^{57}$**, while the remaining 3% patients carried the DRβ **Q^{70}** and/or DQβ **$L^{26}A^{57}$** only, suggesting that these alleles, due to their distinctive amino acids composition, are the main contributors in the disease pathogenesis while other genetic factors may act as disease modifiers.

APPENDIX I

Table 1: General Population 50 most common haplotypes. Red lettering depicts T1DM risk associated haplotypes.

Rank #	A	B	C	DRB1	DQB1	FREQ.
1	33:01:00	14:02	8:02	1:02	5:01	0.019495
2	24:02:00	35:02:00	4:01	11:04	3:01	0.013537
3	2:01	51:01:00	15:02	16:01	5:02	0.013149
4	32:01:00	40:02:00	2:02	16:02	5:02	0.0117
5	24:02:00	18:01	12:03	11:04	3:01	0.009452
6	29:01:00	7:05	15:05	10:01	5:01	0.00792
7	32:01:00	35:03:00	4:01	11:04	3:01	0.007782
8	2:01	51:01:00	14:02	4:03	3:05	0.007433
9	1:01	52:01:00	12:02	15:02	6:01	0.007424
10	2:01	44:05:00	2:02	16:01	5:02	0.007359
11	11:01	35:01:00	4:01	11:01	3:01	0.007355
12	11:01	52:01:00	12:02	15:02	6:01	0.007337
13	2:01	35:01:00	4:01	14:01	5:03	0.007031
14	24:02:00	35:03:00	4:01	16:01	5:02	0.006826
15	**24:02:00**	**8:01**	**7:02**	**3:01**	**2:01**	**0.006682**
16	24:02:00	35:02:00	4:01	10:01	5:01	0.006427
17	24:03:00	18:01	7:01	11:04	3:01	0.006372
18	11:01	35:01:00	4:01	16:02	5:02	0.006307
19	3:01	14:02	8:02	1:02	5:01	0.006307
20	11:01	55:01:00	3:03	16:01	5:02	0.006105
21	**3:01**	**35:08:00**	**4:01**	**3:01**	**2:01**	**0.005152**
22	24:02:00	18:01	7:01	11:04	3:01	0.005079
23	24:02:00	35:03:00	4:01	11:04	3:01	0.004909
24	32:01:00	35:08:00	4:01	16:02	5:02	0.004778
25	68:01:00	35:01:00	4:01	11:01	3:01	0.004587
26	2:01	45:01:00	6:02	1:01	5:01	0.004587
27	26:01:00	38:01:00	12:03	16:01	5:02	0.004587
28	2:01	35:08:00	4:01	13:01	6:03	0.004587
29	**24:02:00**	**35:01:00**	**4:01**	**4:02**	**3:02**	**0.004587**
30	**2:01**	**41:01:00**	**7:01**	**3:01**	**2:01**	**0.004587**
31	31:01:00	35:03:00	4:01	16:01	5:02	0.004569
32	2:01	18:01	12:03	11:04	3:01	0.004515
33	2:17	55:01:00	3:03	16:01	5:02	0.004405
34	32:01:00	35:01:00	4:01	11:04	3:01	0.00433
35	32:01:00	18:01	12:03	11:04	3:01	0.004194
36	24:02:00	35:01:00	4:01	16:01	5:02	0.004159
37	30:01:00	13:02	6:02	7:01	2:02	0.004071
38	1:01	35:02:00	4:01	11:04	3:01	0.004044
39	32:01:00	14:02	8:02	1:02	5:01	0.004014
40	2:01	35:01:00	4:01	11:04	3:01	0.004014
41	11:01	52:01:00	12:02	4:03	3:05	0.004014
42	2:01	51:08:00	16:02	4:08	3:01	0.004014
43	33:03:00	14:01	8:02	4:03	3:05	0.004014
44	29:01:00	7:05	15:05	11:01	3:01	0.003988
45	11:01	35:01:00	4:01	1:01	5:01	0.003839
46	24:02:00	44:02:00	16:04	11:04	3:01	0.003831
47	1:01	13:02	6:02	7:01	2:02	0.003819
48	**3:01**	**35:03:00**	**4:01**	**4:03**	**3:02**	**0.00371**
49	2:01	35:03:00	4:01	16:01	5:02	0.003699
50	32:01:00	40:02:00	2:02	16:01	5:02	0.003684

Table 2: General population haplotypes that carry the diabetes associated risk haplotype

Rank #	A	B	C	DRB1	DQB1	FREQ.
15	24:02:00	8:01	7:02	3:01	2:01	0.006682
21	3:01	35:08:00	4:01	3:01	2:01	0.005152
29	24:02:00	35:01:00	4:01	4:02	3:02	0.004587
30	2:01	41:01:00	7:01	3:01	2:01	0.004587
48	3:01	35:03:00	4:01	4:03	3:02	0.00371
55	3:02	44:02:00	16:04	4:02	3:02	0.00344
61	1:01	35:08:00	4:01	4:03	3:02	0.003106
64	3:01	8:01	7:01	3:01	2:01	0.002867
66	24:02:00	40:02:00	15:02	4:05	3:02	0.002867
72	1:01	41:01:00	17:01	4:02	3:02	0.002867
75	32:01:00	18:01	2:02	4:03	3:02	0.002867
79	2:01	35:08:00	4:01	4:03	3:02	0.002674
86	24:02:00	35:02:00	4:01	3:01	2:01	0.002294
87	1:01	8:01	7:01	3:01	2:01	0.002294
88	30:01:00	44:02:00	5:01	4:03	3:02	0.002294
89	2:01	58:01:00	3:02	4:03	3:02	0.002294
92	2:01	41:01:00	17:01	3:01	2:01	0.002294
107	24:02:00	51:01:00	14:02	4:03	3:02	0.001913
109	2:01	51:01:00	16:02	4:03	3:02	0.001863
122	11:01	35:01:00	4:01	4:05	3:02	0.00172
123	32:01:00	18:01	12:03	3:01	2:01	0.00172
129	33:03:00	58:01:00	3:02	3:01	2:01	0.00172
130	30:01:00	13:02	6:02	3:01	2:01	0.00172
134	24:02:00	55:01:00	3:03	4:01	3:02	0.00172
136	11:01	35:01:00	4:01	4:03	3:02	0.00172
137	3:01	35:14:00	4:01	4:02	3:02	0.00172
143	3:01	35:01:00	4:01	4:03	3:02	0.00164
144	3:02	35:01:00	4:01	4:03	3:02	0.00164
157	11:01	35:01:00	4:01	3:01	2:01	0.001362
158	2:01	35:03:00	4:01	4:03	3:02	0.001361
159	26:01:00	8:01	7:02	3:01	2:01	0.001345
164	3:01	35:02:00	4:01	4:03	3:02	0.00131
172	3:01	51:01:00	16:02	4:03	3:02	0.001248
174	1:01	35:01:00	4:01	3:01	2:01	0.001204
186	2:01	58:01:00	3:02	3:01	2:01	0.001147
187	1:01	51:01:00	15:13	4:02	3:02	0.001147
190	29:02:00	45:01:00	6:02	4:03	3:02	0.001147
193	2:01	18:01	12:03	3:01	2:01	0.001147
199	11:01	50:01:00	6:02	3:01	2:01	0.001147
200	1:01	41:01:00	7:01	3:01	2:01	0.001147
201	32:01:00	55:01:00	3:03	3:01	2:01	0.001147
216	68:01:00	14:02	8:02	3:01	2:01	0.001147
221	32:01:00	35:01:00	4:01	3:01	2:01	0.001147
232	24:02:00	41:01:00	17:01	4:03	3:02	0.001147
240	24:02:00	57:01:00	6:02	4:05	3:02	0.001147
250	30:04:00	35:01:00	4:01	4:02	3:02	0.001147
254	0.142361	39:24:00	7:01	4:03	3:02	0.001129
265	24:02:00	35:08:00	15:04	4:03	3:02	0.000918
268	2:05	15:01	12:03	4:05	3:02	0.00086

Table 3: Analysis of important residues in the DR beta 1 domain

DR Beta 1 domain							
AA POSITION	**AA**	**LOCATION**	**POTENTIAL CONTACT**	**P-CORR.**	**OR**	**Homozygosity Pcorr.**	**Homozygosity OR**
9	**E**	β strand 1	peptide pocket 9	2.40344E-05	6.932		
11	**V**	β strand 1	peptide pocket 6	4.41847E-08	3.905		
13	**H**	β strand 1	peptide pocket 4	8.82016E-17	7.574		
26	**Y**	β strand 2	peptide pocket 4	4.20748E-12	6.563		
33	H			8.82016E-17	7.574		
37	**N**	β strand 3	peptide pocket 9	4.95599E-07	3.799		
	F	β strand 3	peptide pocket 9	4.39895E-06	0.191		
57	**S**	Helix	peptide pocket 9 +hydrogen bond to peptide	1.50714E-07	4.456		
	A	Helix	peptide pocket 9 +hydrogen bond to peptide	0.000287944	0.164		
58	E			6.22453E-08	0.172		
60	**H**			0.000287944	0.164		
67	**L**	Helix	peptide pocket 7+TCR	0.000147905	4.494		
70	**R**	Helix	peptide pocket 7+TCR	3.59023E-18	12.42		
	Q	Helix	peptide pocket 4+TCR	7.42703E-09	0.22	1.40E-17	20.1
	D	Helix	peptide pocket 4+TCR	8.2398E-12	0.04	6.10E-10	0.11
71	**K**	Helix	Peptide pockets 4/7+TCR	3.01784E-11	5.48		
	R	Helix	Peptide pockets 4/7+TCR	5.12009E-06	0.139	3.90E-09	0.1
73	G			2.04621E-06	3.486		
74	**R**	Helix	peptide pocket 4	4.20748E-12	6.563		
	E	Helix	peptide pocket 4	1.36529E-05	0.165		
77	N	Helix	TCR	4.20748E-12	6.563		
	T	Helix	TCR	8.88073E-05	0.021	1.90E-08	0.01

Table 4: Analysis of important residues in the DR beta 2 domain

DR Beta 2 domain							
AA POSITION	**AA**	**LOCATION**	**POTENTIAL CONTACT**	**PCORR.**	**OR**	**Homozygosity Pcorr.**	**Homozygosity OR**
96	Y			8.82016E-17	7.574		
	Q			0.001340297	0.383	2.10E-04	0.03
98	E			4.29412E-08	3.945		
104	A			4.29412E-08	3.945		
112	Y	Extended chain	Homodimer of heterodimers	0.000287944	0.164		
120	N			4.41847E-08	3.905		
140	T	Extended chain	CD4 contact	2.40344E-05	6.932		
180	L			8.82016E-17	7.574		
181	M			0.000527111	0.255		

Table 5: Analysis of important residues in the DQ beta 1 domain

DQ Beta 1 domain							
AA POSITION	**AA**	**LOCATION**	**POTENTIAL CONTACT**	**P-CORR.**	**OR**	**Homozygosity Pcorr.**	**Homozygosity OR**
13	**A**	β strand 1	peptide pocket 4	1.1E-12	0.135		
14	M			2.06E-06	9.826	2.00E-07	10.9
	L			0.0004	0.369		
26	**G**	β strand 2	peptide pocket 4	1.38E-22	18.47	8.68E-06	0.11
	Y	β strand 2	peptide pocket 4	1.1E-12	0.135		
	L	β strand 2	peptide pocket 4	1.86E-05	0.317	4.30E-21	45.8
28	**S**	β strand 2	peptide pockets 4/7	1.61E-14	6.238		
	T	β strand 2	peptide pockets 4/7	3.99E-06	0.085		
30	**S**	β strand 2	peptide pocket 6 + hydrogen bond to peptide	1.61E-14	6.238		
	H	β strand 2	peptide pocket 6 + hydrogen bond to peptide	8.4E-06	0.306		
37	**I**	β strand 3	peptide pocket 9	1.61E-14	6.238		
	Y	β strand 3	peptide pocket 9	1.88E-06	0.082		
45	E			6.19E-12	0.137		
46	E			1.61E-14	6.238		
	V			3.99E-06	0.085		
47	**F**	β strand 4	peptide pocket 7	1.61E-14	6.238		
	Y	β strand 4	peptide pocket 7	3.99E-06	0.085		
52	L	Helix	Homodimerization patch	1.61E-14	6.238		
	P	Helix	Homodimerization patch	3.99E-06	0.085		
53	L	Helix	Homodimerization patch	5.22E-09	10.952		
	Q	Helix	Homodimerization patch	1.71E-08	0.232		
55	L	Helix	Homodimerization patch	1.61E-14	6.238		
	R	Helix	Homodimerization patch	1.5E-08	0.23	1.00E-09	0.07
57	**A**	Helix	peptide pocket 9 + hydrogen bond to peptide	1.33E-27	22.296	1.25E-24	100.6
	D	Helix	peptide pocket 9 + hydrogen bond to peptide	4.5E-25	0.067	3.94E-09	0.03
66	D	Helix	TCR	7.69E-13	5.532		
	E	Helix	TCR	1.11E-05	0.106		

DQ Beta 1 domain (continue)							
67	**V**	Helix	peptide pocket 7+TCR	7.69E-13	5.532		
	I	Helix	peptide pocket 7+TCR	1.11E-05	0.106		
70	**R**	Helix	peptide pockets 4+TCR	1.56E-08	12.079	1.30E-10	17.6
	G	Helix	peptide pockets 4+TCR	1.84E-07	0.263	2.30E-08	0.08
71	**K**	Helix	peptide pockets 4/7+TCR	1.61E-14	6.238		
	A	Helix	peptide pockets 4/7+TCR	0.0004	0.369		
74	**A**	Helix	peptide pocket 4	1.61E-14	6.238		
	S	Helix	peptide pocket 5	0.000393	0.369		
84	Q			5.22E-09	10.952		
	E			1.71E-08	0.232		
85	**L**	Helix	peptide pocket 1	5.22E-09	10.952		
	V	Helix	peptide pocket 1	1.71E-08	0.232		
86	**E**	Helix	peptide pocket 1	5.22E-09	10.952		
	A	Helix	peptide pocket 1	1.3E-08	0.232		
87	L			5.22E-09	10.952		
	Y			0.00088	0.377	3.30E-05	0.14
	F			6.35E-05	0.056		
89	**T**	Helix	peptide pocket 1	5.22E-09	10.952		
	G	Helix	peptide pocket 1	1.71E-08	0.232		
90	T	Helix	peptide pocket 1	5.22E-09	10.952		
	I	Helix	peptide pocket 1	1.71E-08	0.232		

Table 6: Analysis of important residues in the DQ beta 2 domain

DQ Beta 2 domain							
AA POSITION	**AA**	**LOCATION**	**POTENTIAL CONTACT**	**PCORR.**	**OR**	**Homozygosity Pcorr.**	**Homozygosity OR**
116	V			1.04E-06	10.135	1.80E-07	11.1
	I			0.0004	0.369		
125	A			2.59E-09	11.244	1.34E-11	18.57
	S			0.0004	0.369		
167	H	Loop	RGD loop	1.1E-12	0.135		
185	I			1.13E-09	5.134		

Table 7: Analysis of important residues in the DQ alpha 1 domain

DQ Alpha 1 domain							
AA POSITION	**AA**	**LOCATION**	**POTENTIAL CONTACT**	**Pcorr.**	**OR**	**Homozygosity Pcorr.**	**Homozygosity OR**
11	**Y**	β strand 1	peptide pocket 6	4.15E-09	11		
	C	β strand 1	peptide pocket 6	3.04E-08	0.24		
18	S			4.15E-09	11		
	F			3.04E-08	0.24		
26	S			1.61E-15	6.84		
45	V			4.15E-09	11		
	A			3.04E-08	0.24		
47	Q			1.61E-15	6.84		
	R			3.04E-08	0.24		
48	L			4.15E-09	11		
	W			3.04E-08	0.24		
50	L			3.19E-08	3.87		
	E			3.04E-08	0.24		
52	R	Extended chain	peptide pocket 1	3.53E-13	12.3	1.70E-15	29.9
	S	Extended chain	peptide pocket 1	3.04E-08	0.24		
53	R			3.19E-08	3.87		
	K			3.04E-08	0.24		
55	**R**	Extended chain	TCR	4.15E-09	11		
	G	Extended chain	TCR	3.04E-08	0.24		
56	**R**			1.61E-15	6.84		
	G			3.04E-08	0.24		
61	F	Helix	TCR	4.15E-09	11		
	G	Helix	TCR	3.04E-08	0.24		
64	T	Helix	TCR	4.15E-09	11		
	R	Helix	TCR	3.04E-08	0.24		
66	**I**	Helix	peptide pocket 6	4.15E-09	11		
	M	Helix	peptide pocket 6	3.04E-08	0.24		
69	**L**	Helix	peptide pockets 6/9 hydrogen bonds to peptide	4.15E-09	11	1.07E-12	22.86
	A	Helix	peptide pockets 6/9 + hydrogen bonds to peptide	3.04E-08	0.24		
76	**V**	Helix	peptide pocket 9 + hydrogen bonds to peptide	1.61E-15	6.84		

DQ Alpha 1 domain (continue)							
	M	Helix	peptide pocket 9 + hydrogen bonds to peptide	3.04E-08	0.24		
80	S			4.15E-09	11		
	Y			3.04E-08	0.24		

Table 8: Analysis of important residues in the DQ alpha 2 domain

DQ Alpha 2 domain							
AA POSITION	**AA**	**LOCATION**	**POTENTIAL CONTACT**	**Pcorr.**	**OR**	**Homozygosity Pcorr.**	**Homozygosity OR**
129	H	Extended chain	CD4 contact	2.58E-07	9.28	2.70E-09	13.9
	Q	Extended chain	CD4 contact	2.32E-05	0.32	1.70E-05	0.11
175	E	β strand 12	Homodimer of hederodimers	3.19E-08	3.87	1.20E-04	42.96
	Q	β strand 12	Homodimer of hederodimers	3.04E-08	0.24		
187	T			1.61E-15	6.84		

Table 9: The HLA-G promoter SNP profile for T1D cohort and control population.

SNP	Haplotype/Allele	T1D n=182	Frequency %	CTL n=192	Frequency %	p-value	Logit p-value
-1305G>A	A/A	55	30.22%	61	31.77%		
	A/G	84	46.15%	85	44.27%		
	G/G	43	23.63%	46	23.96%	0.928	
	A	43	23.63%	46	23.96%	1.000	
	G	55	30.22%	61	31.77%	0.823	
-1179A>G	A/A	26	14.29%	34	17.71%		
	A/G	92	50.55%	81	42.19%		
	G/G	64	35.16%	77	40.10%	0.259	
	A	118	64.84%	115	59.90%	0.338	
	G	156	85.71%	158	82.29%	0.400	
-1155G>A	A/A	8	4.40%	4	2.08%		
	A/G	59	32.42%	52	27.08%		
	G/G	115	63.19%	136	70.83%	0.193	
	A	67	36.81%	56	29.17%	0.124	
	G	174	95.60%	188	97.92%	0.248	
-1140A>T	A/A	83	45.60%	69	35.94%		
	A/T	76	41.76%	94	48.96%		
	T/T	23	12.64%	29	15.10%	0.163	
	A	159	87.36%	163	84.90%	0.551	
	T	99	54.40%	123	64.06%	0.059	
-1138A>G	A/A	160	87.91%	170	88.54%		
	A/G	21	11.54%	22	11.46%		
	G/G	1	0.55%	0	0.00%	0.485	
	A	181	99.45%	192	100.00%	0.487	
	G	22	12.09%	22	11.46%	0.874	
-1121T>C	C/C	172	94.51%	181	94.27%		
	C/T	10	5.49%	11	5.73%	1.000	
	C	182	100.00%	192	100.00%	NA	
	T	10	5.49%	11	5.73%	0.551	
-1098G>A	G/G	182	100.00%	192	100.00%	NA	
-964G>A	A/A	55	30.22%	63	32.81%		
	A/G	88	48.35%	86	44.79%		
	G/G	39	21.43%	43	22.40%	0.781	
	A	143	78.57%	149	77.60%	0.901	
	G	127	69.78%	129	67.19%	0.656	
-922C>A	C/C	182	100.00%	192	100.00%	NA	
-810C>T	C/C	182	100.00%	192	100.00%	NA	

-762C>T	C/C	39	21.43%	43	22.40%		
	C/T	88	48.35%	86	44.79%		
	T/T	55	30.22%	63	32.81%	0.781	
	C	127	69.78%	129	67.19%	0.656	
	T	143	78.57%	149	77.60%	0.901	
-725G>C	C/C	139	76.37%	140	72.92%		
	C/G	21	11.54%	26	13.54%		
	C/T	15	8.24%	21	10.94%		
	G/G	0	0.00%	2	1.04%		
	G/T	6	3.30%	3	1.56%		
	T/T	1	0.55%	0	0.00%	0.384	
	G	27	14.84%	31	16.15%	0.776	
	C	175	96.15%	187	97.40%	0.566	
	T	22	12.09%	24	12.50%	1.000	
-716T>G	G/G	55	30.22%	63	32.81%		
	G/T	87	47.80%	86	44.79%		
	T/T	40	21.98%	43	22.40%	0.823	
	G	142	78.02%	149	77.60%	1.000	
	T	127	69.78%	129	67.19%	0.656	
-689A>G	A/A	40	21.98%	43	22.40%		
	A/G	87	47.80%	86	44.79%		
	G/G	55	30.22%	63	32.81%	0.823	
	A	127	69.78%	129	67.19%	0.656	
	G	142	78.02%	149	77.60%	1.000	
-666G>T	G/G	40	21.98%	43	22.40%		
	G/T	87	47.80%	86	44.79%		
	T/T	55	30.22%	63	32.81%	0.823	
	G	127	69.78%	129	67.19%	0.656	
	T	142	78.02%	149	77.60%	1.000	
-646A>G	A/A	175	96.15%	187	97.40%		
	A/G	7	3.85%	5	2.60%	0.566	
	A	182	100.00%	192	100.00%	NA	
	G	7	3.85%	5	2.60%	0.566	
-633G>A	A/A	55	30.22%	63	32.81%		
	A/G	87	47.80%	85	44.27%		
	G/G	40	21.98%	44	22.92%	0.783	
	A	142	78.02%	148	77.08%	0.901	
	G	127	69.78%	129	67.19%	0.656	
-546/540A>AG	A/A	172	94.51%	175	91.15%		
	A/AG	9	4.95%	17	8.85%		

	AG/AG	1	0.55%	0	0.00%	0.200	
	A	181	99.45%	192	100.00%	0.487	
	AG	10	5.49%	17	8.85%	0.235	
-541/533GA>G	GA/G	22	12.09%	18	9.38%		
	GA/GA	160	87.91%	174	90.63%	0.408	
	GA	182	100.00%	192	100.00%	NA	
	G	22	12.09%	18	9.38%	0.408	
-539A>G	A/A	182	100.00%	192	100.00%	NA	
-521C>A	C/C	182	100.00%	192	100.00%	NA	
-509C>G	C/C	170	93.41%	185	96.35%		
	C/G	12	6.59%	7	3.65%	0.241	
	C	182	100.00%	192	100.00%	NA	
	G	12	6.59%	7	3.65%	0.241	
-486A>C	A/A	41	22.53%	44	22.92%		
	A/C	87	47.80%	85	44.27%		
	C/C	54	29.67%	63	32.81%	0.758	
	A	128	70.33%	129	67.19%	0.577	
	C	141	77.47%	148	77.08%	1.000	
-483A>G	A/A	175	96.15%	188	97.92%		
	A/G	7	3.85%	4	2.08%	0.370	
	A	182	100.00%	192	100.00%	NA	
	G	17	9.34%	4	2.08%	0.030	0.002
-477C>G	C/C	26	14.29%	34	17.71%		
	C/G	92	50.55%	80	41.67%		
	G/G	64	35.16%	78	40.63%	0.221	
	C	118	64.84%	114	59.38%	0.288	
	G	156	85.71%	158	82.29%	0.400	
-443G>A	G/G	182	100.00%	192	100.00%	NA	
-400G>A	A/A	1	0.55%	0	0.00%		
	A/G	21	11.54%	23	11.98%		
	G/G	160	87.91%	169	88.02%	0.586	
	A	22	12.09%	23	11.98%	1.000	
	G	181	99.45%	192	100.00%	0.487	
-391G>A	A/A	1	0.55%	0	0.00%		
	A/G	21	11.54%	23	11.98%		
	G/G	160	87.91%	169	88.02%	0.586	
	A	22	12.09%	23	11.98%	1.000	
	G	181	99.45%	192	100.00%	0.487	
-369C>A	A/A	64	35.16%	78	40.63%		
	A/C	90	49.45%	80	41.67%		

	C/C	28	15.38%	34	17.71%	0.319	
	A	154	84.62%	158	82.29%	0.580	
	C	118	64.84%	114	59.38%	0.288	
-355G>A	G/G	182	100.00%	192	100.00%	NA	
-284G>A	G/G	182	100.00%	192	100.00%	NA	
-256TC>T	TC/TC	182	100.00%	192	100.00%	NA	
-201G>A	A/A	55	30.22%	63	32.81%		
	A/G	87	47.80%	86	44.79%		
	G/G	40	21.98%	43	22.40%	0.823	
	A	142	78.02%	149	77.60%	1.000	
	G	127	69.78%	129	67.19%	0.656	
-56C>T	C/C	160	87.91%	169	88.02%		
	C/T	21	11.54%	23	11.98%		
	T/T	1	0.55%	0	0.00%	0.586	
	C	181	99.45%	192	100.00%	0.487	
	T	22	12.09%	23	11.98%	1.000	
+15G>A	A/A	55	30.22%	62	32.29%		
	A/G	88	48.35%	86	44.79%		
	G/G	39	21.43%	44	22.92%	0.788	
	A	143	78.57%	148	77.08%	0.804	
	G	127	69.78%	130	67.71%	0.738	

Table 10: The HLA-G 3'-UTR SNP profile for T1D cohort and control population.

SNP	**Haplotype/Allele**	**T1D n=182**	**Frequency %**	**CTL n=192**	**Frequency %**	**p-value**
+2942T>G	T/T	182	100	192	100	NA
+2960G>14bp INS	D/D	62	34	55	29	
	I/D	91	50	99	52	
	I/I	29	16	38	20	0.428
	I	120	66	137	71	0.267
	D	153	84	154	80	0.348
+3001C>T	C/C	182	100	192	100	NA
+3003C>T	C/C	3	2	2	1	
	C/T	34	19	33	17	
	T/T	145	80	157	82	0.809
	C	37	20	35	18	0.694
	T	179	98	190	99	0.678
+3010G>C	C/C	62	34	73	38	
	C/G	92	51	81	42	
	G/G	28	15	38	20	0.241
	G	120	66	119	62	0.452

	C	154	85	154	80	0.280
+3027C>A	A/A	0	0	1	1	
	A/C	18	10	28	15	
	C/C	164	90	163	85	0.233
	A	18	10	29	15	0.160
	C	182	100	191	99	1.000
+3032G>C	G/G	182	100	192	100	NA
+3035C>T	C/C	136	75	135	70	
	C/T	43	24	51	27	
	T/T	3	2	6	3	0.492
	C	179	98	186	97	0.504
	T	46	25	57	30	0.356
+3052C>T	C/C	182	100	192	100	NA
+3092G>T	G/G	177	97	191	99	
	G/T	5	3	1	1	0.113
	G	182	100	192	100	NA
	T	5	3	1	1	0.113
+3121T>C	C/T	4	2	3	2	
	T/T	178	98	189	98	0.717
	C	4	2	3	2	0.717
	T	182	100	192	100	NA
+3142C>G	C/C	24	13	35	18	
	C/G	94	52	82	43	
	G/G	64	35	75	39	0.176
	C	118	65	117	61	0.455
	G	158	87	157	82	0.203
+3177G>T	G/G	182	100	192	100	NA
+3183G>A	G/G	182	100	192	100	NA
+3187A>G	A/A	114	63	112	58	
	A/G	58	32	65	34	
	G/G	10	5	15	8	0.563
	A	172	95	177	92	0.413
	G	68	37	80	42	0.400
+3196C>G	C/C	97	53	99	52	
	C/G	68	37	73	38	
	G/G	17	9	20	10	0.917
	C	165	91	172	90	0.863
	G	85	47	93	48	0.757
+3227G>A	A/G	8	4	6	3	
	G/G	174	96	186	97	0.592
	A	8	4	6	3	0.592
	G	182	100	192	100	NA

Table 11: The HLA-G promoter SNP profile and haplotype interpretation for T1D cohort and control population. The allele presenting the lower frequency (in our population) is represented in shades of grey.

	29794317	29794443	29794467	29794482	29794484	29794501	29794658	29794700	29794812	29794860	29794897	29794906	29794915	29794933	29794956	29794976	29794989	29795076	29795081	29795113	29795136	29795139	29795145	29795222	29795223	29795231	29795253	29795325	29795421	29795566	29795636			
	rs1736936	rs1736935	rs3823321	rs1736934	rs17875389	rs3115630	rs1632947	rs370338057	rs182801644	rs1632946	rs1233334	rs2249863	.	rs2735022	rs35674592	rs17875391	rs1632944	rs201221694	rs368205133	rs17875393	rs1736933	rs149890776	rs1736932	rs17875395	.	rs17875396	rs1632943	rs781618441	rs1233333	rs17875397	rs1630223			
	-1305	-1179	-1155	-1140	-1138	-1121	-964	-922	-810	-762	-725	-716	-707	-689	-666	-646	-633	-546	-541	-509	-486	-483	-477	-400	-399	-391	-369	-297	-201	-56	15		DIABETES	CTRL
	G	A	G	A	A	T	G	C	C	C	G	T	A	A	G	A	G	A	GA	C	A	A	C	G	G	G	C	G	G	C	G		2n	2n
	A	G	A	T	G	C	A	A	T	T	C,T	G	G	G	T	G	A	AG	G	G	C	G	G	A	A	A	A	A	A	T	A		360	384
h3	A	G	G	T	A	C	A	C	C	T	C	G	A	G	T	A	A	A	GA	C	C	A	G	G	G	G	A	G	A	C	A	010102A	118	143
h4	G	A	G	A	A	C	G	C	C	C	C	T	A	A	G	A	G	A	GA	C	A	A	C	G	G	G	C	G	G	C	G	010101A	74	89
h7	A	G	A	A	A	C	A	C	C	T	C	G	A	G	T	A	A	A	GA	C	C	A	G	G	G	G	A	G	A	C	A	0104A	75	59
h1	G	A	G	A	A	C	G	C	C	C	C	T	A	A	G	A	G	A	G	C	A	A	C	G	G	G	C	G	G	C	G	010101F	22	18
h8	G	A	G	A	A	C	G	C	C	C	G	T	A	A	G	A	G	A	GA	C	A	A	C	G	G	G	C	G	G	C	G	010101B	14	22
h2	G	G	G	A	G	C	G	C	C	C	T	T	A	A	G	A	G	AG	GA	C	A	A	G	A	G	A	A	G	G	T	G	0103D	11	17
h5	G	A	G	A	A	T	G	C	C	C	G	T	A	A	G	A	G	A	GA	C	A	A	C	G	G	G	C	G	G	C	G	010101C	12	12
h6	G	G	G	A	G	C	G	C	C	C	T	T	A	A	G	G	G	A	GA	G	A	A	G	A	G	A	A	G	G	T	G	0103E	12	6
h9	G	A	G	A	A	C	G	C	C	C	C	T	A	A	G	A	G	A	GA	C	A	A	C	G	G	G	C	A	G	C	G	010101G	11	5
h10	G	G	G	T	A	C	A	C	C	T	C	G	A	G	T	A	A	A	GA	C	C	A	G	G	G	G	A	G	A	C	A	010102D	4	4
h11	G	A	G	A	A	C	G	C	C	C	C	T	A	A	G	A	G	A	GA	C	A	G	C	G	G	G	C	G	G	C	G	010101D	6	1
h13	G	A	G	A	A	C	G	C	C	C	C	T	A	A	G	A	G	A	GA	C	A	A	C	G	A	G	C	G	G	C	G	010101H	0	2
h15	G	A	G	A	A	C	G	C	C	C	C	T	A	A	G	A	G	A	GA	C	A	A	C	G	G	G	C	G	G	C	A	010101I	0	1
h17	G	A	G	A	A	C	G	C	C	C	C	T	A	A	G	A	G	A	GA	C	C	A	G	G	G	G	A	G	G	C	G	010101J	0	1
h19	G	G	G	A	G	C	G	C	C	C	T	T	A	A	G	A	G	AG	GA	G	A	A	G	A	G	A	A	G	G	T	G	0103A	0	1
h16	A	G	G	T	A	C	A	C	T	T	C	G	A	G	T	A	A	A	GA	C	C	A	G	G	G	G	A	G	A	C	A	010102B	0	1
h18	A	G	G	T	A	C	A	A	C	T	C	G	A	G	T	A	A	A	GA	C	C	A	G	G	G	G	A	G	A	C	A	010102C	0	1
h14	A	G	G	T	A	C	A	C	C	T	C	G	G	G	T	A	A	A	GA	C	C	A	G	G	G	G	A	G	A	C	A	010102E	0	1
h12	A	G	G	T	A	C	A	C	C	T	C	G	A	G	T	A	A	A	G	C	C	A	G	G	G	G	A	G	A	C	A	010102F	1	0

Table 12: The HLA-G coding region SNP profile and haplotype interpretation for T1D cohort and control population. The allele presenting the lower frequency (in our population) is represented in shades of grey. New alleles due to the presence of crossing over and new mutations are shown in green.

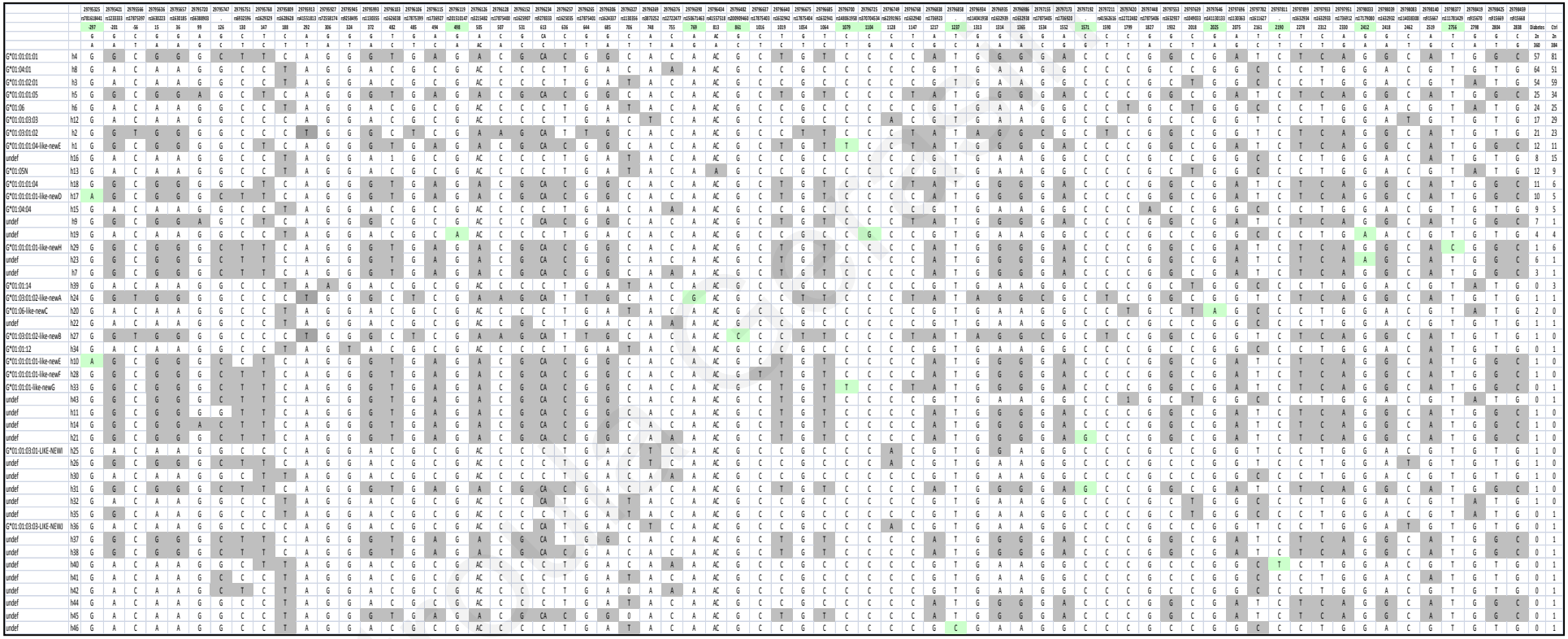

Table 13: The HLA-G 3'UTR region SNP profile and haplotype interpretation for T1D cohort and control population. The allele presenting the lower frequency (in our population) is represented in shades of grey

	INTRON 7 (AFTER STOP CODON)				EXON 8 / 3'UTR																
	29798377	29798419	29798425	29798459	29798581	29798610	29798617	29798634	29798642	29798651	29798699	29798728	29798749	29798794	29798803	29798834					
	rs111781429	rs915670	rs915669	rs915668	rs371194629	rs1707	rs1710	rs17179101	rs17179108	.	rs180827037	rs138249160	rs1063320	rs9380142	rs1610696	rs1233331					
	2756	2798	2814	2838	14bp	3003	3010	3027	3035	3044	3092	3121	3142	3187	3196	3227		Diabetes	Ctrl	Diabetes	Ctrl
	T	G	G	C	G	C	G	C	C	A	G	T	C	A	C	G		2n	2n	2n	2n
	C	A	T	G	GATTTGTTCATGCCT	T	C	A	T	T	T	C	G	G	G	A		360	384	360	384
h1	T	G	G	C	G	T	G	C	C	A	G	T	C	A	C	G	UTR-6	17	15	0.0472	0.0391
h2	T	G	T	G	GATTTGTTCATGCCT	T	C	C	T	A	G	T	G	A	C	G	UTR-5	23	23	0.0639	0.0599
h3	T	A	T	G	GATTTGTTCATGCCT	T	C	C	C	A	G	T	G	A	G	G	UTR-2	92	96	0.2556	0.2500
h4	T	G	G	C	G	T	G	C	C	A	G	T	C	G	C	G	UTR-1	75	89	0.2083	0.2318
h5	T	G	G	C	G	C	G	C	C	A	G	T	C	A	C	G	UTR-4	26	32	0.0722	0.0833
h6	T	G	T	G	G	T	C	C	C	A	G	T	G	A	C	G	UTR-3	71	52	0.1972	0.1354
h7	T	G	G	C	G	C	G	C	C	A	T	T	C	A	C	G	new-A	7	1	0.0194	0.0026
h8	T	G	T	G	GATTTGTTCATGCCT	T	C	A	T	A	G	T	G	A	C	G	UTR-7	19	30	0.0528	0.0781
h9	T	G	T	G	GATTTGTTCATGCCT	T	C	C	C	A	G	T	G	A	G	G	UTR-2	8	18	0.0222	0.0469
h10	T	G	T	G	G	T	C	C	T	A	G	T	G	A	C	G	UTR-13	3	6	0.0083	0.0156
h11	T	G	T	G	GATTTGTTCATGCCT	T	C	C	T	A	G	C	G	A	C	G	new-B	4	4	0.0111	0.0104
h12	T	G	G	C	G	T	G	C	C	A	G	T	C	A	C	A	UTR-18	10	6	0.0278	0.0156
h13	T	G	G	C	G	T	C	C	C	A	G	T	G	A	C	G	UTR-3	1	0	0.0028	0.0000
h14	T	G	T	G	G	T	G	C	C	A	G	T	C	G	C	G	UTR-1	1	0	0.0028	0.0000
h15	C	G	G	C	G	T	G	C	C	A	G	T	C	G	C	G	UTR-1	1	6	0.0028	0.0156
h16	T	G	G	C	G	T	C	C	C	A	G	T	G	A	G	G	UTR-10	1	0	0.0028	0.0000
h17	T	A	T	G	GATTTGTTCATGCCT	T	G	C	C	A	G	T	C	A	C	G	new-C	1	0	0.0028	0.0000
h18	T	A	T	G	GATTTGTTCATGCCT	T	G	C	C	A	G	T	G	A	G	G	UTR-8	0	1	0.0000	0.0026
h19	T	G	G	C	GATTTGTTCATGCCT	T	C	C	T	A	G	T	G	A	C	G	UTR-5	0	1	0.0000	0.0026
h20	T	G	T	G	G	C	G	C	C	A	G	T	C	A	C	G	UTR-4	0	1	0.0000	0.0026
h21	T	G	T	G	G	T	C	C	C	T	G	T	G	A	C	G	new-D	0	1	0.0000	0.0026
h22	T	G	G	C	GATTTGTTCATGCCT	T	C	C	C	A	G	T	G	A	G	G	UTR-2	0	1	0.0000	0.0026
h23	T	A	T	G	G	C	G	C	C	A	G	T	C	A	C	G	UTR-4	0	1	0.0000	0.0026

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