ANALYSIS OF THE HLA-DQ ALLELE IN THE TYPE 1 DIABETES POPULATION AND THEIR UNAFFECTED FIRST DEGREE RELATIVES

by

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ANALYSIS OF THE HLA-DQ ALLELES IN THE TYPE 1 DIABETES POPULATION AND THEIR UNAFFECTED FIRST DEGREE RELATIVES

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Type 1 diabetes mellitus (T1D) is a disease of major public health concern as it is one of the most common diseases of childhood and costs millions of dollars in health care each year in the U.S. T1D is an autoimmune disease and is caused by multiple factors including genetics, autoimmunity, and environment. The genetics of T1D is complex as there are multiple genes thought to play a role in its susceptibility, with the best defined risks associated with the HLA-DQ molecule. This study analyzes the role of the DQ molecule in 265 diabetic children and 1000 unaffected first degree relatives in order to further support the current literature of the presence of specific DQ alleles and haplotypes with a large representative sample from the U.S.

In the diabetic probands, the analysis was supported by the previous literatures for the presence of non-Asp 57 alleles but not for the presence of the DQ2 and DQ8 haplotypes. In this study, 94.14% (96.65% including the DR2 group) have at least one non-Asp allele with a breakdown of 30.54% as non-Asp/Asp (33.05% including DR2) and 63.60% with non-Asp/non-Asp, and 6% Asp/Asp. The distribution of the DQ2 and DQ8 haplotypes includes, 78.63% possessing DQ2 and/or DQ8 haplotypes with 6.49% as DQ2 homozygotes (DQ2/DQ2), 4.96% as DQ8 homozygotes (DQ8/DQ8), and 16.79% as DQ2/DQ8 heterozygotes. These figures are only slightly higher when looking at the Caucasians or the individuals with younger ages of onset. These unexpected results may be the result of clinical or ethnic variability in the population, however further investigation is warranted.

While there is limited information available of the non-Asp alleles and DQ2 and DQ haplotypes for first degree relatives, the results in the study seem to be supported both by previous studies and on the risks associated with each haplotype. Results show that 33.59% non-Asp/non-Asp, 58.2% Asp/non-Asp (with 14.55% of these individuals non-Asp/0602 specifically), and 8.21% Asp/Asp. In the DQ2 and DQ8 analysis 68.34% had DQ2 and/or DQ8 haplotypes, with 31.94% as DQ2 homozygotes or heterozygotes, 30.11% were either DQ8 homozygous or heterozygous, and 6.31% were DQ2/DQ8 heterozygotes.

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1.0 INTRODUCTION

1.1 PURPOSE OF THE STUDY

Type 1 diabetes mellitus (T1DM) is a disease of major public health concern and it is believed that there are multiple genetic, autoimmune, and environmental factors that play a role in its onset. The genetics of T1DM is complex as there are many different genes thought to play a role in susceptibility to developing T1DM. Making up 50-60% of the genetic risk of T1D, one of the best studied is the HLA gene class II molecules DQ. The purpose of this study is to examine the genetic distribution related to DQ in the T1DM population and their unaffected first degree relatives enrolled in the Etiology and Epidemiology of Insulin Dependent Diabetes study through Children's Hospital of Pittsburgh. This analysis is to determine if this study population has similar results to previous studies to further serve as evidence in the role of DQ and T1DM susceptibility.

2.0 BACKGROUND AND SIGNIFICANCE

2.1 DIABETES OVERVIEW

Type 1 diabetes (T1D), formerly known as juvenile diabetes due to its common onset during childhood or adolescence is also known as Type 1a diabetes or Insulin Dependent Diabetes Mellitus (IDDM). Type 1 diabetes is an autoimmune disorder characterized by the T-cell mediated destruction of the β cells of islets of Langerhans in the pancreas. The pancreatic β cells are responsible for the production of the hormone insulin in the body. Therefore, destruction of the β cells leads to absolute insulin deficiency resulting in chronic hyperglycemia [1]. As a result, individuals with T1D require daily exogenous insulin treatment as well as frequent surveillance of blood glucose levels.

It is estimated that over 20.8 million Americans have diabetes, with only 14.2 million actually being diagnosed. Of these individuals, 90-95% have what is known as type 2 diabetes, a non-autoimmune disease of insulin resistance that typically occurs in adulthood. The other 5-10% are affected with type 1 diabetes, with 1 in 400 adolescents being diagnosed with T1D[2]. Diabetes in general thus has huge impact on public health. The annual cost of T1D exceeds 14 billion dollars in Canada and about 10 times more in the United States, figuring in consumption of one in seven health care dollars [3].

The peak incidence of T1D is 11 years, but new cases occur almost as frequently in adulthood. T1D is more common in whites than blacks and differences in incidences vary across the world (Figure 1). The highest annual incidence of type 1 diabetes mellitus is found in Finland with 45 cases per 100,000, 30 per 100,000 in Sardinia, and the lowest is found in Korea with < 1 per 100,000. The incidence of type 1 diabetes is increasing at a rate of approximately 3% a year and the expected annual incidence is expected to exceed 30 per 100,000 by 2010[4].

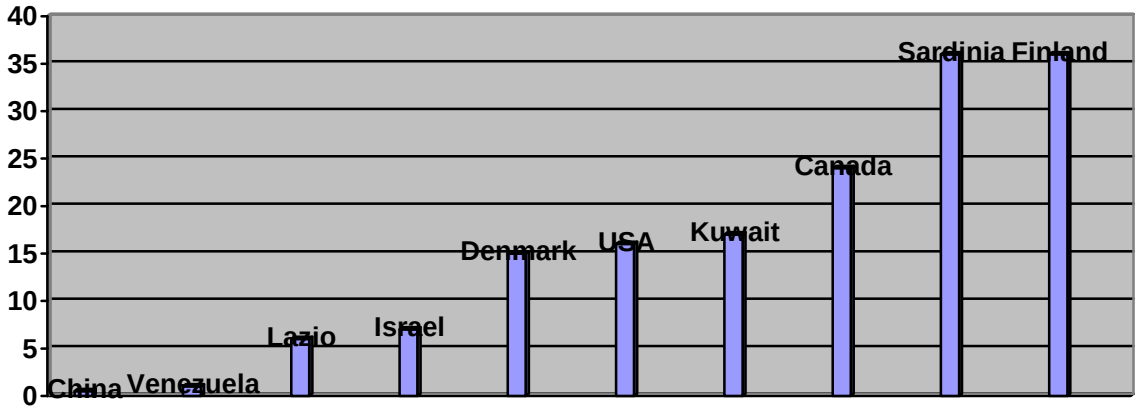

Figure 1: Annual Incidence of Type 1 Diabetes per 100,000 by Country [5]

2.2 PATHOPHYSIOLOGY

As other autoimmune diseases, T1D is believed to result from a combination of genetic, autoimmune, and environmental factors. The exact mechanism in humans has still yet to be elucidated but much has been discovered in the basic pathogenesis in the development in T1D.

The process includes specific haplotypes/genotypes establish a susceptibility to develop T1D, environmental factors that can either initiate an immune response against the β cells or that alter the structure of the β cells which then results in an immune response and destruction against the β cells, β cell infiltration by mononuclear immune cells (dendritic cells, macrophages, T-cells, etc) which directly contribute to β cell destruction, and then the subsequent development of T1D [6]. The specific process of the self-reactive T cells accumulating and targeting the β cells, where they expand and become increasingly efficient β cell destruction is known as progressive prediabetes. Prediabetes is believed to occur in individuals early in life and the process to diabetes development varies in length among individuals both within and across families [3]. While not the focus of this thesis, much work has been done and is still attempting to determine why some individuals develop T1D at a very young age and others in adulthood.

2.3 IMMUNE SYSTEM

The immune system is based on the basic concept of being able to recognize molecules in the body as either "self" or "nonself". Antigens are foreign particles, such as viruses or microbes, which contain markers that indicate to the immune system as "nonself". This initiates an immune response where antibodies are made. When the immune system mistakes "self" molecules as "nonself" and an immune response occurs it is called an autoimmune response. The immune system is made of several organs, including the thymus which is responsible for the maturation of the T lymphocytes, also known as T cells. The T cells contain receptors on their surfaces (T

cell receptors) that recognize antigens on the surfaces of infected cells. Helper T cells are responsible for directing immune responses via cell communication. Through this, antibody production by the B cells is stimulated, phagocytes are involved, and other T cells are activated. Cytotoxic lymphocyte T cells directly attack cells carrying foreign or altered molecules [7]. T cell recognition of exogenous antigens is performed through a process by which the antigens are cleaved into peptides and then coupled to the human leukocyte antigen (HLA) class II molecules of the major histocompatibility complex (MHC). Then, the class II molecules present the antigens to the cell surface of antigen presenting cells for CD4-positive T cell recognition. This process is illustrated in Figure 2.

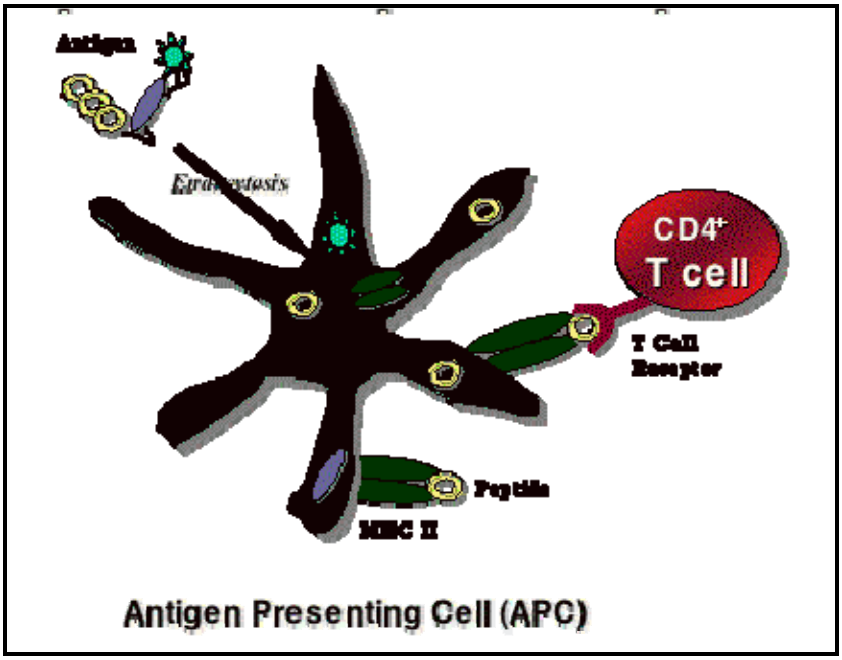

Figure 2: Antigen uptake and presentation by antigen presenting cells (APC) with peptide in the groove of a MHC class II molecule to a CD4+ T cell. Used with permission from Dr. Massimo Pietropaolo. Figure taken from Pietropaolo, M. and D. Le Roith, *Pathogenesis of diabetes: our current understanding.* Clin Cornerstone, 2001. 4(2): p. 1-16 [8].

Conversely, endogenous antigens, such as self proteins, are cleaved and transported into the endoplasmic reticulum of the cell while coupled to class I MHC molecules. Then, they are presented on the cell surface for recognition by CD8 T cells. T cells can only respond to peptide antigens that are bound to class I or class II MHC molecules on antigen presenting cells (APCs). APCs include macrophages, B cells, and dendritic cells and are responsible for pathogen and other particle clearance. This is accomplished through endocytosis in which the antigens are reduced to smaller peptides and are then presented in the groove of the MHC molecule.

The HLA class II genes act as immune response genes and contain highly polymorphic regions which are inherited in a Mendelian manner. Each individual's specific amino acid sequence determines which antigens they can respond to as each amino acid is responsible for binding and presenting specific antigens. During the development of the immune system, the potential exists for a response to almost every foreign antigen as well as every self-antigen. Therefore, depending on which sequences are inherited it can also determine which autoimmune diseases an individual is more susceptible to. Further details of the MHC and HLA class II genes are discussed in section 2.4.2 [9].

2.4 GENETICS

2.4.1 Genetic Susceptibility

The belief that there is a genetic susceptibility to T1D was initially based on observations of diabetes-clustering in families and the indication that relatives of T1D probands appeared to have an increased risk to develop the disease. Among Caucasians in North America, the risk for first

degree relatives ranges from 1% to 15%, dependent on the specific relationship (Table 1), compared with 0.12% in the general population.

Table 1: Empiric Risk of Type 1 Diabetes Mellitus. Modified with permission from Pietropaolo, M. and D. Le Roith, *Pathogenesis of diabetes: our current understanding.* Clin Cornerstone, 2001. 4(2): p. 1-16.[8]

Group at Risk	***Empiric Risk* (%)**
First-degree relatives of T1DM probands*	5–7‡
Individuals without relatives with T1DM*	<1
Children of affected father†	6
Children of affected mother†	2
*Estimates for North American white populations. ‡Estimates for Scandinavian populations. †1% to 15% range depending on the populations.	

These empirical risks illustrate that while there is a genetic susceptibility to developing T1D, it is not inherited in a Mendelian fashion. In fact, 80-90% of individuals with T1D have no family history of the disease. The differences seen in risk estimates for first degree relatives are believed to vary due to the concept of haplotype sharing. Individuals have two copies of each of their chromosomes, one inherited from each parent. They therefore have two copies of each of their genes, alleles, and so forth. A haplotype is the combination of alleles found on one of the chromosomes. Parents and their offspring can at maximum share only one haplotype whereas siblings have the possibility of sharing both haplotypes. Therefore, this supports the increased risk of siblings of diabetic probands versus children of diabetic probands [10].

2.4.2 HLA and DQ

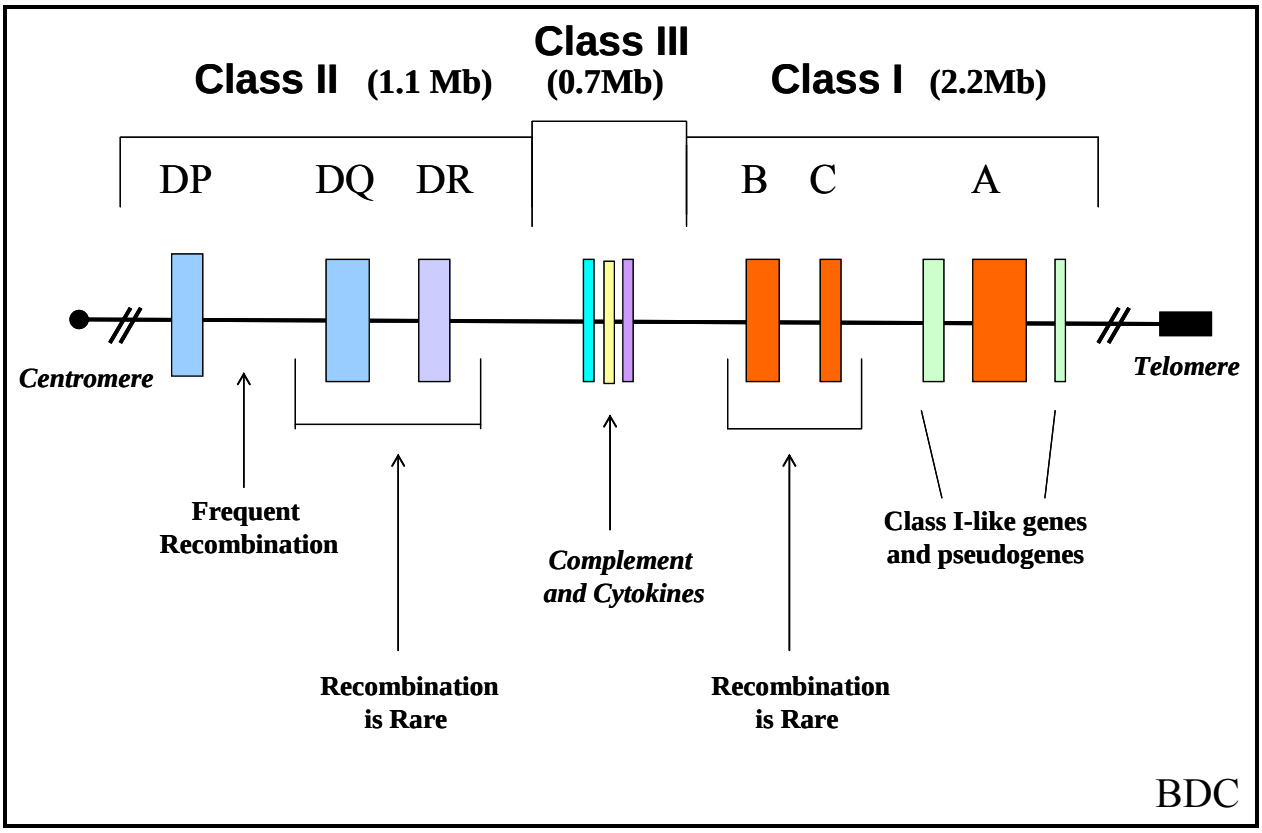

Figure 3: The HLA Complex. Used with permission from *Eisenbarth GS, Lafferty KJ, eds.* Type 1 Diabetes: Molecular, Cellular and Clinical Immunology. *New York, Oxford: Oxford University Press; 1996:3–17. Web site* [9]

In the mid 1970s, the Human Leukocyte Antigen (HLA) genes, were the first to be identified as diabetes susceptibility genes and have since been shown to play the largest role in a genetically heterogenous disease, comprising approximately 50% of the genetic risk [8, 11]. Thus, the HLA genes are known as IDDM1. The HLA genes are located within the major histocompatibility complex (MHC) which is located on the short arm of chromosome 6. The MHC is divided into three subregions, class I, class II, and class III. The class III genes encode a range of molecules

that are not related to immune function. The class I genes are responsible for α peptide chains which associate with β2 microglobulin to form the class I molecules. The class I molecules play a role in the restriction of cytotoxic T cell activity and are expressed on all nucleated cells' surfaces. The molecules bind endogenous antigen-derived peptide fragments. Then, the fragments are presented by the molecules for CD8 positive T cell receptor recognition.

The class II locus is comprised of one A gene and one B gene and are the closest to the centromere on chromosome 6. The A gene encodes for an α peptide chain and the B gene encodes for a β peptide chain, which together form a heterodimer that is the class II molecules. The class II molecules are termed DR, DQ, and DP, with DR and DQ being in high linkage disequilibrium (LD)[6].

Initially it was the class I alleles, in particular, that were associated with T1D due to their cosegregation in families. However, these alleles were also found to be fairly common and did not offer a very high relative risk ratio. Also, these observations were made before the class II molecules had been discovered. Once serological identification of the class II molecules was possible, it was determined that they were much better genetic markers in evaluating the risk for T1D. Following this, between 1979 and 1981, several groups identified the DR3 and DR4 alleles conferred the highest susceptibility and the DR2 alleles provided the highest resistance to the disease. These associations were subsequently confirmed in 1984 at the IX International Histocompatibility Workshop as studies showed that >90% of individuals with T1D were either DR3 or DR4. Of these individuals, only 7.2% were DR3/DR3 homozygous and 7.9% were DR4/DR4 homozygous, but 33.6% were DR3/DR4 heterozygous. Being heterozygous for DR3/DR4 was thus the highest genetic risk factor which initially did not make sense. To explain this situation, Svejgaard et al proposed that one of the α chains in the DR molecule is paired in

trans, with each one coming from a different copy of the two chromosomes, with one of the β chains forming a hybrid molecule. In this formation, the DR3/DR4 heterozygous molecule would be highly involved in presenting antigens to the T cells, initiating an autoimmune process. However, this hypothesis was rejected when it was later determined that actually in the DR molecule a polymorphic β chain pairs with a monomorphic α chain. Since the α chain is the same on both copies of the chromosome, there is no real difference between a *cis* versus *trans* formation.

Around 1987, as restriction fragment length polymorphism (RFLP) technology began to be used in the labs, it became more and more evident that the DQ molecule was more often involved in the risk to develop T1D. This also revived and supported the prior hypothesis of a hybrid molecule facilitating an autoimmune process, as both the α and β chains of the DQ molecule are polymorphic. The DQ molecule as the indicator for diabetes risk was not fully accepted at first, however further studies lent more support including the nonobese diabetic (NOD) mouse which spontaneously develops autoimmune diabetes and only has what is the human DQ complement for a class II molecule and it is negative for what is the human DR complement. Of particular interest, the only difference between the DQ-equivalent molecule in nonobese normal (NON) mice and NOD mice is that at position 57 of the β chain aspartic acid is exchanged for serine in the NOD mice. The relevance of this was subsequently determined when Bjorkman et al. determined the structure of the crystallized HLA-A2 molecule. The structure supported the role of the HLA molecule presenting a processed antigen (i.e. antigenic peptide) to the appropriate T cells. For example, the DQ molecule contains a groove where the processed antigen lodges for T cell presentation (Figures 4 and 5: the asterisks (*) in Figure 4 indicates residue 52 of the α-chain and 57 of the β-chain). Looking into this groove is residue 57 and is

therefore in a position to interact with the process antigen. Considering the prior observations in the NOD and NON mouse and the differing amino acids in position 57, it was hypothesized that an amino acid substitution is critical in mediating the autoimmune reaction via its effect on the lodging of the antigenic peptide in the groove. With the advent of polymerase chain reaction (PCR) techniques and sequence specific oligonucleotide probes, they were able to see the variations in codon 57 in both diabetic and non-diabetic individuals. By the end of 1987, multiple studies showed a strong correlation between having a non-charged amino acid such as alanine, valine, or serine at codon 57 in diabetic patients instead of having aspartic acid as was most often seen in non-diabetic patients. Therefore, being "non-Asp" or "Asp" is describing whether an allele on the β-chain encodes for aspartic acid to be present at position 57 (Asp) or a different non-charged amino acid (non-Asp). How this was explained was that the presence of Asp 57 inhibits "perfect" lodging of an antigenic peptide into the groove, which makes it less likely to be presented to the T cell and initiate an autoimmune process, whereas the absence of Asp 57 would make it more likely for the peptide to lodge into the groove allowing this diabetogenic molecule to be presented for recognition [12].

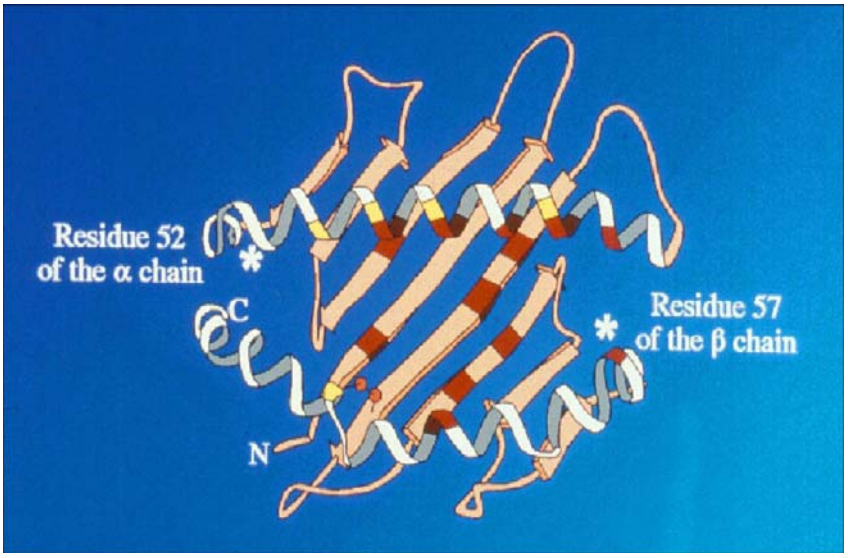

Figure 4: HLA-DQ combining site. Used with permission from Trucco, M., ***To be or not to be Asp 57, that is the question.*** **Diabetes Care, 1992.** 15**(5): p. 705-15. [12]**

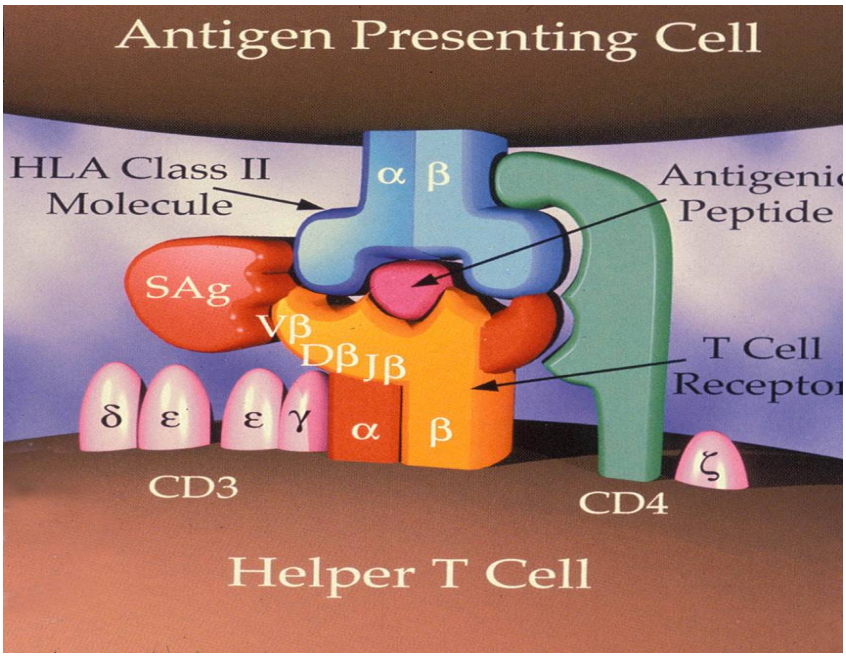

Figure 5: Antigen Presenting Via the HLA Molecule. Used with permission from Trucco, M., *To be or not to be Asp 57, that is the question.* Diabetes Care, 1992. 15**(5): p. 705-15. [12]**

As DQ became accepted as the HLA molecule involved with the genetic predisposition for T1D, the earlier associations of DR3/DR3, DR4/DR4, and DR3/DR4 with the highest level of diabetes susceptibility was revisited. With the DR and DQ molecules in such high linkage disequilibrium it was determined that it was really the DQ haplotypes of DQA1*0501-DQB1*0201 (LD with DR3) and DQA1*0301-DQB1*0302 (LD with DR4) that were involved. Therefore, the genotype with conferring the largest risk of T1D is the DQA1*0501-DQB1*0201/DQA1*0301-DQB1*0302 heterozygote. Again this heterozygous "disadvantage" is explained by the formation of a hybrid molecule that is particularly diabetogenic due to the *trans* formation of the alleles and the resulting effect on antigen binding and presentation [13].

In addition, Khalil et al discovered in 1990 that diabetes susceptibility could be further defined by the presence or absence of arginine at position 52 on the α chain in combination with the presence or absence of Asp 57 on the β chain of the DQ molecule. The combination with the maximum susceptibility to the disease is arginine at position 52 and non-Asp at position 57, and vice versa for the maximum resistance. As these two positions are located on the opposite ends of the groove-containing space, it can be explained that both positions 52 and 57 and their amino acids are in a prime physical location, in addition to the reactions between a neutral non-Asp amino acid and arginine, to affect the presentation of an antigen to the T cell [14]. DQ2 and DQ8 are indeed a particular combination of alleles where one codes for Arg at position 52 on the alpha chain and the other for non-Asp at position 57 on the beta chain.

Today, the DQ2 and DQ8 haplotypes and genotype are still consistently shown to cause an increased genetic risk in developing T1D. Both as a group and individually this is true, however when looking at them within the group the order of risk from highest to lowest is DQ2/DQ8 (~1:15), DQ8/DQ8 (~1:60-200), DQ8/X (~1:60-200), DQ2/DQ2 (~1:60-200), and DQ2/X (~1:300) (where "X" indicates the presence of any other alpha and beta allele with the exception of DQB1*0602). In addition, DQA1*0102/DQB1*0602/DRB1*1501 (DR2) haplotype has shown a high protective effect against the development of T1D, even in the presence of autoantibodies [5, 15].

It had been long observed that monozygotic twin concordance for T1D was not 100%, with estimates ranging from 20 to <40% [16, 17]. This can be explained genetically by the fact that the genes that encode the class II molecules are often subject to somatic rearrangement. Also, with the PCR based methods, HLA typing was performed of multiple populations with differing incidences of T1D. Once genotype frequencies of non-Asp 57 was determined, it was

shown to correlate with the population's corresponding disease incidence thus identifying genetic differences as the primary reason for incidence differences, with the exception of Japanese populations which suggest a different role of non-Asp 57 in this population or discrepancies in study designs [12]. Studies have shown DQ associated susceptibility is only accurate for populations where the disease is common, whereas in populations where the disease is rare as in Asians the same associations are not seen [13].

The following is a table with known DQ alleles and their effect on susceptibility to T1D. The last column lists the associated DR class that is known by linkage disequilibrium.

Table 2: Effect of Human Leukocyte Antigen Alleles on Susceptibility to IDDM. Used with permission from Dr. Massimo Pietropaolo found in Pietropaolo, M. and D. Le Roith, *Pathogenesis of diabetes: our current understanding.* Clin Cornerstone, 2001. 4(2): p. 1-16. [8]

DQ Alleles	***Effect***	***Associated DR Class***
A1*0301, B1*0302	Susceptible	DR4
A1*0501, B1*0201	Susceptible	DR3
A1*0101, B1*0501	Susceptible	DR1
A1*0301, B1*0201	Susceptible(AfricanAmericans)	DR7
A1*0102, B1*0502	Susceptible(Sardinians)	DR2 (DR16)
A1*0301, B1*0303	Susceptible (Japanese)	DR4
A1*0301, B1*0303	Susceptible (Japanese)	DR9
A1*0102, B1*0602	Protective	DR2 (DR15)
A1*0501, B1*0301	Protective	DR5
? B1*0600	Neutral	DR6
A1*0201, B1*0201	Neutral	DR7
A1*0301, B1*0303	Neutral	DR4
A1*0301, B1*0301	Neutral	DR4

2.4.3 Other Genes of Interest

There are many different genetic loci, mostly detected by genome-wide scans, associated with T1D risk. However, no other loci have showed a stronger effect or with such reproducibility as the HLA genes. Table 3 lists the known susceptibility loci as of 2001.

Table 3: Summary of Human IDDM Susceptibility Loci. Used with permission from Dr. Massimo Pietropaolo. Table from Pietropaolo, M. and D. Le Roith, *Pathogenesis of diabetes: our current understanding.* Clin Cornerstone, 2001. 4(2): p. 1-16. [8]

Chromosome	***Locus***	***Linkage Status According to Kruglyak & Lander[l]***
6p21	*IDDM1;* HLA-DQB	Confirmed
11p15.5	*IDDM2;* INS 5 VNTR	Confirmed
15q26	*IDDM3;* IGF1R	Suggestive ($P < .001$, MLS > 2.2)
11q13	*IDDM4;* FGF3	Confirmed ($P < 2.2 \times 10^{-5}$, MLS > 3.6)
6q25	*IDDM5;* ESR1	Confirmed ($P < 2.2 \times 10^{-5}$, MLS > 3.6)
18q21	*IDDM6*	
2q31	*IDDM7;* IL1, HOXD8	Suggestive ($P < .001$, MLS > 2.2)
6q27	*IDDM8;* IGF2R	Confirmed ($P < 2.2 \times 10^{-5}$, MLS > 3.6)
3q21-q25	*IDDM9*	
10p11.2-q11.2	*IDDM10*	
14q24.3	*IDDM11*	Significant ($P < 2.2 \times 10^{-5}$, MLS > 3.6)
2q33	*IDDM12;* CTLA-4	
2q34	*IDDM13;* IGFBP2, IGFBP5	
6p21	*IDDM15* (distinct from HLA)	
10q25[†]	*IDDM17*	Significant (NPL: $P < .002$)
7p	Not assigned; GCK, IGFBP1, IGFBP3	
Xq	Not assigned	
Xp[‡]	Not assigned	Significant ($P = 2.7 \times 10^{-4}$; MLS > 3.6)

Chromosome	*Locus*	*Linkage Status According to Kruglyak & Lander*[l]
Table 3 Continued		
1q[§]	Not assigned	Suggestive (MLS = 3.31)

HLA = human leukocyte antigen; MLS = maximum logarithm of odds score; NPL = nonparametric linkage; VNTR = variable number of tandem repeats.

*The insulin-dependent diabetes mellitus nomenclature is assigned to a locus after linkage has been formally demonstrated, replicated, and confirmed in ≥3 different data sets. Where functional candidate genes are flanked by or are very close to susceptibility markers, they are so indicated.
†The evidence for linkage increased substantially (P = .00004) with higher marker density and the inclusion of data for additional affected relatives and all unaffected siblings.
‡In major histocompatibility complex HLA-DR3-positive patients.
§This locus co-localizes with loci for systemic lupus erythematosus and ankylosing spondylitis.

The following is a description of three additional genes that besides the HLA region, have shown the most reproducibility and effect on T1D risk. The insulin (*INS*) gene region located on chromosome 11p15.5 is believed to be the second major susceptibility locus for T1D in Caucasians. This region is also known as IDDM2. The associations with risk are with the region outside of the coding region, approximately 596 bp upstream of the translational start site, which contains variable number of tandem repeats (VNTR). At this locus there are two common alleles, a shorter class I allele (20-63 repeats) and a longer class III allele (140-210 repeats). It is the former that is associated with T1D susceptibility with class I homozygosity conferring the highest risk for TID. Conversely, the class III alleles have been associated with dominant protection. Of important note, as these areas are also highly polymorphic not all class I alleles are related to susceptibility and not all class III alleles act as protectants. In addition, the roles of the polymorphisms vary by ethnic group. Also, there have been contradictory studies that discuss the possibility of a relationship between the *Ins* gene and HLA-DQ.

Reasons for the VNTR associations are not completely known, however they are believed to have a function related to how insulin mRNA is expressed in the thymus. The protective class

III alleles generate higher levels of insulin mRNA than do class I alleles. It is believed that higher levels of insulin mRNA in the thymus may support the efficient deletion of autoreactive T cells that are specific to the protein. Therefore, this may allow for the individual to have a higher immune tolerance to a key autoantigen that is associated in the pathogenesis of T1D. In contrast, the highest susceptibility combination of being homozygous for the class I alleles is believed to be explained by the fact that these alleles are believed to play a role in complete silencing of insulin transcripts in the thymus and thus results in the predisposition to T1D [6, 13].

The cytotoxic T lymphoctye antigen-4 (*CTLA-4*) gene is another locus that has been recently been associated with the risk of TID. This region is also known as IDDM12 and is located on chromosome 2q33. A possible mechanism of the *CTLA-4* gene and T1D susceptibility is that it codes for an antigen that is expressed on activated T cells and is an important factor in T cell regulation. However, most associations have been weak and studies are still being performed to determine the extent of its relationship to susceptibility to TID [18].

A fourth gene has also shown associations for the risk to develop T1D, the LYP (*PTPN22*) gene. This gene is one of the protein tyrosine phosphatases that modulates T cell activation. It is believed that the LYP-Trp 620 allele is associated with T1D susceptibility as it results in T cell activation via a gain of function mechanism. It has also been associated with other autoimmune disorders including rheumatoid arthritis and lupus erythematosus. However, this association has only been seen in Caucasian populations [18].

2.5 ENVIRONMENTAL FACTORS

A variety of factors support the fact that environmental factors are necessary for the development of T1D including a low MZ twin concordance rate (20-40%), T1D epidemics occurring in different areas of the world, and differing rates of diabetes in inbred diabetic-prone mice. These environmental factors have not completely determined but studies have lent evidence for infectious agents, toxins, and diet [16, 17].

Viruses are one of the more well studied environmental factors believe to play a role in the development of T1D. They are believed to have a variety of roles including initiation, acceleration, or precipitation via either direct or indirect mechanisms. These mechanisms include, but are not limited to, directly causing diabetes by attacking and destroying the β cells, or initiating the autoimmune response associated with T1D. The latter can occur due to molecular mimicry or 'bystander' autoimmune activation. Molecular mimicry is when a particular antigen is similar to the proteins in the body, which causes an autoimmune reaction that is mediated by "cross reactive" T cells and/or circulating antibodies. When this occurs, the T cells will then destroy both the virus and the β cells during the process[19]. Bystander autoimmune activation occurs when inflammatory cytokines are released into the system due to the infection caused by the virus. Enteroviruses, such as Coxsackie B, have particularly gained support in relation to the development of T1D. The role of the Coxsackie B virus has been suggested to be related to the development of autoimmunity and thus accelerating the prediabetes progression stage of autoantibody development. Alternatively, it has been seen in diabetic prone mice that β cell specific toxins, vitamin D3 and nicotinamide, can provide varying degrees of protection against the development of T1D [13, 17].

Infant feeding practices were first discussed as a risk factor in 1984 following a Scandinavian study that showed an increase in the incidence of T1D in the years where breastfeeding was not as common compared to years where it was. From this observation, it was surmised that either the absence of breastfeeding or the presence of cow's milk was related to an increased risk to develop T1D. Since then, there have been many studies trying to prove or disprove this link. Studies on rodent diet and the subsequent development of T1D suggest that the risk of diabetes is increased when early weaning from breastfeeding to a diet containing cow's milk proteins. As further support for the relevance of cow's milk and the development of T1D is that many children with T1D have an increased production of antibodies to cow's milk proteins. These studies also have placed an emphasis on the diet early in life, such as the early introduction of cow's milk (less than 3 months of life) with or without the use of breastfeeding. It is also important to note that many studies have been performed in those considered to be genetically at-risk, such as the Finnish Diabetes Prediction and Prevention (DIPP) Study and currently the Trial to Reduce IDDM in the Genetically at Risk (TRIGR) study. This further lends support to the pathogenic theory of a genetically-susceptible situation that requires multiple environmental (viral, dietary, etc) insults for the development of the disease[3, 20].

It is important to note that the mechanisms for these interactions are still not completely known. Also, even with the animal and human data that we do have that supports these environmental interactions, it is also known that the highest T1D incidence in animals is in diabetic-prone rodents where the amount of infectious agents is lowest and where the young are exclusively breastfed [17]. Therefore, there is still a lot left to be desired in relation to our understanding of the pathogenesis of T1D.

2.6 AUTOANTIBODIES

Over thirty years ago, the presence of autoantibodies in the pancreatic islet cells was discovered in diabetic patients. These are called islet cell antibodies (ICA). ICA is detected by looking at the reactions against either frozen sections of human or rat pancreas. Previously due to the limited availability of human pancreatic samples, rat ICA was more often used. In addition, there are three main antigens that produce autoantibodies in T1D that were discovered in the early 1980s, early 1990s, and mid 1990s. These are GAD65, IA-2, and insulin.

Glutamic acid decarboxylase, with a molecular weight of 65,000, also known as GAD65 was the first to be discovered. GAD65 is found in the islet cells of the pancreas and plays a role in the conversion of glutamic acid to GABA, a major inhibitory neurotransmitter. It is encoded by the *GAD65* gene located on chromosome 10p11.

The second antigen detected was insulinoma-associated protein 2 (IA-2). IA-2 is a protein tyrosine phosphatase–like molecule and the encoding gene is located on chromosome 2q35. It is expressed in neuroendocrine tissues and is found in both the α and the β cells of the pancreatic islets.

The third antigen is insulin which is localized to chromosome 11p15. Measurement of insulin antibodies is only accurate before insulin therapy is introduced in diabetics, as they also can develop antibodies to the exogenous insulin which is indistinguishable upon assay.

The presence of these autoantibodies and their relationship to the development of diabetes has been extensively studied. Up to 90% of newly diagnosed diabetics have at least one of the three antibodies compared to just 1% of individuals in the general population. The statistics for the number and what type of antibodies vary with the age of onset, ethnicity, and duration of disease [21].

With studies of first degree relatives of diabetic probands, it was realized that the development of autoantibodies precedes the development of diabetes and can be use as a predictive tool in assessing the risk to develop T1D. This process of developing autoimmunity is believed to begin with initial activation where the number of islet autoantigens increase and eventually leads to the disease state. This increasing number of autoantigens is associated with overexpression of cytokines and other inflammatory agents and is termed "antigen spreading" or "epitope spreading" (epitope referring to the T cell's molecular code words that interacts with the immune system). This process sets off a sequence of events and results in autoreactivity to numerous autoantigens [8]. It is believed that the development of autoantibodies can precede the development of T1D by years and that the risk does not decrease over time [22].

There have been multiple studies that have estimated the risk of developing autoimmune diabetes in both first degree relatives and individuals from the general population based on the presence of multiple autoantibodies. In the Diabetes Prevention Trial (DPT-1), the presence of 2 autoantibodies conferred a risk of 65% to develop diabetes over 5 years and the presence of 3 conferred a risk of 100% over 5 years [23]. In the Childhood Diabetes in Finland Study Group, the risk was 25% in the presence of two autoantibodies and was 70% with three autoantibodies over 6.6 years [24]. Verge et al showed a 5 year risk estimate of 68% for two autoantibodies and 100% for three autoantibodies [25]. In studies evaluating a 10 year follow up, the presence of two autoantibodies conferred a 57% risk, and three autoantibodies an 80% risk (Figures 6 and 7) [21].

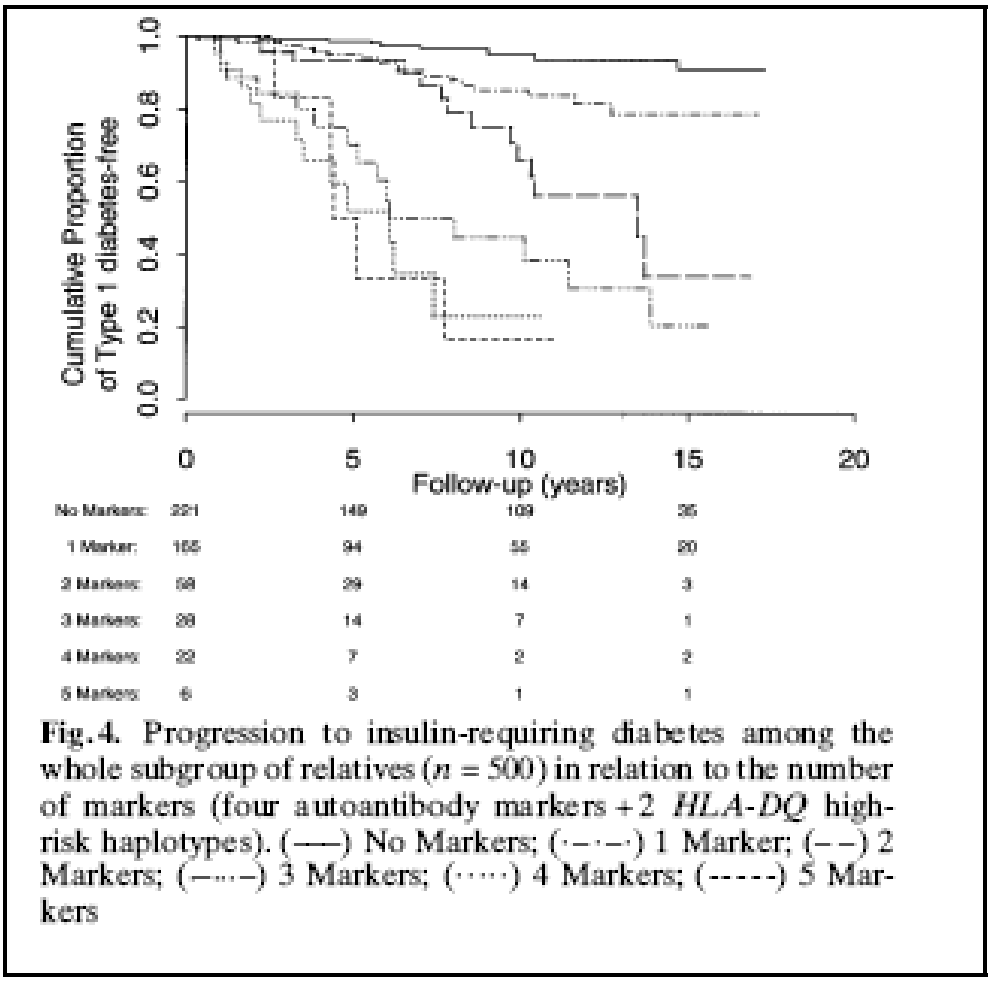

Fig. 4. Progression to insulin-requiring diabetes among the whole subgroup of relatives ($n = 500$) in relation to the number of markers (four autoantibody markers + 2 *HLA-DQ* high-risk haplotypes). (——) No Markers; (·–·–·) 1 Marker; (– –) 2 Markers; (—··—) 3 Markers; (·····) 4 Markers; (-----) 5 Markers

Figure 6: Risk of diabetes in First Degree Relatives in the Presence of Multiple Markers. Used with permission by Dr. Massimo Pietropaolo. Found in Pietropaolo, M., et al.*, Progression to insulin-requiring diabetes in seronegative prediabetic subjects: the role of two HLA-DQ high-risk haplotypes.* Diabetologia, 2002. 45(1): p. 66-76. [26]

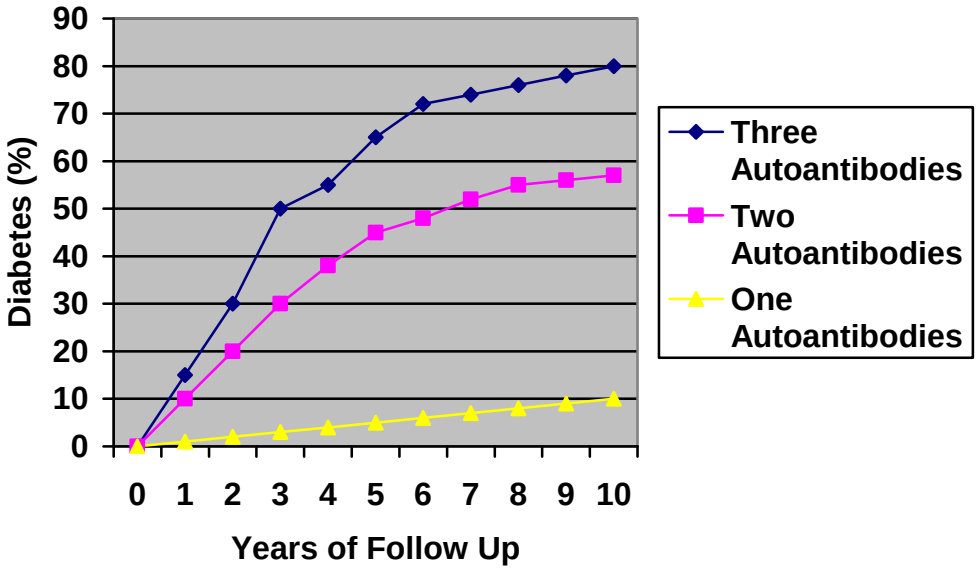

Figure 7: Risk of Diabetes for First Degree Relatives in the Presence of Multiple Autoantibodies (GAD65, Insulin, and IA2) [21]

2.7 THE SSOP METHOD

Before the discovery of recombinant DNA technology, HLA typing was performed by evaluating serologic HLA antigen responses to the introduction of sera containing Class I and II protein antibodies. In addition, polymorphisms in Class II proteins were analyzed by T-cell responses in the Mixed Lymphocyte Reaction (MLR) [27]. In a MLR, two blood samples are mixed together. If the individuals have different alleles, then their lymphocytes will stimulate each other to proliferate whereas if the have the same there will be no proliferation [28].

Once polymerase chain reaction (PCR) techniques became readily available, they were employed in HLA typing. First described in the 1980s, sequence specific oligonucleotide probes

can now be used. This process utilizes PCR to first amplify the DNA which can then be hybridized with the probes that contain known specific sequences of oligonucleotides. This allows for identification of specific HLA alleles, which the probes hybridize to when present. There have been variations with the process, such as using radioactive labeling for the probes which is now being replaced with fluorescent dyes or enzymes[27].

2.8 SPECIFIC AIMS

Specific Aim 1: What is the distribution of the HLA-DQ alleles in the Type 1 diabetic population enrolled in the "Epidemiology and Etiology of Type 1 Diabetes" study compared to the literature?

Hypothesis 1: As our population has been evaluated before and is representative of other populations with similar incidence rates and demographics, this study will continue to support the role of the HLA-DQ molecule and its specific alleles and haplotypes in the development of type 1 diabetes.

Plan 1: Analyze the genetic makeup for each proband as determined by SSOP and code it for the number of non-Asp alleles and the presence of the DQ2 and/or DQ8 haplotypes.

Specific Aim 2: What is the distribution of the HLA-DQ alleles in the first degree relatives enrolled in the "Epidemiology and Etiology of Type 1 Diabetes" study?

Hypothesis 2: The first degree relative population has not been studied before in Pittsburgh, but should be representative of other studies performed on a demographically similar

population. The FDR population should also show significant differences in the distribution of the DQ molecule as compared to diabetic probands and unaffected unrelated controls.

Plan 2: Analyze the genetic makeup for each first degree relative as determined by SSOP and code it for the number of non-Asp alleles and the presence of the DQ2 and/or DQ8 haplotypes.

3.0 MATERIALS AND METHODS

3.1 STUDY POPULATION

The study population are subjects enrolled in the Children's Hospital of Pittsburgh based study "The Etiology and Epidemiology of Type 1 Diabetes" with the current subtitle of AGS (Antigen Spreading) under the direction of Dr. Dorothy Becker. The probands (also known as new onsets) in the study are defined as children age 1 through 18 diagnosed with diabetes mellitus, discharged from the hospital on insulin treatment, had a blood draw for HLA-DQ typing, and had their first study blood draw for autoantibody analysis within 100 days of diagnosis. The latter was done in order to capture their autoantibody status at onset of the disease, as this status is believed to change over time. In addition, it enables the analysis for insulin autoantibody analysis, since this measurement is not accurate once exogenous insulin treatment has been started. All individuals with secondary diabetes were excluded. The probands in the study were recruited from January 2004 until December 2006. As mentioned, 14 of the subjects were part of the original study (also known as called JOD) and these individuals were recruited between January 1980 and November 2003.

The first degree relatives (FDRs) in the study were recruited in the same time frame and in order to be eligible they must be from 1 to 45 years in age, not insulin dependent diabetics at time of enrollment, had a blood draw for HLA-DQ typing and was a first degree relative (parent,

sibling, or child) of an insulin dependent diabetic child. The child with diabetes in their actual family did not necessarily need to be enrolled in the study.

3.2 HLA-DQ TYPING BY REVERSE SSOP

Genomic DNA samples were extracted from either heparinized blood samples with phenol/chloroform or from dried blood spots on filter paper cards. HLA typing for DQA1 and DQB1 alleles was performed on all subjects by PCR and hybridization with sequence specific oligonucleotide probes (SSOP).

DNA extraction and purification is initially performed using the QIAmp DNA Mini and QIAmp Blood Mini Kit. HLA-DQ typing by SSOP is performed using Luminex™ / One Lambda LABType™RSSO**.** The procedure follows as described by the protocol. The method involves the use of Luminex™ microspheres that are incorporated with infrared and red dyes, which gives each microsphere a unique spectral address allowing for 100 different classifications of beads. Each classification of bead then is bound with a particular oligonucleotide probe. PCR is then performed to amplify the DNA samples using primers that are biotinylated. After amplification, the PCR products are hybridized with a mixture of microspheres that have a predetermined set of oligonucleotide probes bound to them. Next, the PCR product finds complimentary sequences and binds with the probe (bead) they recognize. Individual sample reactions are then washed to remove any unbound PCR product. Samples are then stained with R-Phycoerytherin-Conjugated Streptavidin (SAPE), which will bind to the biotinylated primer, and processed through a Luminex™ flow analyzer. The fluorescent intensity of PE (Phycoerytherin) on each microsphere classification is translated into a positive or negative reaction. The assignment of HLA typing is

based on the reaction pattern and analyzed using the current HLA sequence data. Analysis of the results is performed using One Lambda LABType Visual Software.

Any samples that were indicated as DQB1*06 alleles from the reverse SSOP method were further resolved using sequence specific primers (SSP) using the Dynal AllSet™ SSP Procedure. The SSP method is also a PCR-based method but has a higher resolution than SSOP. SSP was used in the case of a DQB1*06 result because it is important to identify the presence of the DQB1*0602 allele, as it is associated with protection from the development of T1D.

3.3 DQ RISK ALLELES AND HAPLOTYPE STRATIFICATION

The HLA-DQ results were stratified by both non-Asp status and the presence of the DQ2 and DQ8 haplotypes. For non-Asp status, each of the probands and FDRs were assessed for the presence of one, two, or zero non-Asp alleles. In addition, for those with one non-Asp allele the presence of *0602 on the other DQB1 allele was recorded separately as Non-Asp/0602. Having no non-Asp alleles is recorded as Asp/Asp. Alleles were considered to be either Asp or non-Asp based on the protocol of the Children's Hospital of Pittsburgh Histocompatibility Center as determined in July 2005. In addition, each of the probands and FDRs were analyzed for the presence of the DQ2 (DQA1*0501-DQB1*0201) and DQ8 (DQA1*0301-DQB1*0302) haplotypes. For each individual, it was recorded if there was one (DQ2/X) or two (DQ2/DQ2) copies of the DQ2 haplotype, one (DQ8/X) or two (DQ8/DQ8) copies of the DQ8 haplotype, or for the DQ2/DQ8 genotype.

3.4 STATISTICAL METHODS

The data was analyzed using Statistical Analysis System (SAS, Cary, NC, USA) software using a Windows operating system. Chi squared analysis was used to compare the proportions of the various studied groups.

4.0 RESULTS

4.1 PROBANDS AND FIRST DEGREE RELATIVES (FDRS)

4.1.1 Demographics

For the HLA-DQ analysis there were samples from 265 probands and 1000 first degree relatives (FDRs). Of these, 14 of the probands were enrolled prior to February 2004 through the continuous original study of Etiology and Epidemiology of T1D whereas the other 251 were part of the Antigen Spreading Branch of the original grant that was approved for February 2004. Four of the probands were part of a multiplex family, all of which had a sibling also diagnosed with T1D. In all of the cases, only one sibling was included in the study and therefore in our analysis group. Tables 4-6 show the distribution of gender, race, and ethnicity for the two groups.

Table 4: Gender Distribution for Probands and FDRs

Gender	Proband Count	Proband Percent (%)	FDRs Count	FDRs Percent (%)
Male	151	56.98	390	39.00
Female	112	42.26	598	59.80
Unknown	2	0.75	12	1.20
Total	265	100	1000	100

Table 5: Race Distribution for Probands and First Degree Relatives (FDRs)

RACE	PROBAND COUNT	PROBAND PERCENT (%)	FDRs COUNT	FDRs PERCENT (%)
White	243	91.70	871	87.1
Black	14	5.28	27	2.70
Other, including multiple races	5	1.89	3	0.30
Unknown	3	1.13	99	9.90
Total	265	100	1000	100

Table 6: Ethnicity Distribution for Probands and First Degree Relatives (FDRs)

ETHNICITY	PROBAND COUNT	PROBAND PERCENT (%)	FDRs COUNT	FDRs PERCENT (%)
Not Hispanic	114	43.02	604	60.40
Hispanic	3	1.13	4	0.40
Unknown	148	55.85	392	39.2
Total	265	100	1000	100

4.1.2 Non-Asp 57 Analysis for Probands and FDRs

For the first part of the HLA-DQ analysis, individuals were classified by their non-Asp status as determined by the presence of 0, 1, or 2 non-Asp alleles and for the presence of 1 non-

Asp allele in combination with DQB1*0602. Twenty-four of the probands and 77 of the FDRs were unable to be determined and will require HLA-DQ typing by SSP for a more detailed analysis of the alleles. The results for all subjects are shown in Table 7, broken by the relationship to the probands (parent or full sibling only) in Table 8, and the same for Caucasian subjects only in Tables 9 and 10.

Table 7: Non-Asp Analysis of Probands and FDRs

ALL SUBJECTS			
Non-Asp Status Frequency Percent Row Pct Col Pct	**Probands**	**FDR**	**Total**
Unknown	0 0.00 0.00 0.00	2 0.17 100.00 0.21	2 0.17
ASP/ASP	8 0.68 9.52 3.31	76 6.47 90.48 8.15	84 7.15
Non-ASP/ASP	74 6.30 15.51 30.58	403 34.30 84.49 43.19	477 40.60
Non-ASP/Non-ASP	154 13.11 32.77 63.64	316 26.89 67.23 33.87	470 40.00
Non-ASP/0602	6 0.51 4.23 2.48	136 11.57 95.77 14.58	142 12.09
Total	242 20.60	933 79.40	1175 100.00
Frequency Missing = 99			

Table 8: Non-Asp 57 Analysis of Probands and FDRs, by Relationship

ALL SUBJECTS				
Non-Asp Status Frequency Percent Row Pct Col Pct	**Probands**	**FDR: parent**	**FDR: sibling**	**Total**
Unknown	0 0.00 0.00 0.00	0 0.00 0.00 0.00	2 0.18 100.00 0.65	2 0.18
ASP/ASP	8 0.73 10.00 3.31	40 3.63 50.00 7.22	32 2.90 40.00 10.42	80 7.25
Non-ASP/ASP	74 6.71 16.52 30.58	249 22.57 55.58 44.95	125 11.33 27.90 40.72	448 40.62
Non-ASP/Non-ASP	154 13.96 35.00 63.64	181 16.41 41.14 32.67	105 9.52 23.86 34.20	440 39.89
Non-ASP/0602	6 0.54 4.51 2.48	84 7.62 63.16 15.16	43 3.90 32.33 14.01	133 12.06
Total	242 21.94	554 50.23	307 27.83	1103 100.00
Frequency Missing = 171				

Table 9: Non-Asp 57 Analysis of Caucasian Probands and FDRs

CAUCASIAN SUBJECTS			
Non-Asp Status Frequency Percent Row Pct Col Pct	**Probands**	**FDR**	**Total**
Unknown	0 0.00 0.00 0.00	1 0.10 100.00 0.12	1 0.10
ASP/ASP	7 0.68 9.46 3.14	67 6.47 90.54 8.24	74 7.14
Non-ASP/ASP	70 6.76 16.91 31.39	344 33.20 83.09 42.31	414 39.96
Non-ASP/Non-ASP	142 13.71 33.49 63.68	282 27.22 66.51 34.69	424 40.93
Non-ASP/0602	4 0.39 3.25 1.79	119 11.49 96.75 14.64	123 11.87
Total	223 21.53	813 78.47	1036 100.00
Frequency Missing = 85			

Table 10: Non-Asp 57 Analysis of Caucasian Probands and FDRs, by Relationship

CAUCASIAN SUBJECTS				
Non-Asp Status Frequency Percent Row Pct Col Pct	**Probands**	**FDR: parent**	**FDR: sibling**	**Total**
Unknown	0 0.00 0.00 0.00	0 0.00 0.00 0.00	1 0.10 100.00 0.36	1 0.10
ASP/ASP	7 0.71 9.72 3.14	37 3.73 51.39 7.52	28 2.82 38.89 10.11	72 7.26
Non-ASP/ASP	70 7.06 17.46 31.39	215 21.67 53.62 43.70	116 11.69 28.93 41.88	401 40.42
Non-ASP/Non-ASP	142 14.31 35.41 63.68	164 16.53 40.90 33.33	95 9.58 23.69 34.30	401 40.42
Non-ASP/0602	4 0.40 3.42 1.79	76 7.66 64.96 15.45	37 3.73 31.62 13.36	117 11.79
Total	223 22.48	492 49.60	277 27.92	992 100.00
Frequency Missing = 129				

4.1.3 DQ2 and DQ8 Analysis for Probands and FDRs

In the second part of the HLA-DQ analysis, individuals were classified as having 0 (X/X), 1 (DQ2/X) or (DQ8/X), or 2 (DQ2/DQ2) or (DQ8/DQ8) copies of the DQ2 or DQ8 haplotypes. Also, individuals with the DQ2/DQ8 heterozygous genotype were classified as such. The results for all subjects are shown in Table 11, broken by the relationship of the FDR to the proband (parent or full sibling only) in Table 12, and the same for Caucasian subjects only in Tables 13 and 14.

Table 11: DQ2 and DQ8 Analysis of Probands and FDRs

ALL SUBJECTS			
DQ2 and DQ8 Status Percent Row Percent Column Percent	**Probands**	**FDRs**	**Total**
X/X	56 4.43 15.05 21.13	316 24.9 84.9 31.60	372 29.41
DQ2/X	59 4.66 16.67 22.26	295 23.33 83.33 29.50	354 27.98
DQ2/DQ2	17 1.34 39.53 6.42	26 2.06 60.48 2.60	43 3.40
DQ8/X	75 5.93 21.43 28.30	275 21.74 78.57 27.50	350 27.67
DQ8/DQ8	13 1.03 35.14 4.91	24 1.90 64.86 2.40	37 2.92
DQ2/DQ8	45 3.56 41.28 16.98	64 5.06 58.72 6.40	109 8.62
Total	265 20.95	1000 79.05	126 100.0
Frequency Missing = 9			

Table 12: DQ2 and DQ8 Analysis of Probands and FDRs, by Relationship

ALL SUBJECTS				
DQ2 and DQ8 Status Frequency Percent Row Pct Col Pct	**Probands**	**FDR: Parent**	**FDR: Sibling**	**Total**
X/X	56 4.72 16.23 21.13	181 15.25 52.46 30.32	108 9.10 31.30 33.23	345 29.06
DQ2/X	59 4.97 17.93 22.26	185 15.59 56.23 30.99	85 7.16 25.84 26.15	329 27.72
DQ2/DQ2	17 1.43 43.59 6.42	14 1.18 35.90 2.35	8 0.67 20.51 2.46	39 3.29
DQ8/X	75 6.32 22.39 28.30	173 14.57 51.64 28.98	87 7.33 25.97 26.77	335 28.22
DQ8/DQ8	13 1.10 37.14 4.91	11 0.93 31.43 1.84	11 0.93 31.43 3.38	35 2.95
DQ2/DQ8	45 3.79 43.27 16.98	33 2.78 31.73 5.53	26 2.19 25.00 8.00	104 8.76
Total	265 22.33	597 50.29	325 27.38	1187 100.00
Frequency Missing = 87				

Table 13: DQ2 and DQ8 Analysis of Caucasian Probands and FDRs

CAUCASIAN SUBJECTS			
DQ2 and DQ8 Status Frequency Percent Row Pct Col Pct	**Probands**	**FDR**	**Total**
X/X	51 4.58 15.74 20.99	273 24.51 84.26 31.34	324 29.08
DQ2/X	54 4.85 17.25 22.22	259 23.25 82.75 29.74	313 28.10
DQ2/DQ2	14 1.26 37.84 5.76	23 2.06 62.16 2.64	37 3.32
DQ8/X	69 6.19 22.77 28.40	234 21.01 77.23 26.87	303 27.20
DQ8/DQ8	13 1.17 35.14 5.35	24 2.15 64.86 2.76	37 3.32
DQ2/DQ8	42 3.77 42.00 17.28	58 5.21 58.00 6.66	100 8.98
Total	243 21.81	871 78.19	1114 100.00
Frequency Missing = 7			

Table 14: DQ2 and DQ8 Analysis of Caucasian Probands and FDRs, by Relationship

CAUCASIAN SUBJECTS				
DQ2 and DQ8 Status Frequency Percent Row Pct Col Pct	**Probands**	**FDR: parent**	**FDR: sibling**	**Total**
X/X	51 4.78 16.56 20.99	161 15.10 52.27 30.43	96 9.01 31.17 32.65	308 28.89
DQ2/X	54 5.07 18.00 22.22	167 15.67 55.67 31.57	79 7.41 26.33 26.87	300 28.14
DQ2/DQ2	14 1.31 41.18 5.76	13 1.22 38.24 2.46	7 0.66 20.59 2.38	34 3.19
DQ8/X	69 6.47 23.47 28.40	147 13.79 50.00 27.79	78 7.32 26.53 26.53	294 27.58
DQ8/DQ8	13 1.22 37.14 5.35	11 1.03 31.43 2.08	11 1.03 31.43 3.74	35 3.28
DQ2/DQ8	42 3.94 44.21 17.28	30 2.81 31.58 5.67	23 2.16 24.21 7.82	95 8.91
Total	243 22.80	529 49.62	294 27.58	1066 100.00
Frequency Missing = 55				

5.0 DISCUSSION

5.1 PROBANDS

5.1.1 Non-Asp 57 Analysis of Probands Compared to Probands in the Literature

Looking at the presence of non-Asp in the probands, 96.65% have at least one non-Asp allele, including the individuals with non-Asp plus DQB1*0602 (DR2) which is a protective haplotype. Not including the DR2 group, 94.14% have at least one non-Asp allele with a breakdown of 30.54% with one non-Asp allele and 63.60% with two non-Asp alleles. For this analysis, we will combine the DQB*0602 individuals with the non-Asp/Asp group as in the Dorman et al. 1990 study, they did not differentiate between the presence of the 0602 allele. Therefore, our estimates for one non-Asp is 33.05% and for two non-Asp alleles is 63.60% compared to the Dorman estimates of 33% and 70% respectively. Looking at just the Whites in this study, 33.18% had one non-Asp allele and 63.68% had two non-Asp alleles. This is compared to approximately 33% and 61%, respectively, in the Dorman study (Table 15).

Table 15: Comparison of Non-Asp Status in Caucasians to Previous Literature on Diabetics

STUDY POPULATION	Caucasian Diabetics (Probands)—Smolnik	Caucasian Diabetics—Dorman et al.	Total
Non-Asp/Non-Asp	142 (63.68%)	30 (61.22%)	172
Non-Asp/Asp	74 (33.18%)	19 (32.65%)	93
Asp/Asp	7 (3.17%)	0 (0%)	7
Total	223	49	316

While the differences between the two non-Asp/non-Asp groups are not significant (chi squared test p-value is approximately 0.975), they may be related to the age differences in the two studies. In the Dorman study, the age at diagnosis in the diabetic population was less than 15, whereas in our study 9.27% of the subjects were diagnosed over the age of 15. This may be important as typically the higher risk haplotypes are associated with younger ages of onset. Since we have individuals with older ages of onset, they may contribute to the overall lower numbers of higher risk haplotypes. Also, this study's population includes some individuals of other or multiple races whereas the Dorman study includes Blacks and Whites only. In addition, while unlikely the fact that in the Dorman study HLA-typing was performed by Dot blot analyses with DQBI allele specific oligonucleotide probes whereas in our study we use a more current and specific technology of reverse sequence specific oligonucleotide probes may contribute some small differences [29].

Seven of the 221 Caucasian probands (3.17%, 3.35% for entire population) had two Asp alleles at position 57 of the DQ beta chain. Again, this lower incidence is to be expected as it is the presence of non-Asp alleles that are associated with the risk of developing T1D. In the

Dorman study of Caucasian probands, 0% were Asp/Asp. Other studies have found incidences of 0% as well [30]. In evaluating these higher than expected results, it was noted that 2 of the 7 Asp/Asp individuals reports themselves as Hispanic (also 2 of the 3 Hispanics in our population) and some studies have suggested that the presence of Asp-57 does not confer protection in the Mexican-American ethnic group [31]. In addition, one of Asp/Asp individuals upon further review of medical records most likely has Type 2 diabetes. He was diagnosed at 17 years, is overweight, has acanthosis and high blood pressure, and did not present classically with diabetic ketoacidosis. This patient was discharged on insulin but has subsequently been switched to oral medications. Another one of the individuals is a female diagnosed at 5 years, no diabetic ketoacidosis, overweight, and is suspected to have either T1D or double diabetes. In addition, HLA is not the only genetic loci linked to T1D, in fact accounts only for 50-60% of the genetic risk, and in these cases the disease may be the result of a combination of other susceptibility genes and environmental interactions. This is also supported by the fact that several of the individuals are overweight. In the situation where an individual is overweight and has T1D, it may be the case that obesity causes insulin resistance, requiring the body to produce more insulin leading to minor beta cell damage and therefore a lower or "less risky" genetic load would be required for disease development. In addition, it may the case in these individuals' disease is related to other genes.

The Asp/Asp individuals' family histories were also evaluated to identify any possible incidences of MODY or other familial situations of the disease. Of the 7, 2 were reported to have a strong family history of Type 2 diabetes. One individual had a family history of thyroid disease. However, three had no family history of Type 1 or 2 diabetes or other endocrine

diseases. Therefore, it may be these variations in clinical features and course as well as ethnicity that can be attributed to the higher incidence Asp/Asp homozygotes found in this study.

Multiple studies both in the United States and Europe, such as Hermann et al [32], have shown that less than 1% of children with type 1 diabetes are DR2 (DQB1*0602). Our study shows about double this amount with 6 of our 239 (2.51%) with the DQA1*0101-DQB1*0602 haplotype that has been associated with near absolute protection from developing T1D [33]. This theory of absolute protection has been disproved before, as in Pugliese et al where 6 children with T1D had the normal DQB1*0602 and DQA1*0102 [15]. In the Swedish Childhood Diabetes Study Group and The Diabetes Incidence in Sweden Study (DISS) Group the haplotype was absent before the age of 10 but the incidence increased with later ages of onset [34]. This is consistent as in our study 4 of the 6 were diagnosed at or over the age of 10 (ages of diagnosis for all six were 5, 8, 10, 10, 13, and 14).

These individuals' family histories were also examined. One individual has a maternal great grandmother with T1D, a mother with hyperthyroidism, and a maternal grandfather with Crohn's disease. Another individual's maternal history was only available. This includes an uncle and grandmother with non-insulin dependent diabetes with the grandmother also having hypothyroidism. Another person had a strong maternal and paternal family history of type 2 diabetes. The fourth person has a strong family history of thyroid disease and type 2 diabetes. The fifth individual has late onset diabetes in the maternal grandparents, with the grandmother also having hypothyroidism. Her father's history is significant for celiac disease. The last individual has a family history of diabetes mellitus in her maternal aunt and paternal great grandmother. It is important to recognize that all of these individuals have a family history of

either diabetes or an autoimmune condition, as this may suggest that there are other genetic and environmental factors playing a role in their case of T1D.

It is also of interesting note that upon review of their medical history, 3 of the 6 individuals with the DQB1*0602 haplotype are suspected to have what is known as type 1.5 diabetes or double diabetes. Double diabetes is when an individual has signs of both autoimmune diabetes and insulin resistance. There is, however, no published information about the relationship between HLA-DQ alleles and double diabetes.

5.1.2 Non-Asp 57 Analysis of Probands Compared to Unaffected Individuals

Our results show that the distribution of non-asp alleles in our probands and FDR population supports the multitude of studies that have shown that the presence of non-Asp at position 57 of DQ beta is associated with the risk of T1D. Using a chi squared test with 2 degrees of freedom to compare the presence of Asp 57 in the diabetic probands and their unaffected first degree relatives, we get a chi-squared value of 8.45 and 2 degrees of freedom we get a significant p-value of 0.0146 (Table 16). This supports the hypothesis that role of the non-Asp alleles are associated with the development of T1D, as there are statistically significant higher amount of diabetics with two non-Asp alleles than unaffected FDRs and lower amounts of diabetics with only one non-Asp allele or two Asp alleles.

Table 16: Comparison of Non-Asp Status for Caucasian Probands vs. FDRs

Study Population	Caucasian Diabetics (Probands)	Caucasian FDRs	Total
Non-Asp/Non-Asp	142 (63.68%)	282 (34.73%)	424
Non-Asp/Asp	74 (33.18%)	463 (57.02%)	537

Table 16 Continued			
Asp/Asp	7 (3.17%)	67 (8.25%)	74
Total	223	812	1035

If you compare this study's probands to the general population statistics in Dorman et al. you will see again that again there is a difference in the presence of non-Asp alleles in the two populations which supports the role of non-Asp in T1D susceptibility. In the Dorman study, the individuals in the general population group were unrelated non-diabetic controls. This information is presented in Table 17 below and the differences are statistically significant with a chi squared value of 11.09 and 2 degrees of freedom gives us a p-value of 0.0039.

Table 17: Comparison of Non-Asp Status in Caucasian Probands and General Population in the Literature

STUDY POPULATION	Caucasian Diabetics (Probands)—Smolnik	Caucasian General Population—Dorman et al.	Total
Non-Asp/Non-Asp	140 (63.35%)	24 (19.51%)	164
Non-Asp/Asp	74 (33.48%)	57 (46.34%)	131
Asp/Asp	7 (3.17%)	42 (34.15%)	49
Total	221	123	344

In addition, if further compare the probands to the FDRs and also to the general population (Table 18), you can see that as described before, the percentages follow the role of non-Asp alleles in T1D risk. This is illustrated by the fact that the majority of probands are non-

Asp/non-Asp, FDRs have one non-Asp allele, and individuals in the general population have mostly either one non-Asp or are Asp/Asp.

Table 18: Caucasian Analysis: Probands vs. FDRs vs. General Population

STUDY POPULATION	Caucasian Diabetics (Probands)—Smolnik	Caucasian FDRs--Smolnik	Caucasian General Population—Dorman et al.
Non-Asp/Non-Asp	140 (63.35%)	282 (34.69%)	24 (19.51%)
Non-Asp/Asp	74 (33.48%)	463 (56.95%)	57 (46.34%)
Asp/Asp	7 (3.17%)	67 (8.24%)	42 (34.15%)
Total	221	812	123

5.1.3 DQ2 and DQ8 Distribution of Probands

In regard to the DQ2 and DQ8 haplotype analysis our results show that 78.63% of the probands possessed DQ2 and/or DQ8 haplotypes. This includes all races and ethnicities. If you evaluate Caucasian probands only, 78.83% possessed the DQ2 and/or DQ8 haplotypes. This is lower than several other studies which have shown an estimates of >90-95% [12, 30]. In the breakdown, 6.49% were DQ2 homozygotes (DQ2/DQ2), 4.96% were DQ8 homozygotes (DQ8/DQ8), and 16.79% were DQ2/DQ8 heterozygotes. If you only look at the Caucasian probands the breakdown is 5.81%, 5.39%, and 17.01%. Again, these estimates are lower than other study results. For example, the IX International Histocompatibility Workshop in 1984

found the haplotypes for Caucasian type 1 diabetics to be 7.2% DR3 homozygous, 7.9% DR4 homozygous, and 33.6% DR3/DR4 heterozygous respectively [12]. In addition, other studies have shown estimates of 30–50% of patients to be DR3/DR4 (DQ2/DQ8) heterozygotes [18, 23]. While our study looks at DQ and these other studies are analyzing DR, the numbers should be comparable due to the high LD between the two loci.

One issue of the differences in the numbers may be due to the fact that the DR3/DR4 statistics are typically higher in diabetics diagnosed under the age of five. The original statistics on the age of diagnosis of the diabetics in those studies were not available for review; however an analysis was still performed on the diabetic probands in this study to see if the estimates are similar to other studies looking at groups in this age at diagnosis range. Of our Caucasian probands, 34 (13.13%) were less than five years at the age at diagnosis. Of these 34, 3 of them required more specific HLA analysis in order to determine the presence of either the DQ2 or DQ8 haplotypes or the number of non-Asp alleles. Therefore, there are 31 diabetics probands diagnosed under the age of 5 available for analysis. Of these, 7 were DQ2/DQ8 heterozygotes (22.58%). However, looking at all 40 diabetic probands that are DQ2/DQ8 heterozygotes only 7 (17.5%) were diagnosed under the age of 5. Again, these numbers are not consistent with studies of those diagnosed before the age of 5 where estimates can range from 50% to ~63% [35, 36].

Looking at the DQ2 and DQ8 (including DQ2 and DQ8 homozygotes and heterozygotes, but not DQ2/DQ8 heterozygotes) frequencies separately, the diabetic probands in our study had frequencies of approximately 29% and of 33%, respectively. Comparing these to the frequencies of DR3 and DR4 among diabetic haplotypes in Allegheny County in 1988 conducted by Morel et al. were 29% and 48%, respectively [30]. These differences are not statistically significant with a chi squared value of .7893 with 2 df and p-value of 0.674 (Table 19).

Table 19: Comparison of DQ2 (DR3) and DQ8 (DR4) Haplotypes in Diabetic Probands in the Study vs. Morel et al

Study Population	Diabetic Probands—Smolnik	Diabetic Probands—Morel et al.	Total
DQ2	76 (28.68%)	20 (28.99%)	96
DQ8	88 (33.21%)	33 (47.83%)	121
Other haplotypes	101 (38.11%)	16 (23.19%)	117
Total	265	69	334

Overall, the differences between the statistics in this study compared to the literature in relation to the DQ2 and DQ8 haplotypes are significant. As discussed before, these differences may be the result of other genetic loci playing a role in this particular population. However, due to having data available from previous populations in Allegheny County, it may be less likely there was any significant change in the genetic distribution of these haplotypes. Also, by controlling for race there are no differences associated with the differences found in race and ethnicity distributions. However, based on other studies which have found an increasing amount of obesity in this T1D population, it may be that the obesity is added strain to cause for a higher insulin requirement and therefore requiring less of a genetic load if you will to develop T1D.

5.2 FIRST DEGREE RELATIVES RESULTS

5.2.1 Non-Asp 57 Analysis of FDRs

In the first degree relatives, the presence of 0, 1, 2 non-Asp alleles as well as the combination of DQB1*0602/non-Asp was determined. Of the 1000 FDRs, 67 were unable to be determined by the reverse SSOP method and will need further analysis by SSP. Therefore, there was 933 FDRs available for the non-Asp 57 analysis. The results include approximately 34% non-Asp/non-Asp, 58% Asp/non-Asp (with about 14.5% of these individuals non-Asp/0602 specifically), and 8% Asp/Asp. Compared to the diabetic probands in the study the amount of Asp/Asp, Asp/non-Asp, and non-Asp/0602 individuals are all higher whereas the amount of non-Asp/non-Asp individuals is lower. This is consistent with the risks associated with each haplotype (i.e. the presence of two non-Asp alleles carries the most risk to develop T1D). Also consistent with what we know about the genetic susceptibility of T1D, the presence of non-Asp alleles is increased, with the exception of Asp/Asp individual which again is to be expected, when compared to the general population who are unaffected and unrelated to an individual T1D. For example, in Dorman et al., the frequency of unaffected, unrelated controls being non-Asp/non-Asp, non-Asp/Asp, and Asp/Asp was 20%, 46%, and 34%, respectively (Table 20) [29].

Table 20: Approximate Non-Asp Status of Caucasian Probands vs. FDRs vs. Controls in Dorman et al.

Non-Asp Status	Probands (%)	FDRs (%)	Controls-Dorman et al (%)
Non-Asp/Non-Asp	64	34	20
Non-Asp/Asp	33	58	46

Table 20 Continued			
Asp/Asp	3	8	34
Total	100	100	100

For a breakdown of the 1000 FDRs, there are 861 known individuals to be a parent or a full sibling, 554 and 307 respectively (Table 21). Comparing these results of non-Asp status we see that there are no significant differences (p-value of 0.551) between the parents and siblings. There is a statistically significant difference between the two FDR categories when they are compared to either the proband or control group. These differences are supported by the fact that probands should have the highest amount of risk genes, followed by FDRs as they share common genes with the proband and thus also have a risk to develop T1D, as well as the fact that there are individuals in the FDR group that will go on to later develop T1D, and with the unaffected, unrelated controls with the lowest amount of risk genes as they have the lowest empirical risk and do not share genes with an affected FDR. However, this data does not support the theory that since siblings more often share haplotypes with their affected proband sibling and are young enough to have still have a chance to develop T1D, they will have higher amounts of risk genes than do the parent FDRs who are in a later age group and if still unaffected, they most likely do not have the higher risk genes. This may be the result of a younger average age of the parents, which the data was unavailable at the time of analysis. If the parents were mostly younger, such as their twenties and not late thirties or early forties, then it may be that the risk of developing T1D is similar to the parent group as it is for the sibling group. It would be interesting to look at the autoantibody results of these two groups as well to examine any possible differences and how this may adjust the risk for the two groups.

Also, one could hypothesize that based on the theory that those with the highest risk genes go on to develop diabetes, then we may expect to see even less of the highest risk categories in the FDR parent population than in a general population group of all ages. In the Dorman study however, the unaffected, unrelated controls were all adults and so such a comparison cannot be made with that study population.

Table 21: Approximate Percentages of Non-Asp Status of Caucasian Probands vs. Siblings vs. Parents vs. Controls in Dorman et al.

Non-Asp Status	Probands (%)	Siblings (%)	Parents (%)	Controls-Dorman et al. (%)
Non-Asp/Non-Asp	64	34.5	33	20
Non-Asp/Asp	33	55.5	59	46
Asp/Asp	3	10	8	34
Total	100	100	100	100

In regard to the presence of DQB1*0602 in first degree relatives, most studies first identify FDRs who are antibody-positive and then follow with DQ haplotype analysis. Therefore, most of the data is first stratified by antibody-positive FDRs and are therefore and not exactly comparable to this study since autoantibody status was unknown to this study at the time. For example, the Diabetes Prevention Trial-1 (DPT-1) aimed at identify relatives at risk for developing T1D in order to design intervention trials to prevent the disease. Through this process, DPT-1 identified a large cohort of antibody-positive DQB1*0602 positive relatives. Of their large group of 81,000 first and second degree relatives, 3.6% were antibody positive and offered genetic analysis. Of the antibody positive group, approximately 7.7% (n=225) first and

second degree relatives were identified with the 0602 allele. In this study, approximately 15% of parents and 14% of siblings were found to have the DQB1*0602 allele. Again, this may be higher because of several reasons including we are looking at just first degree relatives and that we are looking at all FDRs and not just FDRs who are autoantibody positive. In addition, these numbers for the parents and siblings are significantly different than that of the general population which is approximately 25% [37].

5.2.2 DQ2 and DQ8 Analysis of FDRs

For the FDRs, approximately 68% had DQ2 and/or DQ8 haplotypes, whereas fewer than 40% of normal controls have these haplotypes [38]. Specifically, about 32% were either DQ2 homozygotes or heterozygotes, 30% were either DQ8 homozygous or heterozygous, and 6% were DQ2/DQ8 heterozygotes. Evaluating the amount of DQ2/DQ8 heterozygote in the FDRs, we see 6% in this study compared to ~16-17% of probands in this study and 30-50% of probands in multiple other studies as described previously, and compared to an incidence of <1% to 3% in the general population [23, 39]. These results can be supported based on the role of the DQ2/DQ8 genotype as conferring the highest susceptibility to the disease. To be more specific, we would expect less FDRs to have this genotype compared to those affected with T1D. We would also expect there to be more FDRs than unaffected, unrelated controls in this group as FDRs share genes in common with affected individuals who also more often have these risk genes as well as FDRs have a higher risk to develop T1D than do those in the general population and some in this group may still go on to develop the disease. Furthermore, there were no significant differences (p-value of 0.401) between the DQ2 and DQ8 haplotypes between parents

and full siblings. Again, as discussed for the non-Asp analysis, this may be explained by if there is a younger average age of the parents and that some of the parents will go on to develop T1D.

5.2.3 Future Directions

The ultimate goal of this project is to be able to provide families that have participated in the "Epidemiology and Etiology of Type 1 Diabetes" research study more accurate information on their individual risk for developing T1D. From the many studies performed both by the research groups and Children's Hospital of Pittsburgh and around the world, it has already been determined that specific HLA haplotypes confer a risk as well as does the presence of specific autoantibodies.

The next phase of this project would entail analyzing both the probands and first degree relatives to determine if any one haplotype is more associated with any of the autoantibodies that precede the disease state. Therefore, each person would have both their genetic risk in addition to the risk of developing autoantibodies, which even more accurately predict the likelihood of the development T1D. The final phase of the project would then be to construct an appropriate genetic and autoimmunity education session to provide genetic counseling to these individuals at risk for developing T1D. There are already several pilot programs pursuing this based on an individual's genetic risk alone, such as GIFT-D. Being able to provide information with even higher predictability, including both genetics and autoimmunity, would be more beneficial for the participants, as it will allow them to better know their individual risk and possibly pursue the intervention trials that are currently ongoing or new ones in the future.

6.0 CONCLUSIONS

This study has allowed for an analysis of the HLA-DQ molecule in both a large group of probands and first degree relatives of affected individuals. The results for the analysis of the presence of non-Asp alleles in both the probands and FDRs were consistent both with the role of the non-Asp allele in the risk of developing T1D when comparing these groups to each and to unaffected, unrelated controls. Also, these results were mostly consistent with previous results in the literature. It was noted that the percentage of individuals with two Asp alleles was higher in this proband population than expected, 3% compared to multiple other studies showing 0%. When evaluating the individuals personal, medical, and family history we did see of some significance several individuals who were Hispanic, several they have a non-classical picture of T1D suggesting either Type 1.5 ("Double Diabetes") or 2 diabetes, and several overweight individuals. These features may support a hypothesis that these individual's disease development may be attributed to other susceptibility genes, as seen especially in other ethnic backgrounds, or that the presence of obesity may require a higher demand for insulin and thus lowering the need for susceptibility genes for disease development.

In addition, as the DQB1*0602 allele is supposed to be associated with disease protection, we evaluated the ~2.51% probands with this haplotype. After analysis, it was determined that 66.67% were diagnosed after the age of 10, all had a family history of diabetes (either type 1 or type 2) or other autoimmune diseases, and 50% of the individuals were

suspected of having “double diabetes”. Again, these findings are significant in the fact that they represent a non-classical picture of an individual with T1D and while there is no published data yet available on the relationship between HLA-DQ and double diabetes there may be such an association in the future that we could compare this group to.

The non-Asp analysis of the FDRs was consistent with the risks associated with each allele combination both when compared to probands and to unaffected, unrelated controls. When comparing parents versus full siblings, unexpectedly there were no significant differences. Again, this may be explained if there is a younger average age of the parents and that some of the parents will go on to develop T1D.

When assessing the distribution of the HLA-DQ2 and DQ8 haplotypes, the percentage of probands who were DQ2/DQ8 heterozygous was not consistent with previous studies in the literature including studies that were performed previously in Allegheny County. When isolating this group to look at either just Caucasians or those diagnosed under the age of five there were still significant differences. As discussed before, these differences may be the result of other genetic loci playing a role in this particular population. In addition, it may be that the increasing decreased amount of individuals with the highest risk-conferring genotype is the result of the increasing amount of obese individuals, which may require a larger insulin requirement thereby requiring less of a genetic load to develop T1D.

These first degree relative DQ2 and DQ8 results indicated a significant difference between FDRs and the risk categories including fewer individuals in the high risk groups than probands and more individuals than unaffected, unrelated controls. This is supported by the role of the DQ2/DQ8 genotype conferring the highest susceptibility to the disease. Furthermore, the

analysis of the differences between parents and full siblings again was not as expected as there were no significant differences between the DQ2 and DQ8 haplotypes.

This study has provided several important findings as it allows for further verification of the role HLA plays in diabetes as well as allows for more accurate disease prediction. As discussed before, this project plays the initial important role of being able to provide disease risk assessment to research participants. As the morbididy and mortality of T1D affects our society's healthcare and economy, a key to prediction and hopefully prevention will be just as important on the personal level as it will on a large-scale public health level.

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