

GENETIC AND IMMUNOLOGIC BIOMARKERS IN TYPE 1 DIABETES

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2013

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To Lori

ACKNOWLEDGMENTS

Few accomplishments are achieved in isolation. This work is no exception. These efforts represent the product of investments made by many other individuals over a long period of time. I will name a small subset of the most important contributors here, but my gratitude extends to a broad network of people who provided practical, scientific, and moral support through the last several years.

I am tremendously grateful for Drs. Mark Atkinson and Desmond Schatz, my science and clinical mentors, respectively. Dr. Atkinson's patience, scientific guidance, moral support, and hospitality allowed me to make of his lab a second home. The work environment that he has fostered is truly unlike any other I have ever seen, and I will miss the daily positive interaction. Dr. Schatz's depth of thought, forbearance, generosity of time and spirit, and attention to detail were critical to keeping me on the right track. His willingness to educate me as to the practical and medical aspects of diabetes care opened my eyes to the importance of our work.

My appreciation for Clive Wasserfall cannot be overstated. His mentorship and guidance at every step of the way have been absolutely indispensable in the completion of this work. He has been nothing but supportive in every possible way, and his day-to-day instruction is the standard by which I will measure my own quality as a mentor in the future.

My thanks to my committee members. Dr. Charles Baer's thoughts on the genetic aspects of the work has greatly facilitated my efforts. More importantly, Dr. Baer's training during my undergraduate career formed the foundation upon which I built my scientific values and career, and it was his example that showed me what good science could be. Dr. Nick's insight and candor have served to keep me grounded and

motivated, and without his knowledge and the support of his laboratory members (in particular Justin Bickford and Kimberly Aiken), this work would not have been possible.

I am grateful to Maigan Hulme, whose guidance and experience, particularly in the areas of cell culture and immunological assays, made much of the work possible. Similarly, Drs. Todd Brusko, Daniel Perry, and James Thompson have provided indispensable aid throughout this work.

I am grateful to my mentees, undergraduates and postgraduates who have been laboratory assistants, collaborators, and co-conspirators throughout the process. These include Peter Hong, Sarah Green, Adim Moreb, Candace Worsham, Giselle del Valle, Kyle Ziegler, and Andrea Warren.

I am grateful to my lab mates for their moral and logistical support and collaboration; Kieran McGrail, Sean McGrail, Theresa Sumrall, Young Mee Yoon, Courtney Myhr, John Alexander, Song Xue, Matt Parker, Patrick Rowe, Taylor Davis, Ricky, Zack Webster, Zhao Han, Amanda Posgai, Lindsey Bierschenk, and Marcus Moore.

I owe much to the University of Florida Genetics Institute Graduate Program staff, including Hope Parmeter, Mimi Zarate, Diana Nolte, Marta Wayne, Wilfred Vermerris, Connie Mulligan, and Jorg Bungert. I am indebted to the University of Florida Clinical and Translational Science Institute's TL1 graduate training program, and in particular the efforts of Drs. Wayne McCormack and Marian Limacher in my training program.

I am grateful to my wife, Lori, whose patience, support, and dedication have made this work possible and worthwhile.

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Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

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By

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August 2013

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Major: Genetics and Genomics

While human leukocyte antigen typing, autoantibody measurement, and the characterization of many regions affording genetic susceptibility have contributed tremendously to our ability to predict the onset of, comprehend the etiology of, and track the progression of type 1 diabetes, these approaches are far from complete in terms of their understanding. Individuals with high risk human leukocyte antigen, autoantibody, and genotypic profiles still escape overt disease, while, in contrast, individuals with low risk factors can progress to type 1 diabetes. This may be due in part to the influence of environmental factors, but estimates suggest that up to one quarter of the heritability for type 1 diabetes remains unaccounted for by existing knowledge of the genetic architecture. To that end, this effort sought to characterize genetic and immunologic biomarkers that might provide novel insights into type 1 diabetes pathogenesis and progression.

We investigated two pathways: the Vitamin D pathway and the interleukin-2/interleukin-2 receptor pathway; these, based on a variety of efforts that hinted at their potential involvement. We observed an association between Vitamin D Binding Protein levels and disease state; individuals with type 1 diabetes have lower levels of Vitamin D

Binding Protein than controls. Relatives were intermediate between healthy controls and subjects with type 1 diabetes. Investigating the interleukin-2/interleukin-2 receptor pathway, we observed an association between a T>G SNP in the promoter region of Interleukin-2 (rs2069762) and serum levels of the soluble isoform of the Interleukin-2 receptor component CD25. Specifically, the G allele was associated with elevated serum levels of sCD25. Elevated serum levels of sCD25 were significantly associated with type 1 diabetes. However, genotype at the rs2069762 locus did not directly associate with disease state.

We have provided further evidence that the Vitamin D pathway is involved in autoimmune diabetes, potentially not just at the level of Vitamin D itself, but also at the level of its principal transporter, Vitamin D Binding Protein. Further investigation of the role of vitamin D binding protein, sCD25, rs2069762 and disease state in type 1 diabetes will be necessary before we can fully appreciate the meaning of the complex interactions between genetics and environment that lead to type 1 diabetes.

CHAPTER 1 BACKGROUND

Type 1 Diabetes: Etiology and History

Type 1 diabetes is a potentially fatal wasting disease. In the absence of therapy, patients exhibit polydipsia, polyuria, polyphagia, and severe weight loss, all symptomatic of absolute insulin deficiency. The five year survival rate for untreated type 1 diabetes is below 1%, and this survival rate is still observed today in Sub-Saharan Africa, where, in some cases, insulin is available less than 25% of the time [1-3].

Since the 1921 discovery of insulin, most patients in industrialized nations have access to exogenous insulin. This life-saving treatment has afforded many patients the opportunity to live long, healthy lives. Unfortunately, exogenous insulin therapy is not a cure; patients must conform to rigorous therapeutic protocols to minimize risk of any of a series of disease-associated complications, including multiple finger pricks and needle sticks per day for blood glucose monitoring and insulin injection. Even with intensive therapy, patients still face risk of complications including neuropathy, retinopathy, and cardiovascular disease. The medical costs of patients with diabetes are approximately double the national average; diabetes patients incur about \$7,900 of diabetes related expenses per annum [4]. Diabetes is particularly burdensome in part due to its indiscriminate timing; the two peaks of onset for type 1 diabetes are between 5 and 7 years of age and during puberty [5].

In this introduction, we will explore what is known about type 1 diabetes, potential causative factors in the genotype and environment, the important role that biomarkers of

disease could play in detection and prevention of disease, and the relevance of the Interleukin-2 (IL-2) and Vitamin D pathways to the aforementioned factors.

Genotype, Environment, and Models of Pathogenesis

Type 1 diabetes is a complex autoimmune disorder in which aberrant immune recognition of self-antigen(s) is believed to result in a T-cell mediated attack on the insulin secreting pancreatic beta cells located in the islets of Langerhans. This results in a progressive decline in pancreatic beta cell mass concurrent with progressive insulin insufficiency, ultimately leading to a loss of glycemic control.

Clinically, patients typically present with polydipsia, polyuria, and polyphagia [5-8], at which time they are diagnosed via blood sugar and confirmed as type 1 with autoantibody testing. After confirmation of diagnosis, patients are followed closely by health care professionals and are typically prescribed an intensive regimen consisting of frequent blood sugar testing, multiple daily injections or the administration of insulin via an insulin pump. Poor control can lead to long-term complications including nephropathy, neuropathy, vascular disease, and retinopathy and acute, potentially life-threatening complications including hypoglycemic coma and diabetic ketoacidosis.

It is believed that an inciting event in the pancreatic islet leads to a release of self-antigen [9]. These antigens are then picked up by antigen presenting cells, which subsequently migrate to the nearby pancreatic lymph nodes. There, antigen is presented via MHC (major histocompatibility complex) class II molecules to naïve autoreactive T and B cells [10]. These cells then become armed effector and memory cells, proliferating and expanding in the lymph node, secreting inflammatory cytokines and entering circulation through the lymphatic ducts. The immune response expands as effector cells are drawn to the site of inflammation and initiate beta cell destruction,

releasing additional epitopes and driving a broader attack by cells with diverse antigen specificities. Autoantibodies to self-antigens are produced by B cells; in humans, autoantibodies to insulin is most often the first to occur; others including glutamic acid decarboxylase (GAD), insulinoma antigen 2, Zn transporter ZnT8, and likely a host of other epitopes [11] often follow.

These autoantibodies are the most commonly used means of diagnosing and attempting to predict type 1 diabetes. Though the sensitivity of autoantibody assays is greater than 70% in new onset patients, autoantibodies can be found in individuals who do not progress to type 1 diabetes, and there exist individuals who progress to type 1 diabetes but who do not exhibit detectable autoantibodies [12-14]. This fact has led experts in the field to note, “there are no biomarkers of the disease process that are reliably correlated with the pathogenic process” [15]. For this reason, a need exists to develop reliable biomarkers of disease progression and treatment efficacy.

There have been many models of disease pathogenesis and progression over the years, all of which have sought to reconcile the competing pathogenic and protective influences of genetic and environmental factors. Models from the mid-80s to the early 2000s focused on progression through stages, with an environmental trigger acting upon a genetically predisposed individual to incite a progression to overt disease marked by decline of beta cell mass and capacity to regulate blood glucose [8, 16]. Others have expanded upon these ideas, pointing out the possible contributory role of the beta cell itself and the internal cytokine environment to antigen availability, and highlighting the role of internal and external environmental stressors like childhood obesity which could contribute to the release of beta cell antigens [17-19].

With this, it appears clear, the pathogenesis of type 1 diabetes is a complex, multifactorial process that may require tissue and time-specific antigen presentation and T-cell specificity to multiple self-antigens, and further, the relative importance of the various factors involved (i.e., beta cells, immune system, and environment) may vary from patient to patient [20]. To reflect this complexity, a threshold model has been recently proposed which takes into account the relative input of genetic and environmental contributions to total risk and accounts for the possibility that the relative contributions of each may vary on a per-patient basis according to some as yet unknown (and likely highly variable) function [21] (Figure 1-1). The discussion below will consider each of these contributors individually, and will discuss how novel biomarkers might contribute to our understanding of disease progression and treatment efficacy, especially in light of recent developments in our understanding of diabetes.

Genetics of Type 1 Diabetes

General Background

The concordance rate for type 1 diabetes among monozygotic twins has been estimated at 50%, but more recent estimates reveal that, on longer timelines, concordance rates approximate 65% [22]. By comparison, the non-monozygotic sibling risk is 5-6%, suggesting that shared environment does not explain the concordance between monozygotic twins and suggesting a powerful role for genetics [23, 24]. The ratio between sibling risk and general population risk, known as λ_s , for type 1 diabetes is between 12 and 15, suggesting a moderate influence of heritable factors on disease progression [25-27].

Type 1 diabetes does not follow any particular pattern of inheritance. There are more than 60 known susceptibility regions for type 1 diabetes, most of which were

identified through genome wide association studies (GWAS), but the susceptibility in few of these regions has been mapped to a specific variant or even to a specific gene [23, 28, 29] (Figure 1-2). While approximately 80% of the heritability can be explained by the genes identified thus far – a surprisingly high proportion, as compared to other complex disease – there remains considerable unaccounted for (or “missing”) heritability in type 1 diabetes [23, 28, 30, 31]. This heritability could be attributed to as yet uncharacterized structural variation, it could be attributable to rare variants of large effect, similar to the highly protective but extremely rare recently discovered variants of interferon induced with helicase C domain 1 (IFIH1), or it could be a product of gene-gene or gene-environment interactions [31, 32]. Clearly, more work needs to be performed to characterize the regions of disease susceptibility, the genes within them, and the various alleles of these genes.

As noted above, type 1 diabetes involves interaction between the endocrine and immune systems and as such, there are multiple opportunities for genetic defects to influence the course of disease. From antigen presentation to cell signaling molecules, from genes that govern and allow the expansion of self-reactive cells, to those that interfere with immune regulation, as well as those that influence beta cell survival and possible regeneration; there are many pathways that could hypothetically be implicated in the disease process [29]. Among the important known risk regions are the human leukocyte antigen (HLA) region, which contributes an overwhelming majority of risk (i.e., odds ratio of 6.8), insulin, the alpha subunit of the IL-2 receptor, and IL-2 itself [29, 33].

HLA Specific

The HLA region, a 3.6 Mb – 7.6 Mb region in chromosome 6 (6p21.3), accounts for ~ 50% of the genetic risk associated with type 1 diabetes [34-37]. The HLA region

codes for the MHC molecules, and the region is distinguished by high degrees of both polygeny and polymorphism in order to address a broad range of potential antigens. There exist three classes of MHC molecules: class I, found on nearly all cell types and responsible for presentation of antigen to CD8 cytotoxic T-cells, class II, found on professional antigen presenting cells and responsible for presenting antigen to CD4 helper T-cells, and class III, which encodes for other immune (particularly complement) proteins.

In type 1 diabetes, the principal risk is associated with alleles in the MHC class II region. MHC class II alleles are expressed as transmembrane heterodimers composed of an alpha chain and a beta chain (Figure 1-3). These MHC proteins function to present antigen to CD4+ helper T-cells, as T-cells will not recognize antigen and will not activate without the context of MHC.

There are three MHC class II antigen receptors that participate directly in antigen presentation to T-cells; these are HLA-DP, HLA-DQ, and HLA-DR. Genes encoding the HLA-DP alpha and beta chains may have as many as 23 and 120 alleles circulating in a population, respectively. Genes encoding the HLA-DQ alpha and beta chains may have as many as 32 and 68 alleles circulating, respectively. HLA-DR has but one locus for the alpha chain, which is largely invariable, but nine loci for the beta chain, usually two of which (DRB-1 and typically one other) may be expressed in any individual. DRB-1 is highly polymorphic, with over 500 variants known [38].

With respect to how the combination of these complex factors contribute to disease pathogenesis, the late George Eisenbarth has long championed the idea of the trimolecular complex [39]. Here, three molecules are necessary for pathogenicity.

These molecules, the T-cell receptor, the MHC molecule, and the insulin peptide (and in specific, insulin B9-23) are required for the generation of an autoimmune response [39]. While unproven, currently, an extensive effort has been extended to testing this model.

Because there exists a high degree of linkage disequilibrium in the MHC, risk is generally associated with haplotypes; in fact, the term 'haplotype', for 'haploid genotype', was coined due to the study of the HLA region [40, 41]. The HLA DR/DQ haplotypes that account for 30 – 50% of genetic risk of type 1 diabetes are DR3-DQA1*0501*0201 (DR3) and DR4A-DQA1*0301-DQB1*0302 (DR4) [37, 42]. There also exists at least one protective haplotype, DRB1*1501-DQB1*0602 [43, 44], albeit not absolute.

MHC class II related loci are not the only regions of interest within the MHC. Fine mapping data from the Type 1 Diabetes Genetics Consortium indicates that MHC class I regions HLA-A and HLA-B are also independently associated with type 1 diabetes [45]. Finally, careful analysis reveals the likelihood that additional risk variants for type 1 diabetes exist within the extended MHC region, but outside of the class II region; clearly, further study is needed to fully elucidate the level of involvement of the extended MHC region in disease [35, 46, 47].

It is important to note that the strength of association between markers does not necessarily depend upon their map locations and so, the discussed markers may actually be somewhat distant from etiological loci [48]. Further, interaction may be taking place between the HLA locus and non-HLA loci, as evidence suggests that risks at non-HLA loci may be reduced in the presence of high HLA related risk [49]. Despite

the shortcomings, it does remain clear that genotyping in the HLA region continues to be an extremely useful marker for stratifying disease risk [50].

IL-2 Pathway

IL-2 as a target of interest in type 1 diabetes research. IL-2 has been known as an important factor for T-cell proliferation for more than three decades [51-53]. This critical role for IL-2 in T-cell growth, combined with data as early as 1974 suggesting a role for cell mediated immunity and MHC involvement in type 1 diabetes, mean that IL-2 has long been a target of interest in type 1 diabetes, with deficient synthesis of IL-2 observed in patients with type 1 diabetes as early as 1984 [54]. Of the more than 60 known susceptibility genes for type 1 diabetes, three lie within the IL-2 pathway (IL2RA, PTPN2, and IL2), with one in particular, IL2RA, having an odds ratio of over 1.50 and multiple single nucleotide polymorphisms (SNPs) contributing to disease risk [28, 29, 49]. It is likely that IL-2 levels are carefully controlled for proper immune regulation, as a reduction as modest as twofold in IL-2 levels has shown to predispose non-obese diabetic mice to autoimmune diabetes [55]. Translation to the human system has proven to be a challenge, however; high doses of IL-2 in NOD mice have been shown to accelerate autoimmune destruction, leading to concern about the potential for adverse outcomes in human patients under IL-2 therapy [56]. A recent study using IL-2 in combination with Rapamycin (administered with the intent of prevent excessive immune activation) led to a transient decrease in c-peptide levels, suggesting the possibility of harm to beta cell function and further underscoring the need for caution [57].

Structure and Function of IL-2 and its receptor. Human IL-2 is a 15 KDa cytokine that serves as a key regulator of immune function through its secretion by CD4+ and CD8+ T-cells, activated dendritic cells, natural killer cells, and natural killer T

cells [53, 58-60]. The IL-2 receptor is composed of three subunits, an alpha chain (referenced as IL2 α or CD25), a beta chain (referenced as IL2-r β or CD122) and a common gamma chain (IL2 γ or CD127). The beta and gamma chains together form an intermediate affinity receptor that is expressed on T cells, natural killer cells, and monocytes [61]. Low to intermediate concentrations of IL-2 signaling through the receptor will promote memory or effector phenotypes, while high concentrations can drive activation induced cell death in conventional T cells [62].

The alpha chain in combination with the beta and gamma chains forms the high affinity IL-2 receptor, which is transiently expressed on T-effector cells, B cells, dendritic cells, natural killer cells, and eosinophils but which is only constitutively expressed on regulatory T cells [63-66]. Mutations in the human CD25 gene can lead to severe autoimmunity and immune dysfunction [67, 68]. The gene for IL-2, located at 4q27, is composed four exons and codes for four alpha helices (Figure 1). The beta chain is shared between the IL-2 receptor and the IL-15 receptor. The gamma chain, referred to as the common gamma chain, is found in receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 [69].

IL-2 Is a Key Immunoregulatory Cytokine. While early data underscored the relevance of IL-2 to effector T-cell expansion (it was first known as T-cell growth factor), data have subsequently demonstrated that mice deficient in IL-2 or IL-2 receptor experience severe autoimmune reactions but retain their ability to mount an effective immune response once the autoimmunity has been dealt with [70, 71]. Immune dysfunction in IL-2/IL-2 receptor deficiency is rescued by the introduction of purified

CD4+CD25+ cells. These observations suggest that IL-2 signaling is critical for the function of the regulatory T-cells subset [72].

As regulatory T-cells do not manufacture their own IL-2, the primary sources for IL-2 used in regulatory T cell maintenance are likely to be activated CD4+ T-cells and dendritic cells [63, 73, 74]. Further, IL-2 is known to inhibit its own production through STAT and Blimp-1 dependent signaling pathways [75, 76]. IL-2 also appears to promote activation induced cell death after subsequent antigen receptor stimulation in T-cells [77]. These observations, taken together, suggest a powerful role for IL-2 in regulation, and a more redundant role in T-cell growth and proliferation [53]. Given its importance to immune regulation, the frequency with which it appears in association studies, and the diversity of potential targets within the pathway, the IL-2 pathway makes an excellent candidate for investigation in pursuit of biomarkers.

Environmental Influences on Type 1 Diabetes

General Background

Environmental factors make an attractive target for modulation in the interest of disease prevention, as the environmental component of disease may in most cases be more straightforwardly altered or compensated for than the genetic component [78]. The incidence of diabetes varies over 100 fold globally, from 0.1/100,000 cases per year in China to 40.9 per 100,000 per year in Finland, with an average increase in incidence worldwide of 2.8% per annum [79, 80]. Evidence of north-south gradients in incidence along with the presence of seasonal and temperature based variation in incidence all point to a nontrivial role for environmental factors in the pathogenesis of type 1 diabetes [81-83].

An ecological analysis has found associations between incidence of disease and gross domestic product, milk consumption, and coffee consumption, suggesting the potential that multiple factors correlating with the socioeconomic status may influence risk of diabetes; one potential explanation for this observation is the “hygiene hypothesis”, in which children who are not exposed to immune challenge are more vulnerable to disease later in life [84, 85].

These observations are tempered, however, by studies in which children born of parents from high-risk regions move to regions of more modest risk. Sardinia is known to be of remarkably high incidence (37 per 100,000 per year) and interestingly, children of Sardinian parents born in the Lazio region, in the mainland of Italy, had a type 1 diabetes incidence fourfold higher than their Lazio-heritage peers [86]. It is possible, however, that intergenerational environmental effects may be impacting this result, and other studies have demonstrated a marked increase in incidence when moving from low-to-high susceptibility regions [87, 88]. Other studies have shown the converse; second-generation immigrants to Sweden (which has an incidence of 29 per 100,000 per year) showed markedly decreased incidence compared to their Swedish peers, and even lower incidents if their parents were both from outside of Sweden. Results were similar for foreign children adopted by Swedish parents [89].

To further elucidate potential environmental causes of type 1 diabetes, a number of large collaborations have been undertaken, including the Baby Diabetes (BABY-DIAB) study, the Diabetes Autoimmunity Study (DAISY), the Environmental Triggers of Diabetes Determinants in the Young (TEDDY) study, and the Trial to Reduce IDDM in the Genetically At Risk (TRIGR) study [21]. While no environmental factors have thus

far been definitively established as either necessary or sufficient for type 1 diabetes development, much interest has developed in two primary areas: viral infection and nutritional factors.

Viruses

The seasonality of diabetes onset and temporal and spatial clustering suggest a role for infectious disease in diabetes pathogenesis [90]. A viral role in pathogenesis could take place through encouragement of beta-cell directed autoimmunity (for example, through antigenic mimicry), through direct beta cell killing, or via antigen release subsequent to killing (known as bystander effect) [91].

To date, as many as fourteen viruses have been associated with disease pathogenesis in human and animal models [91]. The most prominent of these have been enteroviruses in general and Coxsackievirus B4 (CVB4) in specific. Enterovirus RNA was detected in the blood of five of twelve French new-onset type 1 diabetes patients as compared to none of the controls [92]. CVB4 isolated from the pancreas of a type 1 diabetes patient was found to induce hyperglycemia and beta cell necrosis in laboratory mice, a result that was recapitulated in human pancreatic islet cells in vitro [93, 94]. Coxsackie B4 P2-C protein is known to elicit immune response from PBMC that also respond to GAD peptides, suggesting the possibility of molecular mimicry between the two [95].

It is clear that considerable evidence exists to suggest a potential viral role in type 1 diabetes. This evidence is tempered by the knowledge that incidence of disease is higher in regions with higher socioeconomic status (and better sanitation and hygiene), and lower in areas with higher population density and household crowding; these observations are contrary to what we would expect if virus exposure was a

primary governing factor of disease progression [84, 96]. The importance of viral involvement in disease pathogenesis will depend on how necessary viral infection is to the disease process; it is likely that infection is just one of several pathways by which antigen presenting cells may be exposed to beta cell antigen.

Nutrition

There has been much discussion regarding the relative influences of cow's milk exposure and breastfeeding in the pathogenesis of type 1 diabetes. Dietary exposure to cow's milk proteins or infant formula may increase risk of autoimmunity, while greater duration of breastfeeding has been proposed to offer protection [97, 98]. A recent, large meta-analysis seems to have found broad support for the association between duration of breastfeeding and protection from type 1 diabetes in the literature, possibly due to protection from enterovirus infection [99, 100].

Wheat gluten may also be an important contributor to the pathogenic process, as comorbidity between type 1 diabetes and the autoimmune wheat gluten sensitivity known as coeliac disease is higher than would be expected at random and there are a number of genes that associate with risk to both conditions [101]. In non-obese diabetic (NOD) mice, a wheat gluten diet has a complex relationship with progression to type 1 diabetes, with both low and high levels of wheat gluten apparently protecting from disease [102]. Exposure to a wheat gluten diet appears to promote an inflammatory, interferon gamma driven response in the NOD gut [103]. Finally, introduction of gluten-containing food before three months of age in members of the BABYDIAB study cohort led to an increased risk of islet autoantibodies, with four of seventeen babies who received gluten affected, all of whom had the DRB1*03/04, DQB1*0302 high risk HLA genotype [104].

Vitamin D Pathway

Observations regarding the seasonality of diabetes onset and a north-south gradient in type 1 diabetes incidence, particularly in Europe, led to interest in the role of the Vitamin D pathway in this disease [81, 82, 105].

Vitamin D is received primarily through two means; through dietary sources (primarily fortified milk and fatty fishes), and through ultraviolet B driven synthesis in the skin (Figure 1-4). The vitamin D precursor is 7-dehydrocholesterol, which is converted into its active form via two hydroxylation steps, one in the liver (by CYP2R1) which produces 25-hydroxyvitamin D_3 ($25(OH)D_3$) and the other in the kidney or in other cells like monocytes and macrophages (by CYP27B1), which produces the final active form, $1\alpha 25(OH)_2D_3$. $25(OH)D_3$ is the primary circulating form, around 85% of which is bound to Vitamin D Binding Protein (VDBP), the balance of which is bound to albumin (~15%). Vitamin D acts primarily through the nuclear Vitamin D receptor (VDR), which leads to signaling that regulates gene transcription [78, 106].

Generally, Vitamin D levels in the blood are measured via $25(OH)D_3$ assays, because $25(OH)D_3$ is found at much higher levels in the blood and is generally therefore easier to measure. Further, clinical observations suggest that patients with Vitamin D deficiency can still have normal measurements for $1\alpha 25(OH)_2D_3$ [78, 107]. Consensus on what constitutes a 'normal' Vitamin D level remains elusive; different levels have been measured for optimal calcium absorption (34 ng/mL) [108], optimal neuromuscular performance (38 ng/mL) [109], and reduction of breast cancer incidence (52 ng/mL) [110]. All told, recommendations for adequacy range from the low 30s to 40 ng/mL [111]. If we take deficiency to be serum levels of $25(OH)D_3$ below 20 ng/mL,

evidence suggests that between 30 and 50% of the global population is at risk of Vitamin D deficiency [112].

This widespread deficiency is almost certainly of clinical importance. Most cell types express the vitamin D receptor and Vitamin D is involved in the transcriptional regulation of at least 229 genes [78, 113]. Vitamin D is known to be important in autophagy and the promotion of antimicrobial response [78, 114, 115]. The Vitamin D receptor is found on antigen presenting cells and activated T-cells [116, 117] and the active form, $1\alpha 25(\text{OH})_2\text{D}_3$, is secreted by macrophages and dendritic cells.

$1\alpha 25(\text{OH})_2\text{D}_3$ appears to promote a tolerogenic response, downregulating expression of MHC class II and Th1 cytokine interleukin-12 while inducing Foxp3 expression [116, 118-120]. Vitamin D also appears to act as a necessary component of Phospholipase C- γ 1 induction, modulating TCR signaling and T cell activation, revealing a complex role for Vitamin D in both tolerance and TCR sensitivity [121].

With respect to type 1 diabetes in particular, multiple steps in the Vitamin D pathway are implicated in multiple steps of the etiological pathway. A promoter polymorphism in CYB27B1, which performs the final hydroxylation step to convert Vitamin D to its active form, are associated with type 1 diabetes and other autoimmune conditions [122]. Vitamin D deficient NOD mice demonstrate impaired glucose tolerance followed by a doubling of diabetes incidence [123]. Cohort studies in the UK and in the US military have demonstrated association between Vitamin D deficiency and type 1 diabetes [78, 124]. Studies in Florida have failed to recapitulate these results, possibly due to a masking of the effect by low levels of Vitamin D in both patient and control populations [125]. VDR has been shown to bind intronically to PTPN2, a known

type 1 diabetes susceptibility region, and data suggest an inductive effect [113].

Studies have found association between polymorphisms for the gene encoding the principal transporter of Vitamin D, VDBP, and type 1 diabetes [126, 127].

Summary

Current approaches for type 1 diabetes screening include HLA genotyping and autoantibody screening [29]. For HLA genotyping, an HLA DR3/4 individual with an affected sibling has a 55% chance of becoming concordant for type 1 diabetes by age 12, and an 85% risk of islet autoimmunity by age 15 [29, 128]. For autoantibody screening, the likelihood of progression to disease scales with the number of autoantibodies detected, with three or four islet autoantibody markers (GAD, IA-2A, IAA, and IA-2B) associating with a risk of >60% over 5 years. Specifically, IAA plus one additional marker associating with a risk of >50%, ICA plus one additional marker associating with a risk of ~50%, any two associating with a risk of >25%, with single markers alone ranging from 3 to 9% risk [129, 130]. Combining knowledge of family history with HLA genotype allows up to 1000-fold risk stratification. Some have considered combining HLA-genotype with islet autoantibody status for risk stratification, but HLA genotype partially determines autoantibody risk and so these are not independent measures [50, 131].

While these approaches afford some means of prediction and screening, they are not perfect [12-14]. Researchers have observed that high risk HLA haplotypes are decreasing in their representation among new onset cases, suggesting that the interplay between genotype and environment in autoimmune diabetes is complex and dynamic [132]. Thus, there exists a need for additional reliable biomarkers of disease progression and treatment efficacy that may also serve as novel targets for therapeutic

intervention or tools for the personalization of treatment [15, 29]. In terms of pure genetic/immunological pathways of interest, the IL-2 pathway serves as a remarkable candidate pathway, owing to its multiple representation in association studies for type 1 diabetes and the high degree of biological relevance of the pathway to autoimmune disease [29, 49, 63]. With regard to the relationship between disease state and environment, a compelling case exists for a more detailed understanding of the role of Vitamin D in autoimmune disease in general and type 1 diabetes in specific. With these foci in mind, we sought to identify and characterize biomarkers for type 1 diabetes.

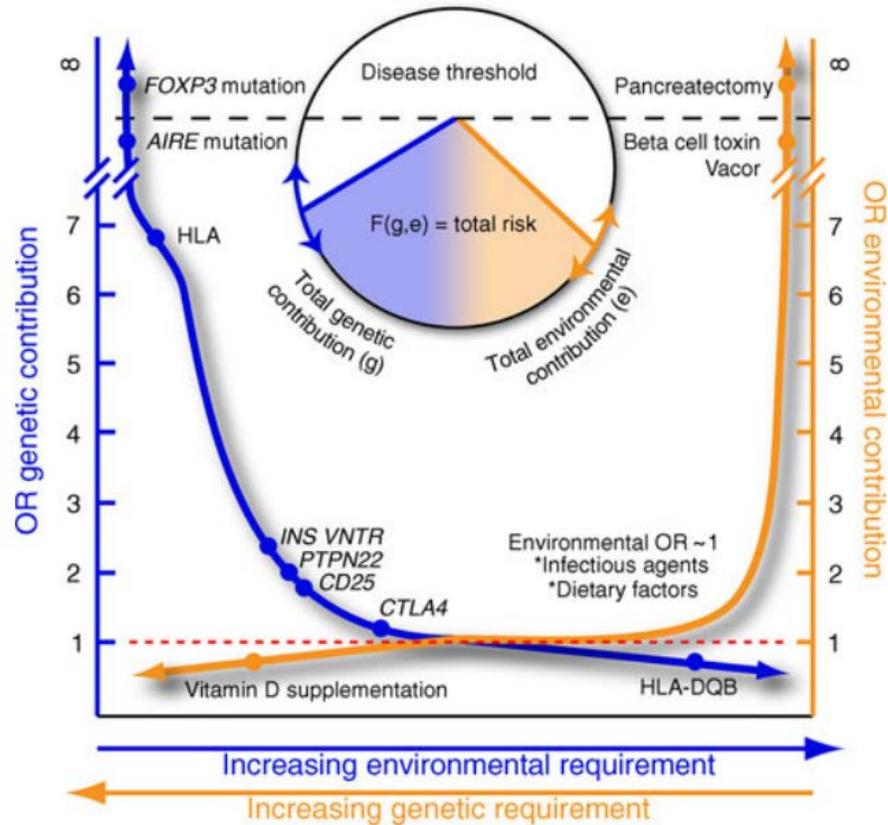


Figure 1-1. The Threshold Hypothesis. T1D likely initiates as a function of the contributions of genetic and environmental risk factors with varying odds ratios. VNTR, variable number of tandem repeats.

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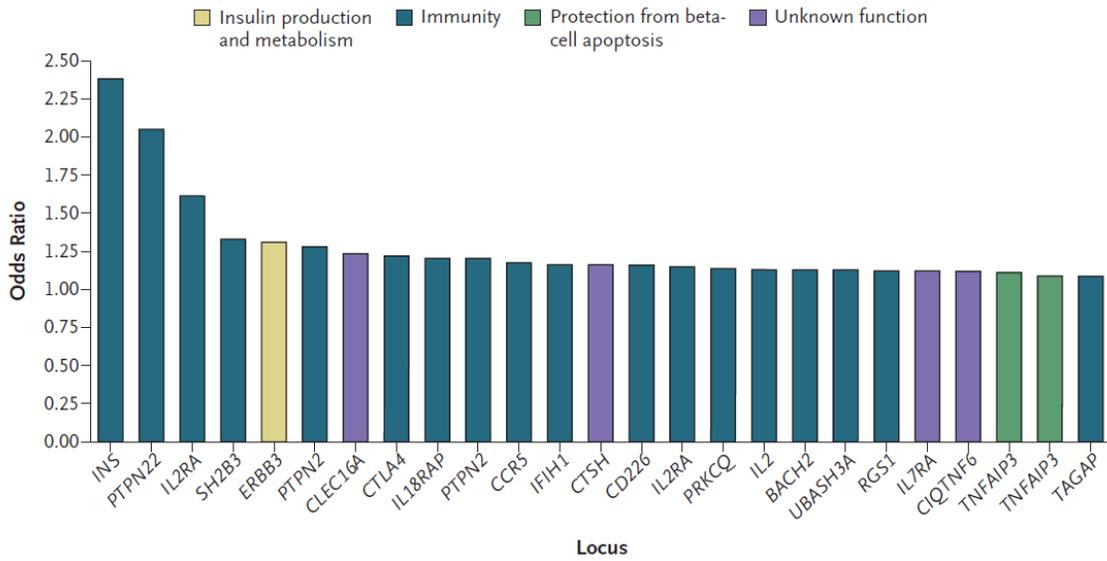


Figure 1-2. Non-HLA associated loci in type 1 diabetes.

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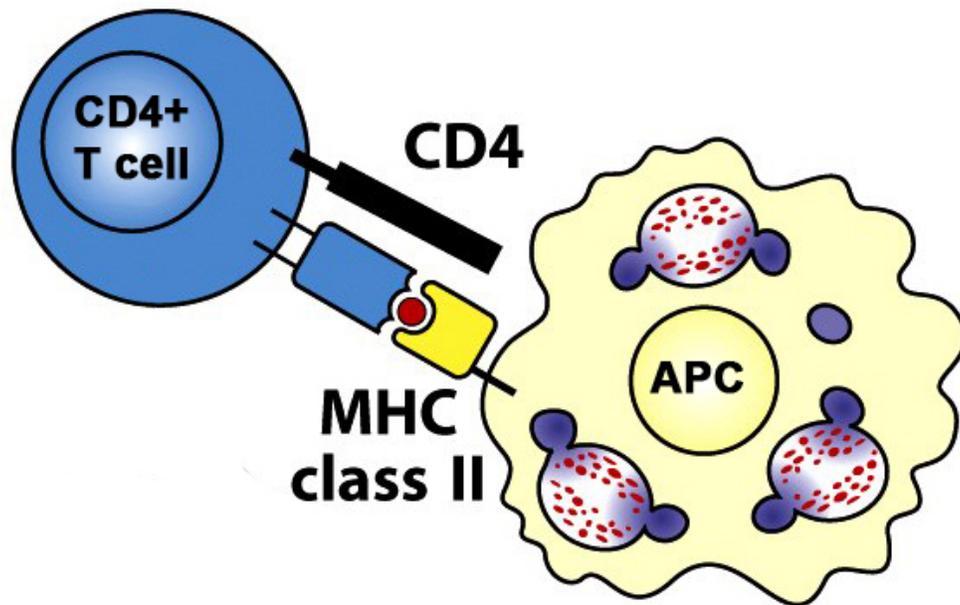


Figure 1-3. CD4+ T-cells recognize antigen in the context of MHC Class II.

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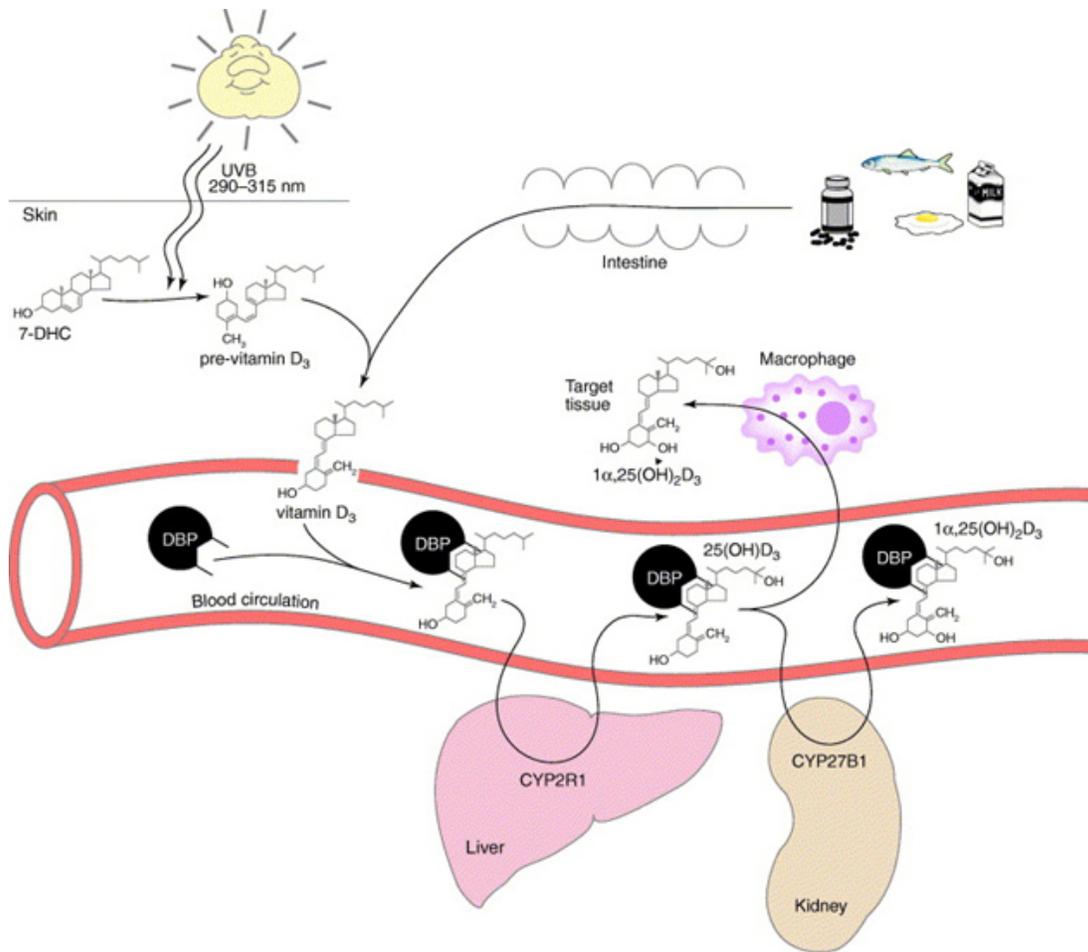


Figure 1-4. The Vitamin D/VDBP system. Vitamin D₃ is synthesized in the skin or absorbed through the intestine, then converted by CYP2R1 in the liver into 25(OH)D₃, and converted by CYP27B1 in the kidneys or in macrophages into its final active form, 1α,25(OH)₂D₃.

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CHAPTER 2
ASSOCIATION BETWEEN VITAMIN D BINDING PROTEIN GENOTYPE AND LEVELS
AND DISEASE STATE IN TYPE 1 DIABETES

Introduction

Various pathways and characteristics of vitamin D metabolism, such as vitamin D analogues and polymorphisms in the vitamin D receptor, as well as genes encoding specific vitamin D enzymes, have recently been associated with type 1 diabetes [133-135]. Reduced serum vitamin D concentrations have been associated with up to 3.5 times greater risk of progression to type 1 diabetes [78, 124, 134, 136], but vitamin D deficiency is not uncommon [125, 137]. Vitamin D therapy (active form) can modulate the development of disease in the nonobese diabetic mouse model of type 1 diabetes [138, 139], and a variety of trials have tested whether vitamin D supplementation has the capacity to modify the development of this disease [134]. To that question, a meta-analysis of trials seeking such a therapeutic benefit suggests that vitamin D supplementation can reduce disease risk [140, 141]. Finally, Vitamin D may be involved in promoting an anti-inflammatory phenotype through upregulation of FOXP3, a key signaling factor in the development of regulatory T-cells, defects in which have been shown in recent years to have a crucial role in the etiology and progression of type 1 diabetes [63, 120] .

Reprinted from Diabetes, 60, Blanton et al., 'Reduced Serum Vitamin D-Binding Protein Levels Are Associated With Type 1 Diabetes, pp. 2566-2570, copyright 2011, with kind permission from the American Diabetes Association.

This said, despite our current understanding of the vitamin D pathway, including its capacity to modulate the immune system [137], the causal relationship between impaired vitamin D constituents and the development of type 1 diabetes remains uncertain. This is largely due to the intricate nature of vitamin D metabolic processes, as well as the extensive biological effects exhibited by its components. Therefore, understanding the influence of the vitamin D pathway on the pathogenesis of type 1 diabetes requires a systematic examination into the distinct roles of its various components.

One essential component of the vitamin D pathway is the polymorphic vitamin D binding protein (VDBP), also known as group specific component (Gc). Aside from its main function of vitamin D transport and preservation, VDBP has been shown to scavenge actin, bind fatty acids, activate macrophages, stimulate osteoclasts, enhance chemotactic activity of C5-derived peptides, and associate with immune cell surfaces, such as T and B cells [142]. Even after ligand binding, 98-99% of VDBP binding sites remain unoccupied, which suggests a function beyond vitamin D transport [142]. While several studies have associated specific *VDBP* gene polymorphisms with the presence of diabetes (i.e., type 1 diabetes and type 2 diabetes) [126, 127], we sought to confirm this association and identify differences in VDBP levels in patients with type 1 diabetes.

Methods

Participants

Banked serum samples from a total of 472 individuals in Florida who participated in studies on the natural history of type 1 diabetes were grouped into the following cohorts: controls (n=153, median age 21.0 years, range 5.0-56.0 years, 85 female), patients with type 1 diabetes (n=203, median age 14.5, range 4.0-62.6; 96 female), and

first-degree relatives of those with type 1 diabetes (n=116, median age 21.0, range 1.0-62.6, 56 female). Of this study group, DNA from 53 controls, 81 patients, and 38 relatives were further analyzed by SNP genotyping for *VDBP* polymorphisms (SNPs rs4588 and rs7041). Previously banked DNA samples from a second study cohort consisting of 1,502 patients and 1,880 healthy controls collected at the Georgia Health Sciences University, obtained from a national population from the U.S., were also genotyped. All samples were collected under informed consent with the approval of the University of Florida and Georgia Health Sciences University's Institutional Review Boards.

VDBP levels

VDBP levels were quantified in duplicate with a commercial EIA kit (ALPCO; Salem, NH) using 10 μ L of banked serum from each subject. Levels of VDBP were interpolated from a standard curve after reading the absorbance on a M5 Spectramax plate reader using Softmax Pro 4.8 software (Molecular Devices, Sunnyvale, CA). The intra- and inter-assay coefficients of variation for this assay were 5.0% and 12.7%, respectively. The published normal reference range for VDBP levels is 300-600 μ g/mL [142]. Correlation analysis was also performed for a subset of the VDBP serum samples (n=386), which had previously been measured for 25-OH vitamin D levels [125]. This subset included 152 controls, 141 type 1 diabetes patients, and 93 first-degree relatives.

SNP Genotyping

For the initial University of Florida data set, 200ng of genomic DNA was used to amplify an 809 bp fragment with primers 5'-CAAGTCTTATCACCATCCTG-3' and 5'-GCCAAGTTACAATAACAC-3' as previously published [126]. The amplicons were gel

purified using GENE CLEAN Turbo kit (MP Biomedicals; Aurora, OH) to ensure no inhibition of restriction enzymes. For verification of the rs4588 and the rs7041 SNPs, PCR products were digested with *StyI* and *HaeIII* respectively and the genotypes were determined by gel electrophoresis on a 1.5-2% agarose gel. For the data set collected under the auspices of the University of Georgia, the SNPs were genotyped using TaqMan PCR genotyping, with modifications, as previously described [143]. All primers and probes used in this study were designed and validated by Applied Biosystems (Foster City, CA). Amplification reactions were performed in a 5 μ L final volume in optical 384-well plates. PCR was carried out with 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C using an ABI9700 Real-Time PCR system (Applied Biosystems). To validate the TaqMan assays, five SNPs were also genotyped using standard amplified restriction fragment length polymorphism (ARFLP) analysis.

Statistical Methods

Analysis of multiple, unpaired group comparisons was achieved using the non-parametric Kruskal-Wallis test. Dunn's post-test was used for multiple testing corrections if the Kruskal-Wallis test was significant. The association of age and disease duration with VDBP levels was analyzed by linear regression. To determine the relationship between VDBP levels and gender, the non-parametric Mann-Whitney test was used. All analyses were performed using GraphPad Prism software ver 5.00 (Software Inc.; San Diego, CA). The association between each *VDBP* SNP and type 1 diabetes was assessed by calculating the odds ratios (OR) separately for each genotype, as well as for total allelic frequency (i.e., heterozygous and homozygous minor allele combined). The Pearson's chi-square (χ^2) and Fisher's exact tests were

used to test the differences in genotype and allele (respectively) distribution between patients and control subjects. Statistical significance was defined as $p < 0.05$. We used the Breslow-Day test to examine heterogeneity in the ORs between subsets stratified for age at onset, sex, and HLA risk status. Hardy–Weinberg equilibrium (HWE) of the genotypic frequencies among cases and controls was examined separately.

Results

Levels of Serum VDBP Associate with Type 1 Diabetes

Serum VDBP levels (median, range, interquartile range [IQR]) for the three study groups were as follows: healthy controls (423.5 $\mu\text{g/mL}$; 193.5-4345.0; 354.1-586.7), first-degree relatives (402.9 $\mu\text{g/mL}$; 204.7-4850.0; 329.6-492.4), and type 1 diabetes patients (385.3 $\mu\text{g/mL}$; 99.3-1305.0; 328.3-473.0) (Fig. 2-1). Median VDBP serum levels were significantly lower in patients with type 1 diabetes than controls ($p=0.003$), type 1 diabetes patients. Due to a lack of availability, serum VDBP levels were not measured for the Georgia samples.

In finding VDBP levels were significantly lower in the presence of type 1 diabetes, we then questioned whether the duration of disease influenced VDBP levels. Linear regression analysis indicated that disease duration did not associate with VDBP levels ($p=0.516$, $r^2 = 0.003$).

Serum VDBP Levels Associate with Gender, but Not Age or Serum 25-OH Vitamin D Levels

It has previously been shown that gender influences VDBP levels [144]. Therefore, we performed gender analysis with regard to VDBP levels to identify any correlations within our study participants (Fig. 2-2). The 472 study participants distributed into two near identical gender based cohorts of 238 females and 233 males.

Serum VDBP levels were significantly higher in females (433.4 $\mu\text{g}/\text{mL}$; 99.32-4850.0; 359.4-567.8) versus males (374.7 $\mu\text{g}/\text{mL}$; 188.9-1602.0; 326.9-449.9; $p < 0.0001$).

Gender distribution between type 1 diabetes patients, relatives, and controls was not significantly different ($p = \text{ns}$), reducing the likelihood that group composition contributed to the aforementioned association between type 1 diabetes and serum VDBP levels.

We next sought to determine whether age influenced serum levels of VDBP. Linear regression analysis revealed that age did not associate with VDBP levels in our study population ($p = 0.164$, $r^2 = 0.004$) (Fig. 2-3).

We previously noted that individuals with type 1 diabetes had insufficient serum levels of 25-OH vitamin D, though this finding was not specific for those with the disease [125]. To determine whether a correlation exists between serum 25-OH vitamin D and VDBP levels, we analyzed VDBP levels on banked serum from a large subset ($n = 386$) of the 25-OH vitamin D cohort (Fig. 2-4). Interestingly, we found no association ($p = 0.557$, $r^2 = 0.001$) between these two analytes, irrespective of study group (152 controls, 141 patients, 93 relatives).

VDBP Genotypes Do Not Associate with Type 1 Diabetes.

The rs4588 and rs7041 *VDBP* genetic variants were analyzed in the type 1 diabetes patients and controls whose DNA had been banked at the Georgia Health Sciences University for studies regarding the genetics of this disease [145]. Cases and controls were found to be in Hardy-Weinberg equilibrium for the two SNPs analyzed (Table 2-1). There were no significant differences in the allele and genotype frequencies of the *VDBP* rs4588 and rs7041 genetic variants among patients with type 1 diabetes and controls (Table 2-2).

We next tested whether the association between these SNPs is dependent upon other covariates, such as gender of the subjects, age of disease onset (i.e., early onset being under 18 years of age), or HLA-DQB1 genotypes (Table 2-3). No significant association was detected when patients were stratified by gender or age of disease onset. A heterogeneity test further showed no difference in odds ratios between males and females or between early and late onset subsets (Table 2-4).

We then determined whether the high-risk HLA-DQB1 genotype was associated with the SNPs. All subjects were classified into two subsets, a high-risk DQB1 genotype subset (i.e., 0201/0201, 0302/0302 or 0201/0302) and a low-risk DQB1 genotype subset (i.e., all others). There was no significant association of the rs4588 and rs7041 SNPs in either the low or high-risk HLA-DQB1 subsets after correction for multiple testing (Table 2-3). Since the p-values for these tests are heavily influenced by the smaller number of control subjects carrying the high-risk HLA genotypes, we performed heterogeneity tests to determine whether the odds ratios differed between the high and low-risk subsets. We found no significant difference between the odds ratios of the HLA-DQB1 risk subsets (Table 2-4).

Finally, we investigated the possibility of an association between genotype at rs4588 and rs7041 and levels of serum VDBP. No such association was observed (Fig. 2-5).

Discussion

The major finding in our study adds further credence to the concept that the vitamin D pathway may play a significant role in the development of type 1 diabetes. While previous studies have associated the rs4588 and rs7041 *VDBP* SNPs with the disease [126, 127], we were unable to support that conclusion in this large-scale study,

and we were unable to associate levels of VDBP with genotype at either of these loci. However, we were able to associate the phenotype of lower VDBP levels with type 1 diabetes. While the exact consequence of this association remains unknown, the fact that VDBP has immunomodulatory characteristics and is responsible for transport of vitamin D metabolites supports, in theory, a model whereby the impact of reduced serum levels may be significant enough to lend itself, directly or indirectly, to the autoimmune destruction of pancreatic β cells in the disease. Our findings also suggest another means by which VDBP levels are influenced, beyond the *VDBP* SNPs tested here, must exist, and will certainly be subject to future investigations.

A recently published study assessing VDBP levels in 100 healthy, middle-aged and older participants found that women had higher mean VDBP levels than men, yet no associations were observed between VDBP levels and age, body weight, BMI, fat mass, or fat percentage [144]. While the aforementioned study measured VDBP levels among individuals greater than middle-age, the majority of our samples derived from individuals much younger than middle-age; however, we were able to confirm gender differences in the younger study cohort.

Despite our having noted this interesting correlation, additional studies are required to address its biological significance. For example, it could be argued that the presence of reduced VDBP levels in the type 1 diabetes study group may not have a biologically significant effect on the transport of vitamin D metabolites (i.e., reduced transport), since VDBP circulates at a much higher concentration than its ligands [146]. Additionally, as mentioned previously, in a prior study, we found that 25-OH vitamin D levels did not specifically associate with type 1 diabetes, but were low in similar

proportion among those with and without the disease [125]. In the current investigation, we did not find a significant correlation between those previously measured 25-OH vitamin D levels and serum VDBP in the same patients. This we interpret to imply that lower VDBP levels in type 1 diabetes are independent of 25-OH vitamin D, which is one of its ligands.

While our study is novel in its simultaneous assessment of 25-OH vitamin D, VDBP, and genetic polymorphisms, we would note a very recently published study where type 1 diabetes patients were noted to have exaggerated urinary loss of VDBP [147]. However, the study did not find a significant difference in plasma VDBP levels, which may, in part, be due to the smaller cohort size. Though we did not measure urine levels of VDBP in our study, it is possible that exaggerated VDBP urinary loss may be a factor contributing to lower serum VDBP in type 1 diabetes patients.

Overall, our findings warrant further investigation into the role of VDBP, as well as the contribution of other vitamin D pathway components, in type 1 diabetes. The various contributions of this pathway to innate immune function are the topic of much discussion and may be multifaceted, with each individuals' risk being the sum of different pathways to disease; hence, the reason for the historical difficulty in describing a specific mechanisms for involvement of vitamin D in the pathogenesis of type 1 diabetes.

Table 2-1. Hardy-Weinberg equilibrium of patients and controls from Georgia samples

Gene	SNP	Allele (Major>Minor)	HWE Cases	HWE Controls
VDBP	rs4588	C>A	0.2897	0.6740
VDBP	rs7041	G>T	0.8386	0.4072

Table 2-2. Association analysis of VDBP SNPs rs7041 (G>T) and rs4588 (C>A) with type 1 diabetes.

SNP	Genotype	Type	1	Odds Ratio	<i>P</i>
		Diabetic Patients	Controls	(95% CI)	
rs7041	GG	441 (30.33%)	579 (31.67%)	1.00 (reference)	
	GT	723 (49.72%)	884 (48.36%)	1.07 (0.92-1.26)	0.3774
	TT	290 (19.94%)	365 (19.97%)	1.04 (0.86-1.27)	0.6755
	GT + TT	1013 (69.67%)	1249 (68.33%)	1.07 (0.92-1.24)	0.4086
rs4588	CC	723 (50.45%)	929 (51.58%)	1.00 (reference)	
	CA	578 (40.33%)	734 (40.76%)	1.01 (0.87-1.17)	0.8745
	AA	132 (9.21%)	138 (7.66%)	1.23 (0.95-1.59)	0.1163
	CA + AA	710 (49.55%)	872 (48.42%)	1.05 (0.91-1.20)	0.5235

Table 2-3. Association analysis of *VDBP* SNPs rs7041 (G>T) and rs4588 (C>A) after stratification for sex, onset of type 1 diabetes, and HLA risk .

SNP	Subset	Genotype	Type 1 Diabetic		Odds Ratio (95% CI)	<i>P</i>
			Patients	Controls		
rs7041	Early Onset	GG	285 (30.94%)	579 (31.67%)	1.00 (reference)	
		GT	474 (51.47%)	884 (48.36%)	1.09 (0.91-1.31)	0.3527
		TT	162 (17.59%)	365 (19.97%)	0.90 (0.71-1.14)	0.3842
		GT + TT	636 (69.06%)	1249 (68.33%)	1.03 (0.87-1.23)	0.6974
	Late Onset	GG	152 (29.23%)	579 (31.67%)	1.00 (reference)	
		GT	245 (47.12%)	884 (48.36%)	1.06 (0.84-1.33)	0.6409
		TT	123 (23.65%)	365 (19.97%)	1.28 (0.98-1.68)	0.0710
		GT + TT	368 (70.77%)	1249 (68.33%)	1.12 (0.91-1.39)	0.2884
	Male	GG	212 (30.55%)	302 (31.10%)	1.00 (reference)	
		GT	343 (49.42%)	483 (49.74%)	1.01 (0.81-1.27)	0.9193
		TT	139 (20.03%)	186 (19.16%)	1.07 (0.80-1.41)	0.6629
		GT + TT	482 (69.45%)	669 (68.90%)	1.03 (0.83-1.27)	0.8092
	Female	GG	229 (30.13%)	277 (32.32%)	1.00 (reference)	
		GT	380 (50.00%)	401 (46.79%)	1.15 (0.92-1.44)	0.2329
		TT	151 (19.87%)	179 (20.89%)	1.02 (0.77-1.35)	0.8870
		GT + TT	531 (69.87%)	580 (67.68%)	1.11 (0.90-1.37)	0.3431
	High Risk HLA	GG	244 (33.15%)	58 (29.44%)	1.00 (reference)	
		GT	346 (47.01%)	99 (50.25%)	0.83 (0.58-1.20)	0.3166
		TT	146 (19.84%)	40 (20.30%)	0.87 (0.55-1.36)	0.5379
		GT + TT	492 (66.85%)	139 (70.56%)	0.84 (0.60-1.19)	0.3228
Low Risk HLA	GG	195 (27.50%)	520 (31.90%)	1.00 (reference)		
	GT	373 (52.61%)	785 (48.16%)	1.27 (1.03-1.56)	0.0239	
	TT	141 (19.89%)	325 (19.94%)	1.16 (0.89-1.50)	0.2665	
	GT + TT	514 (72.50%)	1110 (68.10%)	1.24 (1.02-1.50)	0.0338	
rs4588	Early Onset	CC	472 (52.10%)	929 (51.58%)	1.00 (reference)	
		CA	350 (38.63%)	734 (40.76%)	0.94 (0.79-1.11)	0.4612

Table 2-3. Continued.

SNP	Subset	Genotype	Type 1 Diabetic Patients	Controls	Odds Ratio (95% CI)	<i>P</i>
		AA	84 (9.27%)	138 (7.66%)	1.20 (0.89-1.61)	0.2263
		CA + AA	434 (47.90%)	872 (48.42%)	0.98 (0.83-1.15)	0.8004
	Late Onset	CC	244 (47.38%)	929 (51.58%)	1.00 (reference)	
		CA	225 (43.69%)	734 (40.76%)	1.17 (0.95-1.43)	0.1401
		AA	46 (8.93%)	138 (7.66%)	1.27 (0.88-1.82)	0.1964
	Male	CA + AA	271 (52.62%)	872 (48.42%)	1.18 (0.97-1.44)	0.0924
		CC	340 (49.78%)	487 (50.78%)	1.00 (reference)	
		CA	281 (41.14%)	401 (41.81%)	1.00 (0.82-1.23)	0.9718
		AA	62 (9.08%)	71 (7.40%)	1.25 (0.87-1.81)	0.2324
	Female	CA + AA	343 (50.22%)	472 (49.22%)	1.04 (0.86-1.27)	0.6891
		CC	383 (51.07%)	442 (52.49%)	1.00 (reference)	
		CA	297 (39.60%)	333 (39.55%)	1.03 (0.84-1.27)	0.7855
		AA	70 (9.33%)	67 (7.96%)	1.21 (0.84-1.73)	0.3105
	High Risk HLA	CA + AA	367 (48.93%)	400 (47.51%)	1.06 (0.87-1.29)	0.5694
		CC	381 (52.62%)	98 (50.26%)	1.00 (reference)	
		CA	272 (37.57%)	80 (41.03%)	0.87 (0.62-1.22)	0.4310
		AA	71 (9.81%)	17 (8.72%)	1.07 (0.61-1.91)	0.8067
	Low Risk HLA	CA + AA	343 (47.38%)	97 (49.74%)	0.91 (0.66-1.25)	0.5569
		CC	337 (48.01%)	829 (51.68%)	1.00 (reference)	
		CA	304 (43.30%)	654 (40.77%)	1.14 (0.95-1.38)	0.1573
		AA	61 (8.69%)	121 (7.54%)	1.24 (0.89-1.73)	0.2044
		CA + AA	365 (52.00%)	775 (48.32%)	1.16 (0.97-1.38)	0.1041

Table 2-4. Heterogeneity tests to determine whether the odds ratios for homozygous genotypes significantly differ between stratified groups.

SNP	Subset	Genotype Analyzed	Odds Ratio (95% CI)	Heterogeneity
rs7041	Early Onset	GG > TT	0.90 (0.71-1.14)	
	Late Onset	GG > TT	1.28 (0.98-1.68)	0.0529
	Male	GG > TT	1.07 (0.80-1.41)	
	Female	GG > TT	1.02 (0.77-1.35)	0.8338
	High Risk HLA	GG > TT	0.87 (0.55-1.36)	
	Low Risk	GG > TT	1.16 (0.89-1.50)	0.2778
rs4588	Early Onset	CC > AA	1.20 (0.89-1.61)	
	Late Onset	CC > AA	1.27 (0.88-1.82)	0.8084
	Male	CC > AA	1.25 (0.87-1.81)	
	Female	CC > AA	1.21 (0.84-1.73)	0.8891
	High Risk HLA	CC > AA	1.07 (0.61-1.91)	
	Low Risk	CC > AA	1.24 (0.89-1.73)	0.6713

Odds ratio of patients with type 1 diabetes to controls. Heterogeneity determined by Breslow-Day test.

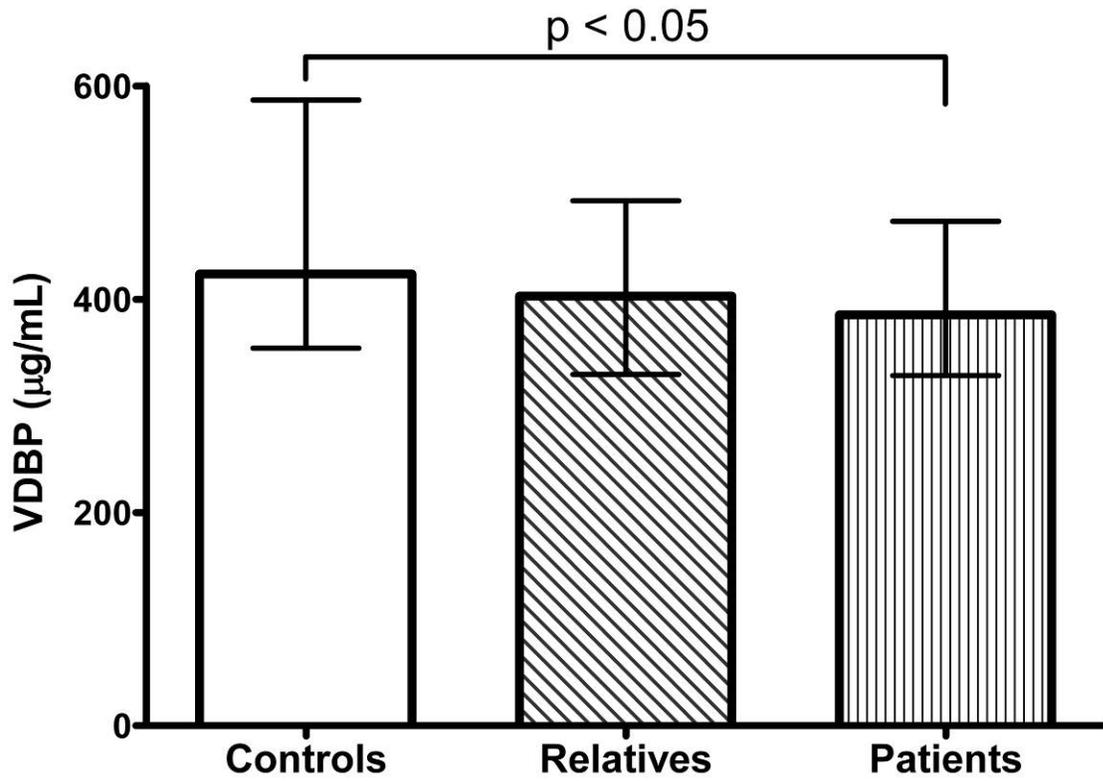


Figure 2-1. Serum levels of vitamin D binding protein. VDBP levels in controls (n=153), first-degree relatives (n=116), and type 1 diabetic patients (n=203). Serum VDBP in males (n=233) and females (n=238), total of all study groups. Median with IQR shown.

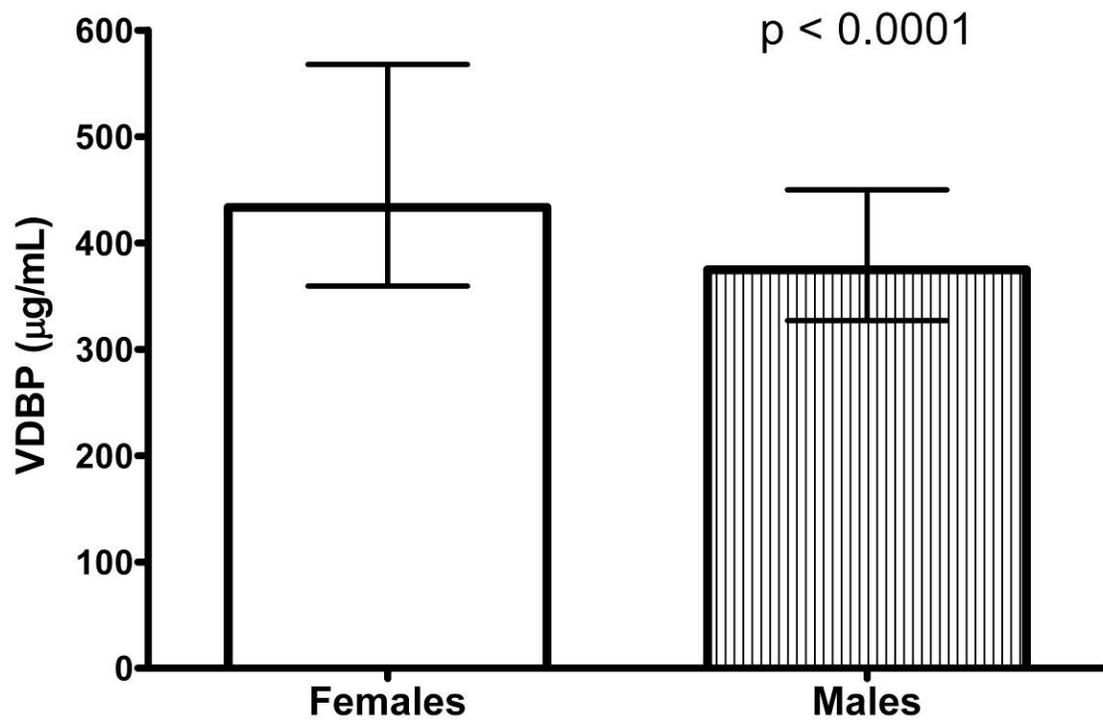


Figure 2-2. Serum levels of vitamin D binding protein. Serum VDBP in males (n=233) and females (n=238), total of all study groups Median with IQR shown.

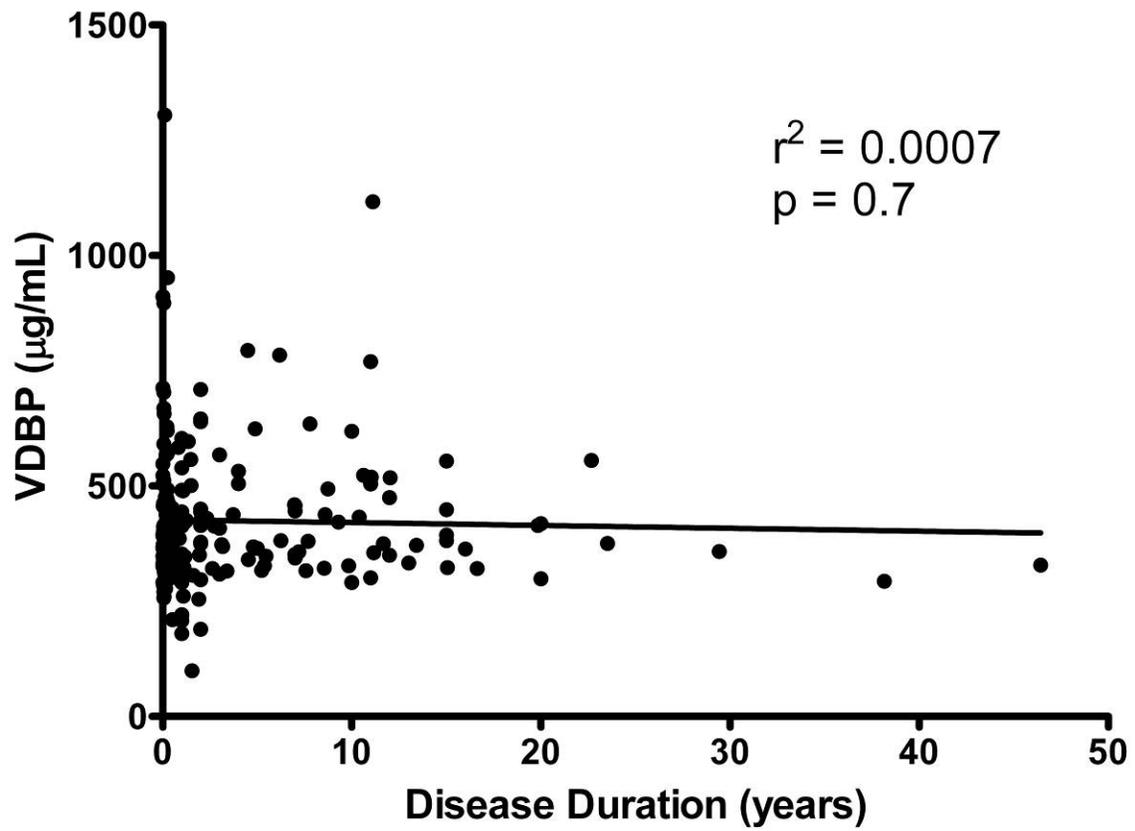


Figure 2-3. No association between VDBP levels and disease duration by linear regression analysis.

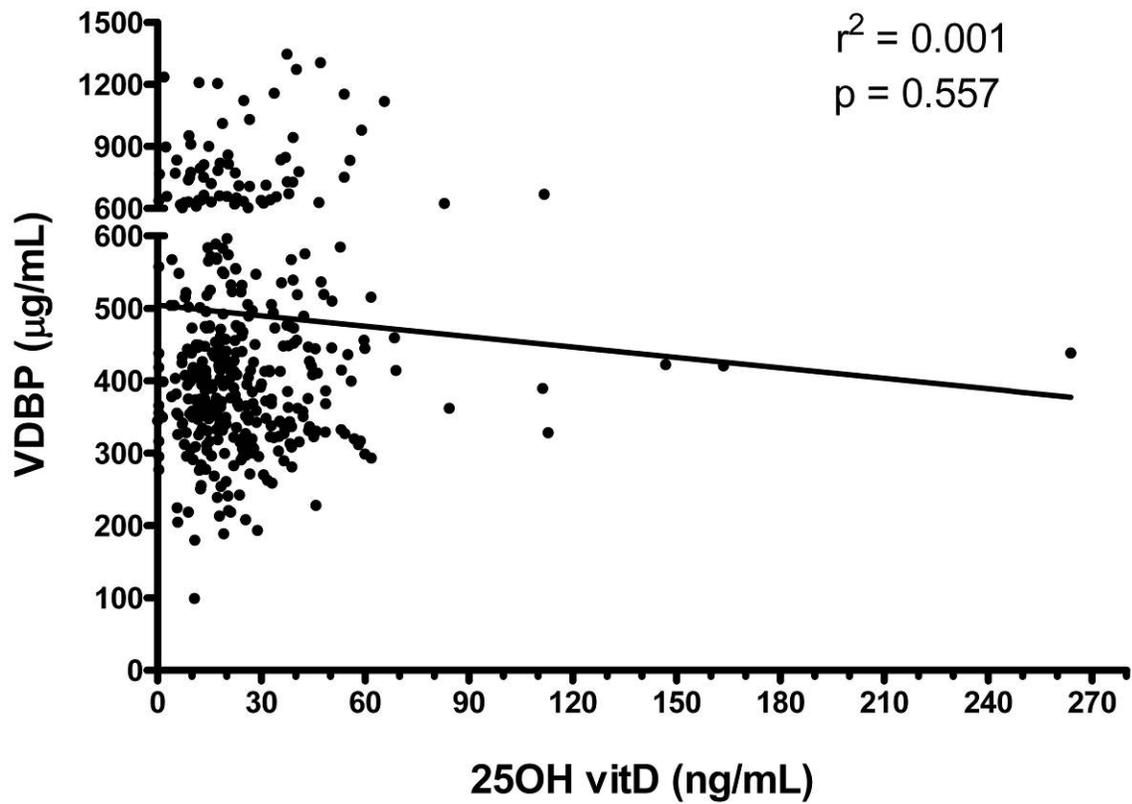


Figure 2-4. Linear regression of serum 25-OH vitamin D levels and vitamin D binding protein levels. There was no significant correlation between these two parameters in the study cohort (n=386).

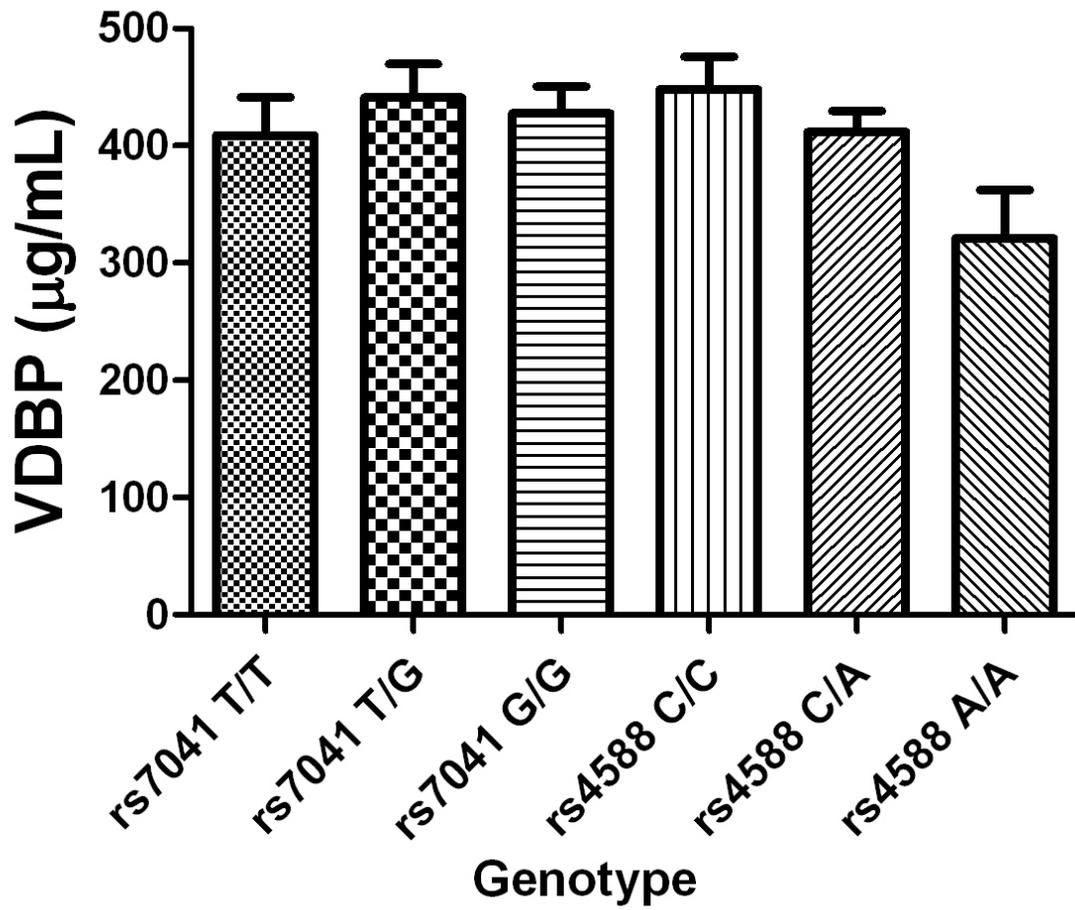


Figure 2-5. No statistically significant association between VDBP levels and genotype at rs7041 and rs4588.

CHAPTER 3
A SNP IN THE PROMOTER REGION OF THE INTERLEUKIN-2 GENE IS
ASSOCIATED WITH MODULATION OF HUMAN SERUM SCD25 LEVELS

Introduction

IL-2 is a key immunoregulatory cytokine. Originally thought to be important to T-cell proliferation, it has since been found to be largely dispensable for that role, but indispensable in its role in maintaining populations of the anti-inflammatory regulatory T cell. Regulatory T cell defects are powerfully implicated in the pathogenesis and progression of type 1 diabetes, and so the IL-2 pathway represents a key piece of the etiological puzzle of autoimmune diabetes.

SNPs in promoter regions can affect gene splicing, binding of transcription factors, abundance of messenger RNA, or the structure of non-coding RNAs [148-152]. These polymorphisms can also have an impact in *trans*, through effects on downstream genes [153]. GWAS and linkage studies have long demonstrated the utility of SNPs as biomarkers of disease risk, possible targets for therapeutic intervention, and targets for the investigation of pathogenesis.

Rs2069762 is a T>G SNP located 330 base pairs upstream of IL-2, in the promoter region [154]. This SNP is located within a region shown to be important for the inducible expression of IL-2, for when deletion of the region from 289 to 361 bp upstream of IL-2 occurs, IL-2 expression is observed to be considerably inhibited [155]. Rs2069762 has already been shown to modulate levels of IL-2 in treated peripheral blood lymphocytes, with a study in Maryland associating the G allele with higher levels of IL-2 in CD3/CD28 72 hour bead stimulated in-vitro assays [152]. Interestingly, this locus is far less polymorphic in African American populations, with a T/G distribution of 94%/6% (n=81) in African Americans and 74%/26% in Caucasians (n=150) [156].

It is important to note that association with disease does not necessarily mean that there are functional implications due to the associated SNP. rs2069762 is in linkage disequilibrium with 55 other SNPs in the region, and is likely in linkage disequilibrium with many other types of polymorphisms that could have functional relevance to disease. Disentangling the connection between these SNPs and disease will be a challenging process, for but now, rs2069762 could serve as a biomarker through its association with particular, disease relevant phenotypes.

We were primarily interested in the effect of the rs2069762 polymorphism in the promoter of IL-2 on levels of IL-2 in stimulated peripheral blood mononuclear cell populations, and of the effect of polymorphisms in the IL-2 promoter on levels of downstream, immunologically relevant factors such as soluble CD25 (sCD25).

CD25, also known as IL2 α , is the high affinity subunit of the IL-2 receptor, encoded by a gene located on chromosome 10 in humans. It is transiently expressed on many cell types, including T-effector cells, B cells, dendritic cells, natural killer cells, and eosinophils, but is only constitutively expressed on regulatory T-cells [63-66]. IL2 α mutation or knockout can lead to severe autoimmunity, suggesting an important role for the high affinity IL-2 receptor component in immune regulation [67, 68].

Interestingly, CD25 does not exist exclusively as a membrane bound receptor component. Instead, it can be cleaved (releasing the extracellular domain and reducing the protein in size from 55 KDa to 45 Kda) and can take a soluble form (sCD25) that may have important immune effects [157]. This shedding is associated with activation and proliferation [157-159] and appears to occur in all cells that express CD25 [158, 160, 161]. In vitro studies suggest that the primary source of sCD25 is activated

effector T-cells, as these cells are far more likely than regulatory T-cells to shed sCD25 into the culture medium after activation [159].

Hypotheses for the role of sCD25 are many and varied. It has been proposed that the cleaved sCD25 is simply a byproduct of a cleavage process regulating the amount of membrane bound CD25. However, it is known that sCD25 can bind the IL-2 ligand, albeit with affinity orders of magnitude lower than the complete receptor [161, 162]. Given this, functions could include presentation of IL-2 to the IL-2 receptor in trans, the 'soaking up' of free IL-2 to deprive effector T-cells or regulator T-cells of the cytokine, or the preservation of IL-2 bioavailability through keeping it free from forming complexes with α 2-macroglobulin [162, 163].

Serum sCD25 levels range between 1 and 2 ng/mL in healthy individuals [164]. High sCD25 expression appears to be associated with a number of pathologies, including infection, leukemia and lymphomas, rheumatoid arthritis, type 1 diabetes, celiac disease, multiple sclerosis, Graves' disease, and Sjogren's syndrome. In many cases, sCD25 levels correlated positively with disease activity [161, 165-169]. In the case of celiac, sCD25 level elevation reliably accompanied gluten challenge and remitted in the absence of gluten, making sCD25 a noteworthy potential marker of immune activation [169].

Studies of the effects of sCD25 on the immune process reveal complex results, and its role remains a source of controversy. In spite of demonstrating higher levels of in vivo sCD25 secretion as compared to controls, PHA stimulated PBMCs from patients with type 1 diabetes were observed to produce lower levels of sCD25 than those of healthy controls in vitro [161, 166, 170]. sCD25 has been found in some studies to

inhibit IL-2 driven STAT5 signaling in a fashion similar to anti-IL-2 mAb, while at the same time promoting T-cell activation and expansion [171]. Other studies have found Stat5 signaling (and Foxp3 expression) to be enhanced by sCD25 in the presence of IL-2 [172]. sCD25 appears to enhance the suppressive effects of regulatory T cells in a dose dependent fashion at high doses, possibly by decreasing CD25 receptor expression, but may have no effect when administered alone or at physiologic doses [158, 159, 173]. At lower doses that do not affect regulatory T cell suppression, sCD25 alone may still be able to decrease proliferation in effector T cells, though these findings conflict with earlier, contrary reports [159, 171]. Results of in vitro studies using sCD25 have been found to be highly context dependent, with measures of proliferation, sCD25 expression, and IL-2 signaling activity changing depending upon the timing of measurement, what culture medium is used, whether IL-2 is present, and whether the medium is serum free or contains serum [159, 172]. Extensive biological variation in sCD25 levels is undoubtedly also present, as sCD25 levels have been observed to vary according to circadian rhythms and could also vary according to other factors such as blood glucose levels [174].

Brusko et al. recently hypothesized that the role of sCD25 may be to interrupt the negative feedback loops by which the IL-2 pathway downregulates inflammatory activity (through promotion of regulatory T cell activity and sensitization of effector T cells to AICD) [159]. How sCD25 carries out this role may be dependent upon the immunochemical milieu in which sCD25 is being expressed. Further study is needed to fully understand the role of sCD25 in immune response [159].

Given that IL-2 itself is a key regulator of immune response, and given that IL-2 expression has been shown to influence sCD25 expression, variation in the IL-2 promoter region could play a crucial role in multiple facets of the immune dysfunction that leads to autoimmune diabetes [175]. Therefore, we hypothesized that levels of IL-2 and soluble CD25 would be different between individuals based upon their genotype at the rs2069762 locus.

Methods

Participants

Human subjects consisted of those with type 1 diabetes, relatives of those with type 1 diabetes, and healthy controls. Peripheral blood was obtained via venipuncture after informed consent and in accordance with approved protocols. Fresh PBMC, DNA and serum were obtained from peripheral blood. DNA and peripheral blood were stored at -20 degrees.

SNP Genotyping

DNA for SNP typing was extracted using the QiaAmp Blood Mini commercial kit (Qiagen; Venlo, Netherlands) according to manufacturer specification and using the Qiacube instrument. SNP genotype for all subjects were determined using a commercial (Life Technologies; Carlsbad CA) Taqman SNP typing kits on a Roche (Penzberg, Germany) Lightcycler 480 instrument, according to manufacturer directions.

Cell Culture Experiments

Freshly isolated human PBMC were plated at 10^6 /mL, 100 microliters per well, in 96 well round bottomed plates. Cells were cultured with 5 ug/mL CD3 and 2.5 ug/mL CD28 or 2.5 ug/mL CD3 and 1.25 ug/mL CD28 or a mitogenic stimulus, PHA at 1 ug/mL

for a period of 24 or 48 hours. Supernatants were then separated by centrifugation and frozen at -20 until analysis by ELISA.

The culture medium was RPMI 1640 (Corning Cellgro, Manassas, VA) with 10% FBS (Hyclone), 50 µg/mL penicillin/streptomycin, 2 mM L-glutamine, 1% sodium pyruvate, 5 mM HEPES, and 50 µg/mL beta-mercaptoethanol. Incubation of cells took place in a humidified incubator, 37 C, 5% CO².

IL-2 Protein Detection by ELISA

IL-2 protein was detected using the eBioscience Ready Set Go IL-2 ELISA according to manufacturer specifications in 96 well flat-bottomed plates. Based on preliminary experiments, standards from 2 ng/mL through 31 µg/mL gave a linear range, and we used this standard curve to measure IL-2 in our test samples. Dilutions took place in RPMI 1640, formulated as specified in 'Cell Culture Experiments', above. Samples were analyzed in duplicate.

sCD25 Protein Detection by ELISA

sCD25 was measured using the BD OptEIA kit (BD Pharmingen) according to the manufacturer's specifications. Any necessary dilutions were performed in PBS with 10% FBS. Measurements were conducted in duplicate.

Statistical Methods

Data analyses were performed in GraphPad Prism (San Diego, CA). One-way ANOVA with Dunnet's correction for multiple testing were performed for all analyses with the exception of the genotype frequency by disease state analysis, which was performed via a Chi-squared test, and age and disease duration correlations, which were calculated using the Spearman's R method.

Results

rs2069762 Genotype Frequencies by Disease State

No significant association was observed between genotype at rs2069762 and disease state (between type 1 diabetes, relatives, and controls) ($X^2 = 2.2$, $df=4$) (Table 3-1). Allele frequencies were 0.337 G and 0.663 T (Table 3-2).

IL-2 Levels in Cell Culture and rs2069762

In order to investigate the potential effect of the IL-2 promoter SNP rs2069762 on IL-2 levels, human PBMCs from a mixed population of type 1 diabetes patients, relatives, and controls were cultured under differential stimulation conditions for either 24 or 48 hours (Figure 3-1).

For samples cultured under anti-CD3/anti-CD28 at 2.5 ug/mL and 1.25 ug/mL for 24 hours (4 GG patients, 22 GT patients, 18 TT patients) (Figure 3-1A), GG patients trended toward much lower expression levels of IL2 (median 23.67 pg/mL) than either TT patients (median 69.62 pg/mL) or GT patients (median 111.5 pg/mL) ($p=ns$). For samples cultured under anti-CD3/anti-CD28 at 2.5 ug/mL and 1.25 ug/mL for 48 hours (1 GG patients, 17 GT patients, 15 TT patients) (Figure 3-1B), a similar trend was observed, with the GG patient again trending toward extremely low levels of IL-2 expression (36.59 pg/mL) as compared to TT patients (45.22 pg/mL) or GT patients (82.37 pg/mL) ($p=ns$).

Under anti-CD3/anti-CD28 at 2.5 ug/mL and 1.25 ug/mL at either 24 or 48 hours, extremely high outliers were observed.

Culturing under anti-CD3/anti-CD28 at 5 ug/mL and 2.5 ug/mL for 24 hours (Figure 3-1C)(4 GG patients, 12 GT patients, 10 TT patients), a similar pattern of expression was observed to that at the lower anti-CD3/anti-CD28 stimulation, with the GG group

(median 22.82 pg/mL) falling substantially below either the GT (69.71 pg/mL) or TT (73.27 pg/mL) groups. Medians were comparable to those at the lower stimulation, but the upper limit of expression was substantially lower at the higher stimulation.

In the case of anti-CD3/anti-CD28 culture at 5 ug/mL and 2.5 ug/mL for 48 hours (3 GG patients, 9 GT patients, 7 TT patients)(Figure 3-1D), the pattern continued, with the GG group (median below limit of detection, mean 12.43 pg/mL) again falling below either the GT (median below limit of detection, mean 40.38 pg/mL) or TT (median 50.82 pg/mL, mean 111.1 pg/mL) groups. We again observed decreased maximum levels of expression as compared to the lower stimulation condition.

We additionally cultured PBMCs in PHA at 1 ug/mL to examine the effect on IL-2 production when stimulation was not processed through the T-cell receptor (Figure 3-1F). At 24 hours (4 GG, 12 GT, 10TT)(Figure 3-1E) and 48 hours (4 GG, 9 GT, 10 TT), IL-2 expression was modest and there were not noticeably different trends between genotypes.

sCD25 Levels in Human Serum and rs2069762

We next investigated whether genotype at rs2069762 correlated with levels of sCD25 in 181 (16 GG, 83 GT, 82 TT) human subject serum samples (Figure 3-2). We found that the presence of a G allele at rs2069762 seemed to correlate with higher levels of sCD25 (GG median = 2213 pg/mL, GT median 2098 pg/mL, TT median 1752 pg/mL). The GT/TT difference was statistically significant with a p-value of 0.0082. Normal range for sCD25 levels are between 1 and 2 µg/mL.

sCD25 Levels by Disease State

We sought to determine whether levels of serum sCD25 in our population correlated with disease state, as has been observed in other studies (Figure 3-3). In

133 subjects (31 controls, 53 relatives, 97 patients with type 1 diabetes), we found statistically significant differences ($p < 0.0001$) between controls (median = 1384 pg/mL) and relatives (median = 2009 pg/mL) as well as between controls and patients with type 1 diabetes (median = 2077 pg/mL), with disease and relative status associating with higher levels of sCD25. We additionally investigated whether a correlation existed between levels of sCD25 and number of autoantibodies (Figure 3-4). We found no significant difference in sCD25 levels between those with no autoantibodies, one autoantibody, and two autoantibodies. GAD and IA-2 were considered for this analysis.

Associations with Age and Type 1 Diabetes Duration

There was a statistically significant association between age and disease state in type 1 diabetes in our sample population, with patients being significantly younger than either first degree relatives or controls, likely due to sampling bias ($p < 0.0001$) (Figure 3-5). We observed a statistically significant correlation between age and sCD25 levels (Spearman $r = -0.31$, $p < 0.0001$) (Figure 3-6). No significant association was observed for either sCD25 by type 1 diabetes duration (Figure 3-7) or rs2069762 genotype by age (Figure 3-8).

Discussion

Here we sought to determine an association between rs2069762 and disease as well as between rs2069762 and disease related phenotypes (IL-2 expression and expression of the secreted IL-2 receptor component soluble CD25). Previous, unpublished data from other groups suggest that the G allele is protective with an odds ratio of 0.89. rs2069762 G has also been observed to afford resistance to new-onset diabetes after transplantation (NODAT) in renal transplantation[176]. We observed no association was observed between genotype at rs2069762 and disease state. It is

probable that our sample size of 297 individuals was inadequate to detect a difference, owing to the modest effect size of the SNP. The allele frequencies observed in our population for the rs2069762 were not out of line with prior expectations [152] .

While we obtained no statistically significant results in our correlations between stimulated IL-2 samples and genotype at rs2069762, marked trends were observed suggested than under anti-CD3/anti-CD28 stimulation, presence of the G allele seems to drive a trend toward lower levels of IL-2 expression. These findings directly conflict with a previous study by Hoffman et. al that associated the G allele with higher IL-2 expression levels [152]. It is possible that the level of mode of stimulation is different, as these prior studies used a 3:1 bead stimulus rather than our soluble stimulus; it is known that the effect of IL-2 on immune cell populations can differ depending on the level and context of exposure [63]. The observed effect may also be due to the fact that the Hoffman study used peripheral blood lymphocytes instead of peripheral blood mononuclear cells; the exclusion of monocytes and macrophages may have had an impact on the outcome.

Interestingly, higher levels of stimulation through CD3 and CD28 seemed to inhibit the maximum expression of IL-2, with maxima in the lower stimulation conditions being considerably higher than in the high stimulation conditions. High levels of stimulation may be driving cells into anergy or may be pushing an earlier peak of IL-2 expression, such that the 24 and 48 hour time points now catch declining expression levels. It is important to note, however, that while the range was altered, the medians were similar between the two stimulation levels, so the effect may only apply to outlying samples.

We additionally used a T-cell receptor independent stimulus, PHA. The trend toward suppression of IL-2 expression observed in the G genotype seemed to be dependent upon signaling through the T-cell receptor. Circumvention of the T-cell receptor through use of PHA produced levels of IL-2 expression that were modest and uniform across groups in comparison to what was observed with anti-CD3 and anti-CD28 stimulation.

For our investigation of rs2069762 and its association with serum levels of soluble CD25, we found that presence of the G allele seemed to be associated with higher levels of expression. This result seems counterintuitive if the G allele is protective, as the unpublished OR of 0.89 would suggest; high sCD25 expression is generally associated with inflammation and disease (even in these same samples (Figure 3-3)), and the current best model for the role of sCD25 would suggest that it functions in disrupting the negative feedback loops by which IL-2 expression reins itself in [159].

Our observation of a correlation between sCD25 and disease state led us to question the role of age in our results. While onset of type 1 diabetes may occur at any age, the primary peaks of onset are between 5 and 7 years of age and during puberty. Because parents of children within these age groups are more likely to bring their children in to participate in studies, sampling bias effects cause type 1 diabetes to be associated with young age in our sample set with a p-value <0.0001 (Figure 3-5).

We investigated the correlation between soluble CD25 and age, and observed that a correlation exists (Figure 3-6). However, we observed no correlation between sCD25 and disease duration (Figure 3-7). These results, and the lack of a correlation with

autoantibody presence (Figure 3-4), raise the possibility that our correlation of sCD25 with disease state is confounded by an association between sCD25 and age.

Additionally, there remains the possibility of confounding by other unmeasured metabolic factors such as blood glucose and circadian rhythm. However, we lack sufficient sample sizes to determine with certainty what role such potential confounding relationships play in our analysis; further study will be needed to verify that observed correlations between sCD25 and disease state are not confounded by associations between sCD25 and age or other factors.

A lack of correlation between age and genotype at rs2069762 suggests that we did not recruit an unusual number of patients of any genotype in any age group (Figure 3-8), and that our association between genotype at rs2069762 and levels of serum sCD25 is not confounded by age.

Our results would suggest that the relationship between sCD25, rs2069762, and disease state in type 1 diabetes is complex, that this complexity cannot be reduced to methodological problems related to culture conditions (as our investigation was done in serum), and more work needs to be done in elucidating the role of the soluble IL-2 receptor alpha subunit before definitive conclusions can be made as to its role in the etiology or progression of type 1 diabetes. It should also be noted that groups were within the normal physiologic range for sCD25 expression, so the biological relevance of this finding is unclear.

Rs2069762 may be associated with factors that play an important role in the etiology or progression of autoimmune diabetes. Further studies are needed to ascertain its utility as a meaningful biomarker. For IL-2, more in-depth comparisons and

titrations of bead stimulations and soluble stimulations in larger sample groups might help to clarify discrepancies between our study and previous work. Once the relationship between rs2069762 and IL-2 expression levels is definitively worked out, it may lend some insight into the interaction between rs2069762 and sCD25 levels.

For soluble CD25, it would be most beneficial to definitively establish a connection between genotype at rs2069762 and disease state. Once this has been worked out, we can determine whether our result; that the G allele is associated with higher levels of sCD25, is truly counter-intuitive and if so, begin to determine why this is the case. Further functional studies using sCD25 may lend insight.

At low levels, it has been observed that sCD25 does not augment regulatory T-cell mediated suppression of T effector proliferation but does affect proliferation directly, but at high levels, it appears to impact suppression [158, 159, 173]. sCD25 has been associated with both upregulation and downregulation of T-cell signaling factor STAT5 [171, 172]. Its effects are highly context dependent; presence or absence of IL-2, choice of culture medium, and presence or absence of various cell subsets have all been shown or speculated to play a role in the downstream effects of sCD25 [159]. These results add additional layer to the complex story of the IL-2/IL2R pathway in type 1 diabetes.

Table 3-1. rs2069762 Genotype by Disease State (Counts).

	T1D	Relative	Controls	
GG	10	9	12	31
GT	55	45	38	138
TT	49	44	35	128
Totals	114	98	85	297

Table 3-2. rs2069762 Allele Frequencies

	Count	Freq
G	200	0.3367
T	394	0.6633

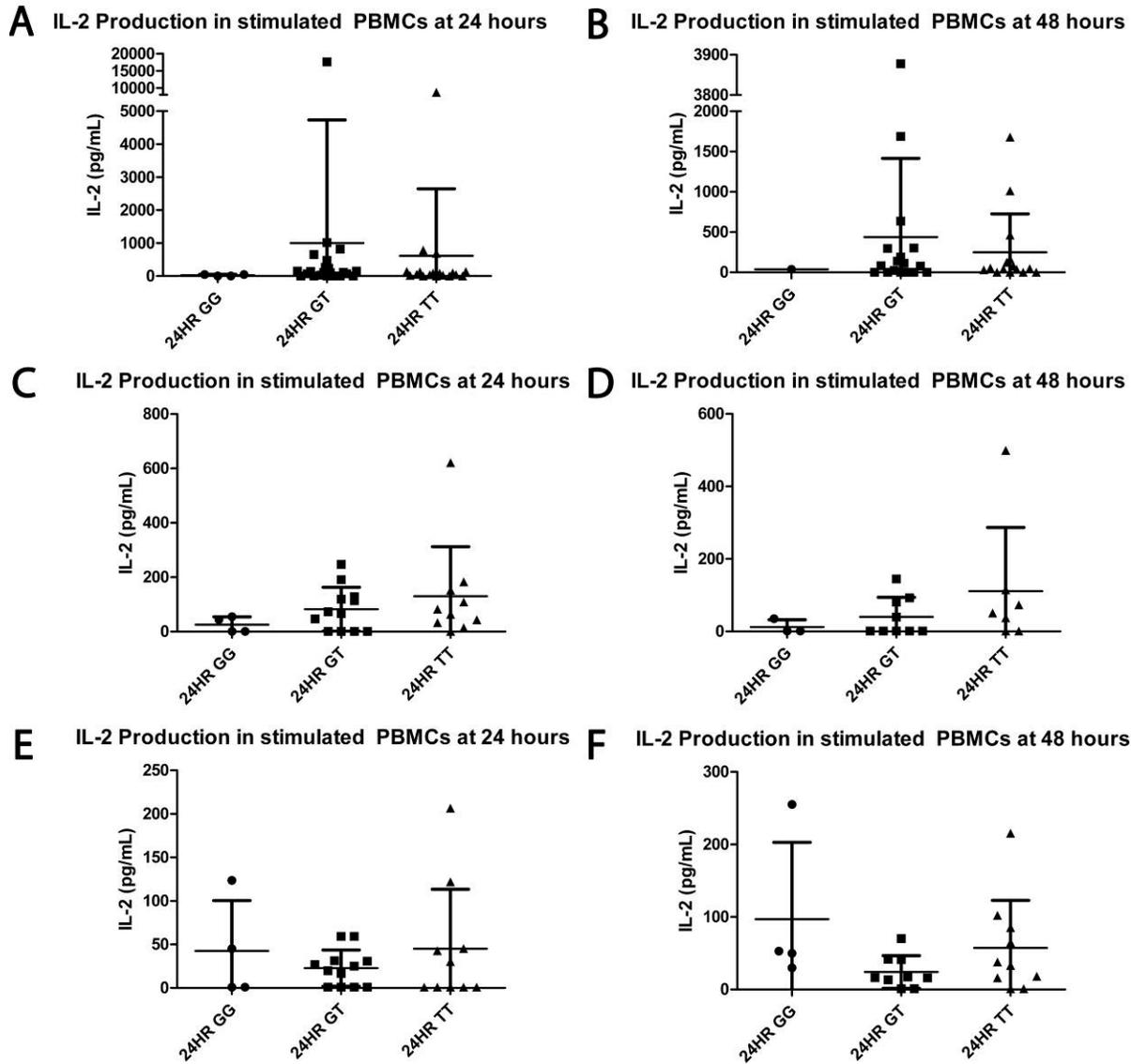


Figure 3-1. IL-2 production measured by ELISA in stimulated PBMCs by rs2069762 genotype. (A) IL-2 levels at 24 hours with 2.5 μ g/mL anti-CD3 and 1.25 μ g/mL anti-CD28 stimulation. (B) IL-2 production at 48 hours with 2.5 μ g/mL anti-CD3 and 1.25 μ g/mL anti-CD28 (C) IL-2 levels at 24 hours with 5 μ g/mL anti-CD3 and 2.5 μ g/mL anti-CD28. (D) IL-2 production at 48 hours with 5 μ g/mL anti-CD3 and 2.5 μ g/mL anti-CD28. (E) IL-2 levels at 24 hours with 1 μ g/mL PHA (F) IL-2 production at 48 hours with 1 μ g/mL PHA.

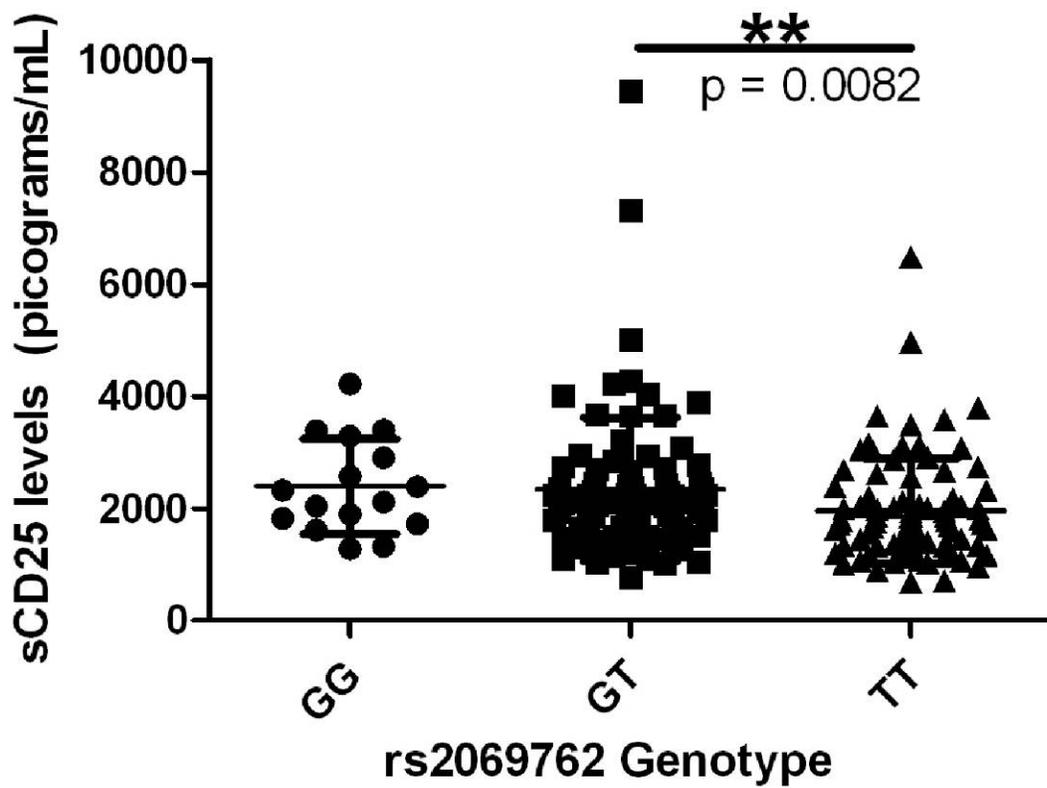


Figure 3-2. sCD25 levels by rs2069762 genotype. (p=0.0082)

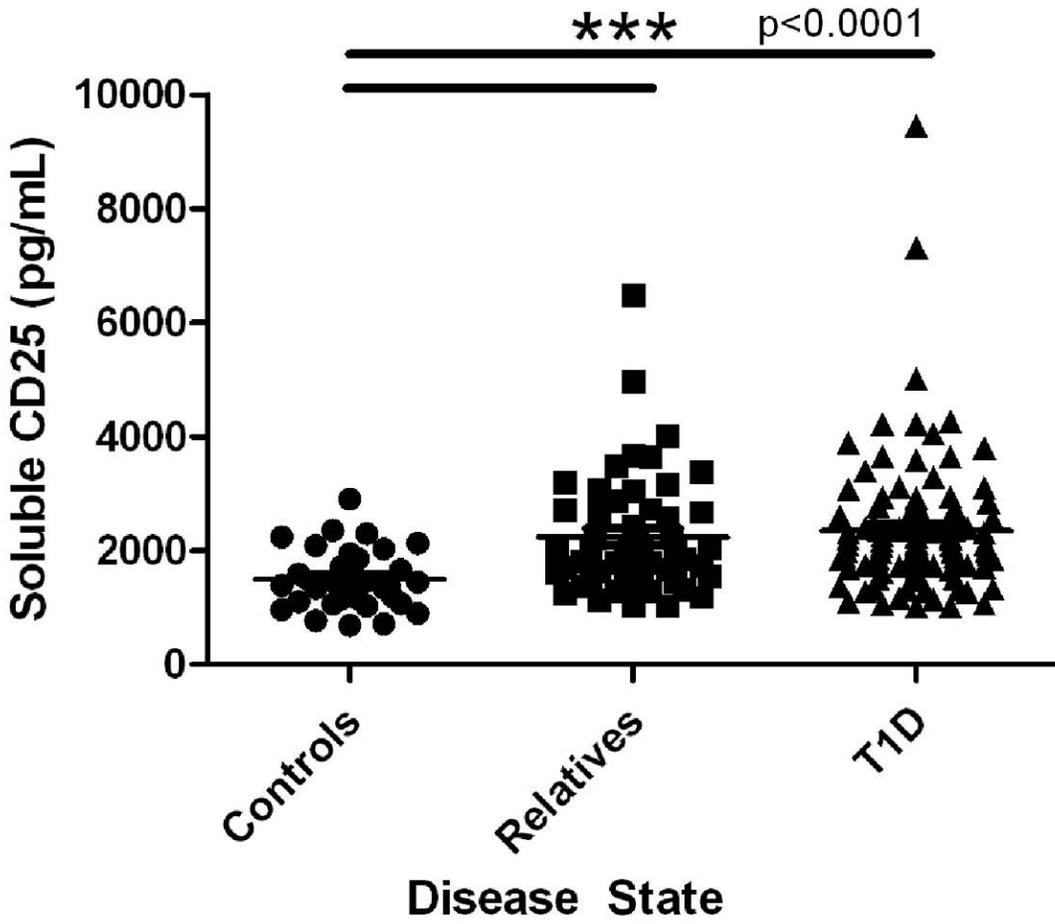


Figure 3-3. Soluble CD25 levels by disease state in type 1 diabetes. (p<0.0001)

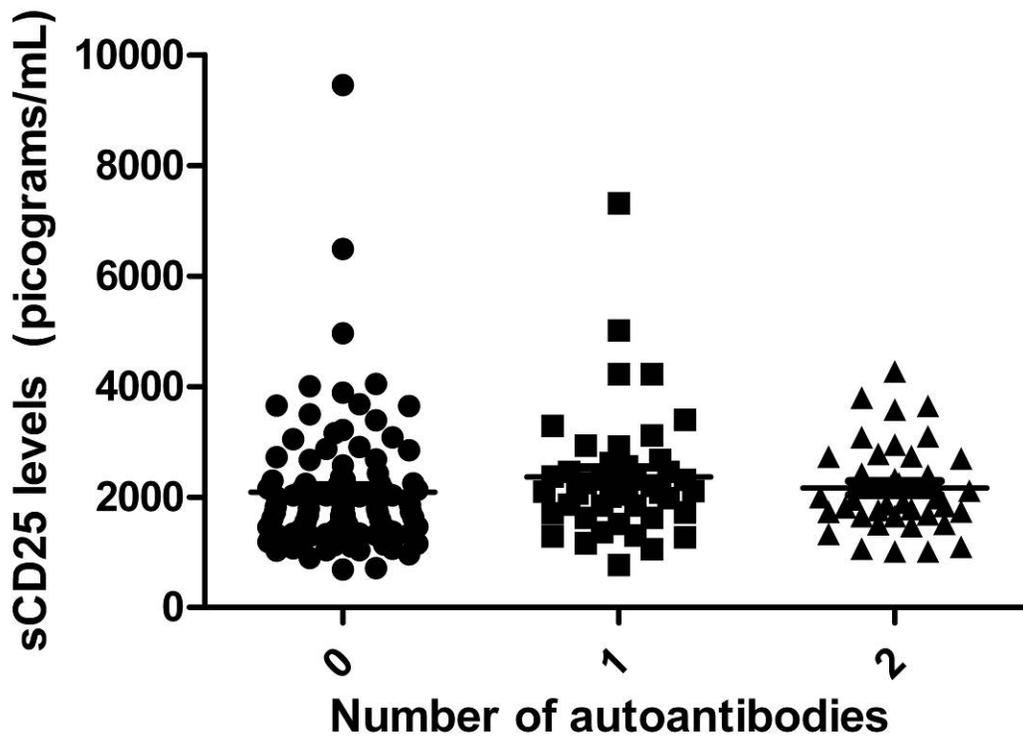


Figure 3-4. sCD25 by autoantibody positivity (GAD and IA-2) ($p=ns$).

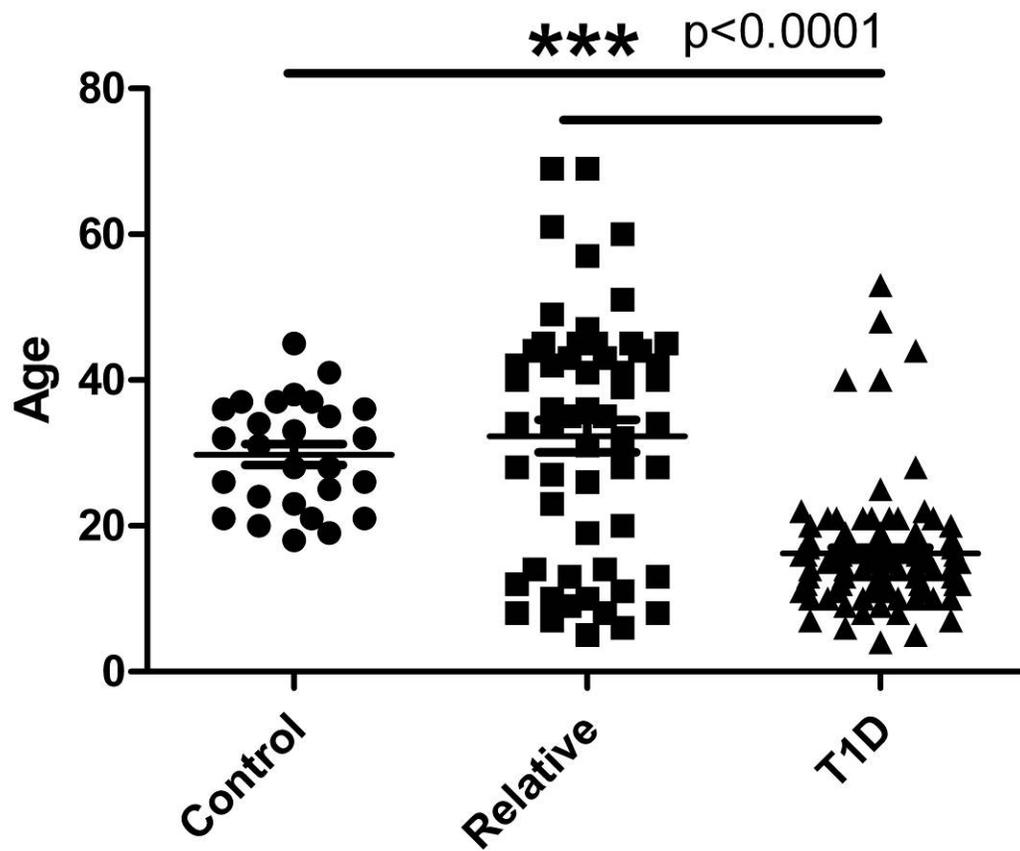


Figure 3-5. Disease state by age in type 1 diabetes ($p < 0.0001$).

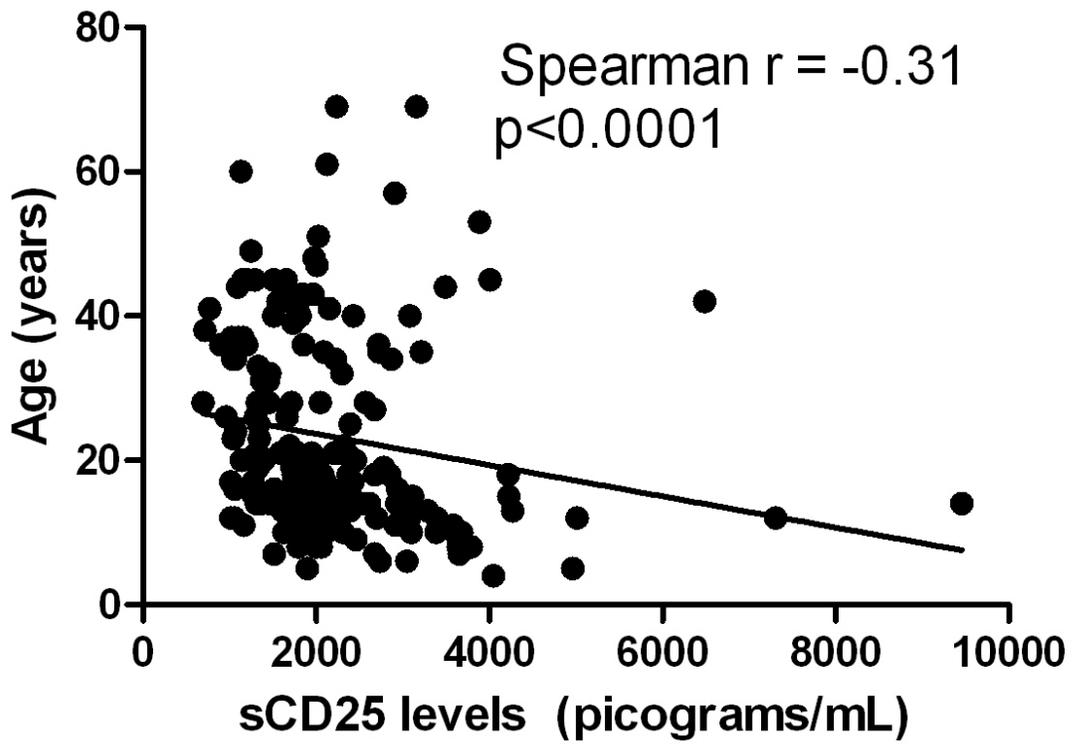


Figure 3-6. Soluble CD25 levels by age in type 1 diabetes. ($p < 0.0001$)

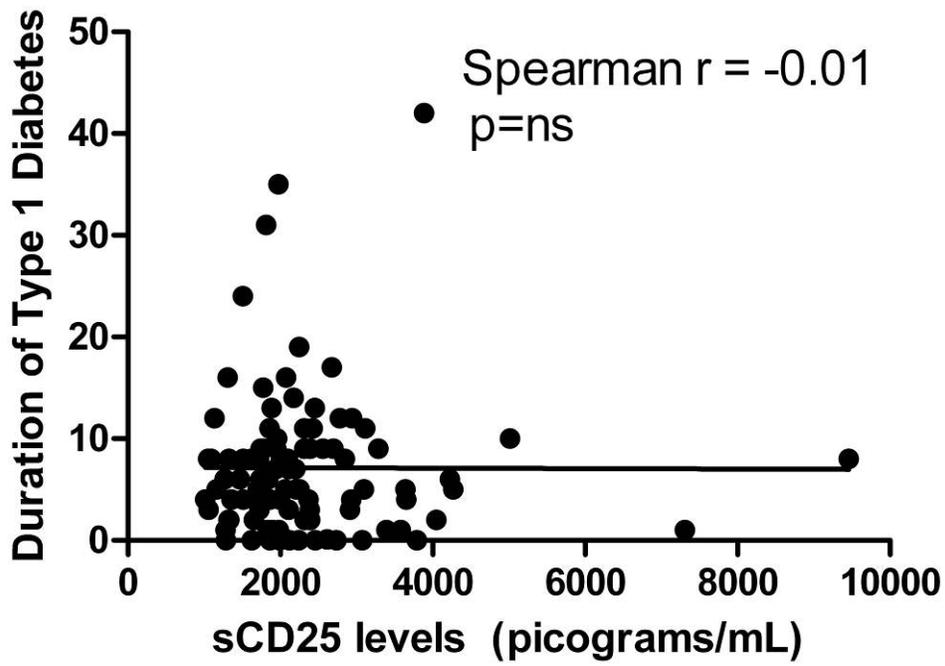


Figure 3-7. Soluble CD25 levels by disease duration in type 1 diabetes. ($p < 0.001$)

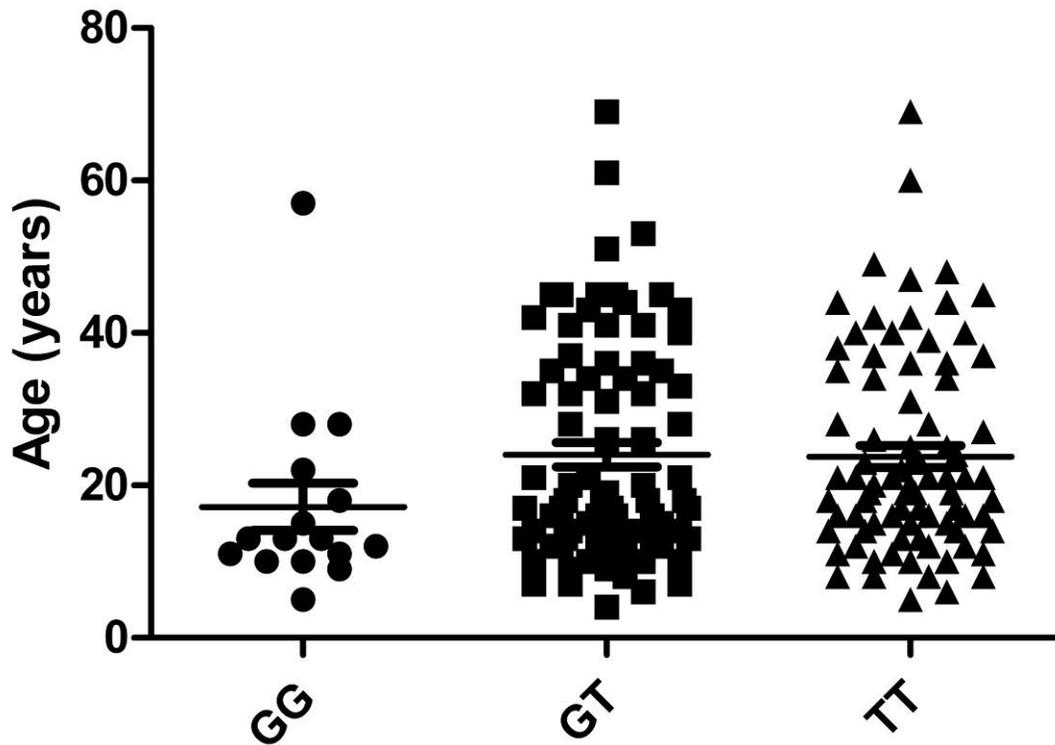


Figure 3-8. Genotype by age in type 1 diabetes. (p=ns)

CHAPTER 4 IMPLICATIONS AND FUTURE DIRECTIONS

We sought to identify biomarkers that would inform our understanding of the etiology, or aid with treatment of type 1 diabetes. To this end, we investigated two pathways known to be relevant to autoimmune type 1 diabetes: the Vitamin D pathway and the IL-2/IL2R pathway. In each case, our findings were informative, and form a strong basis for future investigations.

In the case of the Vitamin D pathway, we found that VDBP SNPs rs4588 and rs7041 did not have any correlation with disease. We did, however, find an association between lower VDBP levels and type 1 diabetes. This, in spite of the fact that, in our patient population, Vitamin D levels were low in all studied groups and consequently, no distinctions in Vitamin D levels were observed between the groups.

VDBP exists in levels far in excess of those necessary to bind Vitamin D; 98-99% of VDBP binding sites remain unbound [142]. This and the diverse immunological activity of VDBP suggest that it may play an important role in the immunobiology of type 1 diabetes independent of its connection to Vitamin D. It is also possible that interaction between Vitamin D and VDBP independent of SNPs rs4588 and rs7041 may be important to type 1 diabetes. It is unclear whether the lower VDBP levels observed in those with type 1 diabetes are a cause of autoimmunity, an effect of immune dysregulation, or simply share common causative factors with disease. We have identified a potentially important role for VDBP as a marker in autoimmune diabetes; further investigation of the more than 120 known polymorphisms at the VDBP locus and their relationship to disease state may help to elucidate the importance of this pathway to the etiology of type 1 diabetes.

The IL-2/IL-2 receptor pathway is heavily implicated in the etiology of type 1 diabetes, with polymorphisms in both IL-2 and the IL-2 receptor being associated with susceptibility. We sought to characterize the association between rs2069762, a T>G SNP 330 bp upstream of IL-2 in the promoter region, and disease relevant phenotypes. Our failure to find an association between rs2069762 and disease state stands in contrast to previous findings suggesting an OR of 0.89 for the G allele. It is possible that our sample size was too small to detect a difference.

Our data trended toward lower IL-2 expression in the presence of the G allele. While not statistically significant, these data are contrary to previous findings suggesting higher IL-2 expression in the presence of the G allele; the differences may be a result of the cell subsets used in the experiments (the previous work excluded monocytes and macrophages) or the culture conditions (the previous work used beads rather than soluble stimulus)[152]. More study will be needed to definitively resolve this conflict.

We observed an association between rs2069762 and serum levels of sCD25, with the presence of the G allele being associated with higher levels of sCD25. This would seem to contrast with previous data on rs2069762; the G allele is generally associated with protection, while high sCD25 expression is generally associated with inflammation and disease. Because the impact of sCD25 expression is so highly context dependent, varying based on cell subsets present, levels of sCD25 present, and possibly levels of IL-2 expression, it is likely that we are observing the end result of a highly complex set of interactions. sCD25 can promote regulatory T cell mediated suppression or have no impact at all, can promote or inhibit Stat5 expression, and can inhibit T-effector proliferation when expressed alone. Perhaps high levels of sCD25 observed in

inflammation and disease represent a failed attempt at counter-regulation that, for whatever reason, is more effective in those with the G allele at rs2069762.

While SNPs have been known to have functional effects on their associated proteins, and rs2069762 is in the promoter region, it lies upstream of the majority of the transcription factor binding sites. It is unclear what, if any, functional impact this SNP may have, and any hypotheses about functional impact outside of mere association would be speculation at this point. Future investigations with larger samples sizes could confirm the observed trend in IL-2 expression related to rs2069762. With regard to sCD25, a key component will be determining, in a larger sample population, what, if any association rs2069762 has with disease state; from there, we may have more basis for understanding the possible relevance of the association between levels of sCD25, the rs2069762 SNP, and disease state in type 1 diabetes.

These biomarkers provide novel insight into two pathways implicated in autoimmune diabetes. The mechanism of the association between VDBP and disease and rs2069762 polymorphism and sCD25 levels require further study. More detailed understanding of the Vitamin D and IL-2 pathways may be informative for our understanding of disease etiology and progression in type 1 diabetes.

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BIOGRAPHICAL SKETCH

Dustin Blanton was born to Cecil C. and Garnette Blanton in Ft. Worth, Texas. He grew up in Branford Florida from the age of two through the age of eighteen, graduating from Branford High School. In June of 2005, he married his wife, Lori. He graduated with a Bachelor of Science in Interdisciplinary Studies: Biology in 2006, completing a senior honors thesis under the supervision of Dr. Charles F. Baer. He subsequently taught high school for two years at Bradford High School in Starke, Florida. Upon completion of his second year teaching high school and following a suggestion from Dr. Baer, he applied to the Genetics and Genomics Graduate program at the University of Florida and was enrolled in August of 2008. He began work under the mentorship of Drs. Mark Atkinson and Desmond Schatz in early 2009. He has mentored six undergraduate and postgraduate students during his time in the laboratory. Upon completion of this work, he hopes to pursue a career in post-secondary education.