The Role of Genetics and Environmental Factors on Autoimmune Disease Incidence With a Focus on Gender Bias in a Family Case Study

The Role of Genetics, Environmental Factors and Gender Bias on Autoimmune Disease Incidence in a Family Case Study

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Abstract

Most autoimmune diseases disproportionately affect women more than men, with women comprising about 80% of autoimmune disease incidence. The cause for this discrepancy is not known, although there are both genetic and environmental hypotheses. This thesis project aimed to elucidate whether the increased prevalence in women is due to a genetic or environmental factor by analyzing single nucleotide polymorphism (SNP) data from a family of five individuals with a mix of autoimmune diseases (systemic lupus erythematosus (SLE), psoriasis and Hashimoto's thyroiditis). The overarching hypothesis was that the increased prevalence of autoimmune diseases in women is caused by differing levels of sex hormones and estrogen receptors, which can in turn affect various pathways that play a role in the development of immunological disease. As such, in the current study, pathways that have been shown to modulate the immune system, such as vitamin D and calcium levels and affect tight junctions and epithelial barrier integrity were examined in the context of the current known literature and SNPs. For each member of the family in the case study, a genetic analysis was carried out to search for the presence of SNPs that are known to increase risk of developing each autoimmune disease. By comparing and contrasting the male father, who has lupus, to the female members of his family, two of which have separate autoimmune diseases and two of which are healthy, differences and similarities in the SNP profile led to the finding of a SNP in the estrogen receptor alpha. Prior studies showed that disease associated with SNPs in the estrogen receptor alpha is correlated with environmental factors, such as

smoking (A. Zhou et al., 2017). The male subject, who has lupus, is heterozygous for this polymorphism and so is his female daughter, who does not have lupus. The male subject was a heavy smoker at the time of disease onset and his daughter has never smoked, which may be a reason for the similar SNP profiles but difference in disease onset. In sum, the findings of this study suggest genetic reasons for autoimmune disease incidence in this family. This data also correlates with a previous study that shows that a polymorphism in the estrogen receptor alpha may be influenced by environmental factors, such as smoking, and may have contributed to disease incidence in a member of this family. Further studies will need to be completed in larger cohorts to see which SNPs and environmental factors are the most important factors for disease onset among the population as a whole.

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Chapter I.

Introduction

Autoimmune disease incidence has been on the rise for the past few decades without clear evidence as to why. Autoimmune diseases are hard to diagnose and are responsible for an estimated $100 billion annually in direct health costs, according to the National Institute for Health (NIH). The NIH estimates that there are over 50 million people in the US living with autoimmune diseases and of this, about 80% are women. There is no clear evidence as to why women have a higher risk of developing an autoimmune disease although many hypotheses have been proposed.

Are genetic factors or environmental factors to blame for the increased prevalence of autoimmune diseases among women? Most of the literature points towards both genetic and environmental factors being important for autoimmune disease onset, where a genetically predisposed individual encounters an environmental agent that then triggers the disease (Somers & Richardson, 2014). What is less understood are the factors that distinguish males from females i.e. are females more likely to have genes that cause autoimmune susceptibility, or are they more likely to respond to environmental triggers in a more negative fashion. Are there environmental stimuli that both males and females are equally likely of encountering, but have a much more negative effect on women due to differing levels of hormones or other factors?

If environmental factors are to blame, what are they and why are women more prone to them? Many environmental factors have been hypothesized to induce different

autoimmune diseases, including vitamin D deficiency for multiple sclerosis (Mahon, Gordon, Cruz, Cosman, & Cantorna, 2003) and rheumatoid arthritis (Als, Riis, & Christiansen, 1987a); agents causing oxidative stress (diet, smoking, UV light exposure, infections) for lupus (Marder, Vinet, & Somers, 2015a); and cumulative epigenetic changes for lupus and other autoimmune diseases (Somers & Richardson, 2014).

What is the basis for the differing immune responses between men and women? Women and men have differing immune responses, with women tending to respond to infection, vaccination and trauma with an increased Th2 response, and men tending to respond with an increased Th1 response (Fairweather, Frisancho-Kiss, & Rose, 2008). This has been replicated in other studies including one where Th1/Th2 ratios were determined for healthy men and women and women had a predominantly Th2 cytokine profile (Girón-González et al., 2000). It is not evident as to why this is the case and this difference is generally overlooked in the experimental design of autoimmune disease studies, especially animal studies. This thesis project aimed to look at these questions in the context of different genetic components, single nucleotide polymorphisms, and the environmental factors such as, differing sex hormones, levels of vitamin deficiency and other environmental risk factors that may be acting on genetic susceptibility to autoimmune diseases.

Autoimmune Diseases

A major pathway for autoimmune disease incidence is the body producing antibodies that cause the immune system to attack itself. This attack injures and/or destroys organs, tissues or cells depending on which autoimmune disease is present.

Other autoimmune disease pathways include inefficient central tolerance, activation of autoreactive cells, strongly self-reactive Treg cells and defective nucleic acid sensing (Theofilopoulos, Kono, & Baccala, 2017). According to the National Institute of Allery and Infectious Diseases (NIAID), there are more than 80 chronic diseases classified as autoimmune and more than 8% of the US population is currently living with one or more of them (NIH, 2016). Autoimmune diseases cost over $100 billion dollars in direct health care costs per year in the US (NIH, 2016) and are hard to diagnose. Most autoimmune diseases don't have a clear cause and the symptoms can come on slowly and be indicative of a lot of different diseases. Many patients have alternating periods of flare-ups and remission and they can live in pain for years before they are diagnosed and symptoms can be brought under control. There are no known cures for autoimmune diseases, however there are treatments that can be used to bring down inflammation and control overactive immune responses, such as immune suppressing drugs and nonsteroidal anti-inflammatory drugs (NSAIDS).

Incidence of Autoimmune Diseases in Women

Autoimmune diseases are more prevalent in women than men with estimates of nearly 80% of all incidences occurring in women (NIH, 2016). In an analysis of the age of onset and percent incidence of women vs. men of 40 autoimmune diseases, diseases that occurred after puberty (with appearances during early adult life or during mature

adult life) correlated with a higher disease incidence in women. Some of these diseases, such as systemic lupus erythematosus (SLE), Erythema nodosum, Sjogren's syndrome and Primary biliary cirrhosis, occur in women 90% (or more) of the time (Beeson, 1994). There is not a clear reason as to why women are more prone to autoimmune diseases but various hypotheses have been proposed including genetic variations and environmental factors such as: varying Th1 and Th2 responses between men and women, sex hormone differences, fluctuating vitamin D levels and differences in the microbiome.

Lupus

Lupus is an autoimmune disease that is characterized by hyperactivity of T cell and B cell responses and the formation of autoantibodies against nucleic acids and their binding proteins. The immune system becomes hyperactive, attacks healthy tissues and causes a global loss of self-tolerance (J. Choi, Kim, & Craft, 2012). The most studied autoantibody in lupus is anti-dsDNA although there are others that are also indicative of disease (Fu, Dai, Zhao, & Gaskin, 2015). There are thought to be two forms of lupus, systemic lupus erythematosus (SLE) and cutaneous lupus erythematosus (CLE or discoid lupus), however current biomarkers are the same between the forms and the only known difference is where the disease manifests (SLE manifests systemically and CLE manifests mainly in the skin). Lupus used to be diagnosed by meeting four of eleven criteria established by the American College of Rheumatology (ACR) in 1997, however the ACR came out with new guidelines in 2018 (Figure 1) that has 22 criteria with each given

varying weight. A percentage of patients who have discoid lupus later develop SLE, some of which involve mostly skin related symptoms, but some of them develop symptoms in other organs (Wieczorek, Propert, Okawa, & Werth, 2014), so it is possible that all forms of lupus are genetically related but certain factors prevent milder forms from becoming more severe. Symptoms of lupus can be seen in a wide range of tissues including joints, skin, kidneys, blood cells, brain, heart and lungs (Mayo Clinic, 2019). It is estimated by the Center for Disease Control (CDC) that 1.5 million Americans have lupus and of these nine out of ten cases are women.

Figure 1. ACR and EULAR Criteria For SLE Classification

New ACR and EULAR criteria for classification of SLE

All patients classified as having systemic lupus erythematosus must have a serum titer of antinuclear antibody of at least 1:80 on human epithelial-2–positive cells or an equivalent positive test. In addition, a patient must tally at least 10 points from these criteria. A criterion is not counted if it has a more likely explanation than SLE. Occurrence of the criterion only once is sufficient to tally the relevant points, and the time when a patient is positive for one criterion need not overlap with the time when the patient is positive for other criteria. SLE classification requires points from at least one clinical domain, and if a patient is positive for more than one criterion in a domain only the criterion with the highest point value counts:

Clinical domains	Points
Constitutional domain	
Fever	2
Cutaneous domain	
Nonscarring alopecia	2
Oral ulcers	2
Subacute cutaneous or discoid lupus	4
Acute cutaneous lupus	6
Arthritis domain	
Synovitis in at least two joints or tenderness in at least two joints, and at least 30 min of morning stiffness	6
Neurologic domain	
Delirium	2
Psychosis	3
Seizure	5
Serositis domain	
Pleural or pericardial effusion	5
Acute pericarditis	6
Hematologic domain	
Leukopenia	3
Thrombocytopenia	4
Autoimmune hemolysis	4
Renal domain	
Proteinuria >0.5g/24 hr	4
Class II or V lupus nephritis	8
Class III or IV lupus nephritis	10

Immunologic domains	Points
Antiphospholipid antibody domain	
Anticardiolipin IgG >40 GPL or anti-β2GP1 IgG >40 units or lupus anticoagulant	2
Complement proteins domain	
Low C3 or low C4	3
Low C3 and low C4	4
Highly specific antibodies domain	
Anti-dsDNA antibody	6
Anti-Smith antibody	6

MDedge News

Source: Dr. Johnson

Figure 1: Criteria for Classification of SLE by the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR).

Genetic Factors in Autoimmune Diseases

Most autoimmune diseases seem to have a genetic component, as they tend to run in families, however, members of the same family might not necessarily have the same autoimmune disease, just an inherited susceptibility. Some autoimmune diseases show higher genetic susceptibility than others, but most show some variation in the gene that codes for the human leukocyte antigen (HLA). The genetic susceptibility for genes other than those related to the HLA have been studied in some autoimmune diseases, including rheumatoid arthritis (RA), SLE, and primary biliary cholangitis, however more work is needed to show definitive correlation between these genes and diseases (Ceccarelli, Agmon-Levin, & Perricone, 2017). Single nucleotide polymorphisms (SNPs) are genetic variants that are inherited differences in DNA sequence which are involved in the phenotypic variation seen within species. In humans over 1.42 million SNPs have been found (Waterson & McPherson, 2001) .

MHC Genetic Loci

Most of the early genetic loci studied in autoimmune diseases involved the major histocompatibility complex (MHC). Studies taking place as early as 1970 showed correlation of certain MHC alleles with autoimmune diseases. The MHC gene cluster is also known as the HLA in humans, but it is homologous with the MHC in other higher vertebrates, and as such can be used interchangeably when referencing humans. The

HLA gene cluster is expressed on chromosome six and it contains many genes which are related to the immune response, current literature describes anywhere from 100-150 genes depending on the study, as well as genes for some other related functions. The genes of the HLA are highly polymorphic (they have many different forms) and are split into three categories; HLA (MHC) class I, HLA (MHC) class II and HLA (MHC) class III genes. In humans, the class I genes are HLA-A, HLA-B and HLA-C; the class II genes are HLA-DR, HLA-DP and HLA-DQ; and the class III genes are the complement protein genes as well as some genes that encode cytokines (Matzaraki, Kumar, Wijmenga, & Zhernakova, 2017).

The MHC is expressed on the surface of cells which allows the cell to be recognized as "self" by other cells. Class I molecules are displayed on the cell surface of all nucleated cells, while class II molecules are primarily displayed on professional antigen presenting cells (APCs), such as macrophages, B cells and dendritic cells (DCs). HLA class I genes are polymorphic and humans can express up to six different class I gene products at the cell surface. HLA class II has six main genes in humans: HLA-DP1, HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRA and HLA-DRB1. The HLA class II genes are also polymorphic, but with varying degrees. Both MHC I and MHC II have an alpha and beta chain that combine to form functional proteins. MHC II is made up of two membrane spanning chains (alpha and beta) that are produced by MHC genes, while MHC I is made up of one membrane spanning chain (alpha) and one light chain (beta) that is produced by the β2- macroglobulin gene, which is located on chromosome 15 and not chromosome 6 like the rest of the MHC genes (Figure 2). The MHC genes have a

plethora of possible variants that allows for each human to react to wide ranges of foreign peptides, which is necessary for normal immune functioning.

Figure 2. MHC Class II and MHC Class I Molecular Structures

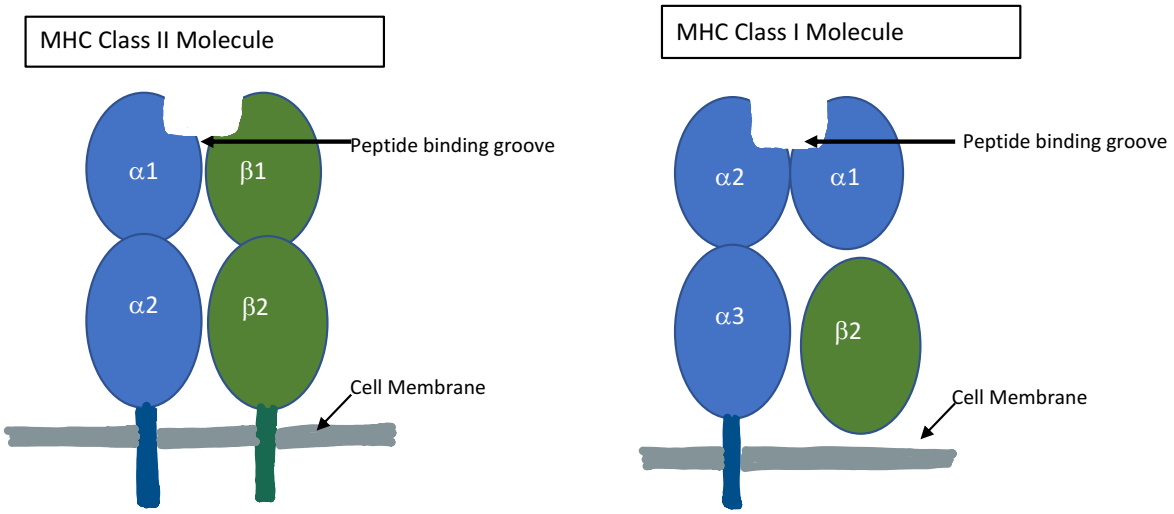

Figure 2: Schematic drawing of the molecular structures of MHC Class II (left) and MHC Class I (right) molecules. Blue denotes alpha chains and green denotes beta chains.

There are several associated genes that are located close to the MHC genes on chromosome six (Figure 3). These genes are involved in antigen processing, including TAP1 and TAP2 genes, the HLA-DMA and HLA-DMB genes (which are nonclassical class II genes), and the LMP-2 and LMP-7 proteasome genes.

Figure 3. MHC Class II

Figure 3: MHC Class II (Ting & Trowsdale, 2002).

Schematic map of the MHC class II region humans. The classical class II molecules are shown in yellow. The nonclassical class II genes are in pink (DO) and dark blue (DM). Pseudogenes are shown in red and yellow stripes. Antigen processing molecules are shown in purple (LMP) and green (TAP).

TAP Proteins in Antigen Processing

In order for MHC dependent T cell recognition to occur the cell must break down proteins into their peptide components. This occurs by proteolysis, either in the cytosol where it is mediated by the proteasome, or in the lysosomal compartments where it is mediated by different compartments (endosomes, lysosomes etc.) with varying levels of acid used to break down the proteins or protein pieces. Once the proteins are processed into short peptides they are transported into the endoplasmic reticulum (ER) by the Transporter Associated with Antigen Processing (TAP). TAP is a heterodimer made up of the proteins TAP1 and TAP2, which are encoded in the class II region of the HLA locus (6p21.32), and is a member of the ATP-binding cassette family (D. L. Zhou &

Blum, 2002). If cells do not have functional TAP1 or TAP2 genes they will express very little surface MHC-I because peptides are not able to be imported into the ER.

CLIP Affinity

MHC gene variants are closely linked with certain autoimmune diseases based on MHC class II antigen processing. Class II-associated invariant chain peptide (CLIP) is important in making sure that self-peptide fragments are not able to bind to the MHC receptor during development, by binding to the peptide binding groove. Once the receptor is assembled CLIP is released, peptides are bound and the MHC II molecule with antigen is transported to the cell membrane for presentation. Although class II antigen processing can occur without CLIP, it is beneficial in enhancing the efficiency and precision of the process (Berger & Roche, 2008). There are some MHC II alleles with a low affinity for CLIP that are associated with different autoimmune diseases, especially in the case of rheumatoid arthritis where certain HLA-DR alleles were shown to correlate with disease incidence. One theory is that if CLIP is released prematurely from a disease-associated MHC II allele with low affinity to CLIP, it may allow for the selection of epitopes from autoantigens or allow the peptide groove to capture self-peptide in certain endosomal compartments (Berger, 2008). However, other studies have directly challenged this hypothesis using animal models of transgenic mice expressing human CLIP regions with either normal affinity or low affinity for MHC class II (Honey, Forbush, Jensen, & Rudensky, 2014). Their study showed that the peptide repertoire was not substantially

altered with transgenic mice that had low affinity CLIP and autoimmune susceptibility was not significantly increased.

MHC II Associated Proteins

A non-classical MHC II gene, HLA-DM has also been implicated in autoimmune disease onset. It is encoded in humans by HLA-DMA, of which all humans express at least two, and HLA-DMB, of which all humans also express at least two. HLA-DM is oligomorphic with only seven known allelic variants of DMA and only eleven known allelic variants of DMB (Alvaro-Benito, Morrison, Wieczorek, Sticht, & Freund, 2016). HLA-DM functions as a peptide editor for classical MHC II proteins and chaperone of empty MHC II molecules by regulating which peptides can bind to them and by preventing them from breaking down. HLA-DM is beneficial in selecting for peptide-MHC II complexes that are highly immunogenic and so a decrease in HLA-DM activity is shown to be correlated with the inability to fight infections and risk of autoimmune diseases (Alvaro-Benito, 2016).

HLA-DO is another non-classical oligomorphic MHC II gene whose function is less well known but has been shown to interact with HLA-DM in the process of epitope selection (Alvaro-Benito et al., 2016).

Immunoproteasome

The immunoproteasome is involved in proteolysis (the breakdown of proteins into peptides and amino acids) and is expressed in immune cells. It is derived from the constitutive proteasome, which is expressed in the cytosol and nucleus of most cells, where it functions to maintain cell homeostasis. When cells receive inflammatory stimuli the subunits of the constitutive proteasome are substituted for different subunits and thus it becomes the immunoproteasome (Ho, Bargagna-Mohan, Wehenkel, Mohan, & Kim, 2007). Two of the most important subunits that are exchanged are Beta-1 replaced by the large multifunctional peptidase 2 (LMP2 aka proteasome subunit beta type 9 (PSMB9)) and Beta-5 is replaced by the large multifunctional peptidase 7 (LMP7 aka proteasome subunit beta type 8 (PSMB8)). The immunoproteasome is thought to be involved in autoimmune diseases by influencing the polarization of T cells, signaling through the NF-kB pathway and producing inflammatory cytokines (Kimura, Caturegli, Takahashi, & Suzuki, 2015).

Common SLE Risk Loci

The genetic component of SLE is significant, as seen by high levels of familial clustering and higher concordance rates among monozygotic twins than dizygotic twins (Kelly, Moser, & Harley, 2002) and as such there have been several genetic risk factors found through genome-wide association studies (GWAS). The SLE loci that have been

analyzed the most and clearly confirmed through numerous studies are for MHC class II alleles, low-affinity receptors for the constant fraction of IgG and the genes PTPN22, IRF5, ITGAM, STAT4 and C8orf13-BLK. In 2009, Suarez-Gestal et. al ran a large GWAS looking to confirm the latter five genetic loci as well as others that were previously shown to have some correlation. Their study confirmed the five previously mentioned loci, as well as six others: TYK2, MECP2, 1q25.1, PXK, BANK1 and KIAA1542 (Suarez-Gestal et al., 2009).

Immunomodulatory Receptors in SLE

Lymphocyte antigen 9 (LY9) belongs to the Signaling Lymphocyte Activation Molecule (SLAM) family of immunomodulatory receptors which are involved in the regulation and communication of the innate and adaptive immune response. It is significantly associated with a decrease in the proportion of naïve CD4+ T cells and activated T cells and an increase in CD8+ memory T cells (D. S. C. Graham et al., 2008). LY9 is also known as SLAMF3 and is expressed on T cells, B cells, macrophages DCs and granulocytes (Chatterjee et al., 2012). LY9 plays a role in promoting Th2 polarization and is involved in enhancing T cell activation (D. B. Graham et al., 2006). LY9 can interact with an adaptor molecule named SLAM-associated protein (SAP). Deficiency of SAP, as seen in a mouse model using SAP KO mice, leads to a strong skewing of CD4+ T cells toward Th1 responses, with defects in Th2 cytokines, deletion

of NKT cells, and defective class switching to IgE (D. B. Graham et al., 2006). Deficiency of LY9, as seen in a mouse model using LY9 KO mice, showed a muted Th2 defect as compared to SAP KO mice, but with T cells that proliferated worse and produced less IL-2, normal macrophage cytokine production and normal NKT cell development (Graham, 2006).

The Role of IL-21 in SLE

Another biological process that is known to be dysregulated in SLE is IL-21, which has also been shown to have a genetic component. IL-21 is a cytokine that has an effect on B cell differentiation into plasma cells, an effect on dendritic cell maturation and T cell responses. IL-21 is produced by activated CD4+ T cells, and its receptor, IL-21R, is expressed on T cells, B cells, NK cells and DCs. The effects of IL-21 on these cell types has been shown to both promote and suppress immune responses and it signals through a number of pathways, including Jak1, Jak3, Stat3 and Stat5. SLE patients that are homozygous for the GG allele, in the SLE and IL-21 associated SNPs, rs907715 and rs2221903, suffer from less central nervous system involvement. However, this finding is preliminary and will need to be repeated in future studies (Sawalha et al., 2008).

There are several mouse models that support the hypothesis that IL-21 is involved in SLE incidence. Lupus prone BXSB-yaa mice have elevated levels of IL-21 in their serum compared to BXBS wild type mice which suggests that IL-21 may be involved in the pathogenesis of SLE (G. Wang et al., 2014). In the sanroque mouse strain, a mutation

in the RING-type ubiquitin ligase protein family member, roquin, results in excessive production of IL-21 and a severe lupus-like autoimmune phenotype (Cornall et al., 2005)

Environmental Factors in Autoimmune Diseases

While there is definitely a genetic component to autoimmune diseases, that alone does not fully explain why some people are afflicted with autoimmune diseases and some are not. There are numerous cases where one identical twin may have an autoimmune disease while the other doesn't, which signifies that there must be environmental triggers as well genetic susceptibilities (Gourley & Miller, 2007). There has been a marked increase in incidence over the past few decades which must be attributed to changing environmental factors over latent genetic factors, as our genetics as a species are not changing that abruptly (i.e. through evolution) (Gourley & Miller, 2007). One of the leading general hypotheses about the increased incidence is the "Hygiene Hypothesis" which states that the decrease in early exposure to infectious agents, parasites etc. leads to a decreased programming of the immune system and an increased risk for allergies and autoimmune diseases (Okada, Kuhn, Feillet, & Bach, 2010). Another hypothesis is that the current western diet (high-fat and high-sugar) is increasing inflammation in the gut which leads to systemic inflammation (Manzel et al., 2014). These hypotheses are not mutually exclusive, and microbiome modulation, whether it be through lack of encountering microbes, diet changing the formulation of microbes in the gut, or overuse

of antibiotics, may be a significant reason for the higher incidence of autoimmune diseases in the past decades.

In accordance with the hygiene hypothesis, there has been a selection for SNPs or other variants that partially compensate for immunoregulation in parts of the world where there are heavy loads of organisms causing immunoregulation (ie helminths). When these organisms are deleted from populations by higher standards of cleanliness the genetic variants that were once beneficial in combating helminth etc. infection are now leading to excessive levels of inflammation (Rook, 2012). This inflammation becomes widespread and can lead to a plethora of disorders, including allergic and autoimmune diseases, which are categorized by chronic inflammation. This harmful phenotype from ancestral alleles has been shown for SNPs correlated with pro-inflammatory cytokines (Riva et al., 2009), IgE (Mehta et al., 2008) and STAT6 (Rook, 2012). In patients with multiple sclerosis, those that are infected with helminths have significantly lower rates of disease progression. These patients develop myelin-specific Tregs that circulate through the body and release the modulatory cytokines IL-10 and TGF-beta when they encounter a peptide from myelin-basic protein. This leads to less chronic inflammation than patients without these Tregs (Rook, 2012).

Other environmental factors have been better studied in the context of specific autoimmune diseases but can be extrapolated to broader autoimmune disease prevalence as well. Vitamin D deficiency (Mahon et al., 2003) (Als, Riis, & Christiansen, 1987), diet, smoking and infections (Marder, Vinet, & Somers, 2015) and cumulative epigenetic changes from past generations (Marder et al., 2015) have been shown to be important in diseases such as multiple sclerosis, rheumatoid arthritis, and SLE.

Cytokine Production and Immune Response

Cytokines are small glycoproteins that are involved in the regulation of both innate and adaptive immunity and serve an important role in normal immune system functioning. They are produced mostly by T helper cells and macrophages and their effects are exerted on receptors on the cell surface of target cells over short time periods and distances, however they can produce a cascade by one cytokine stimulating its target cell to produce more cytokines. Cytokines induce inflammatory responses, cellular proliferation, cellular differentiation, and innate and adaptive immune responses (D. L. Zhou & Blum, 2002). Cytokines are important in the pathogenesis of autoimmune diseases, including during SLE where aberrant expression of certain cytokines, IL-2, IL-17, IFN-α, IL-12, IL-15 and IL-21, are thought to activate signaling pathways and lead to disease induction (Moudgil & Choubey, 2011)

T Lymphocyte Helper Cells

T lymphocyte helper cells (Th cells) are CD4+ T cells and along with cytotoxic CD8+ T cells, they make up the lymphocyte population that are mainly derived from the thymus (Luckheeram, Zhou, Verma, & Xia, 2012). Th1 and Th2 responses refer to T lymphocyte helper cells which can be divided into two functional subsets (T helper 1 and

T helper 2) based on their cytokine profiles. Th subsets are activated and differentiated through antigen presentation by antigen presenting cells (APCs). Th1 lymphocytes typically secrete Th1-type cytokines, such as IL-2, IFN-gamma, and TNF-beta, while Th2 lymphocytes typically secrete Th2-type cytokines, such as IL-4, IL-5, IL6 and IL-13 (Girón-González et al., 2000). Th cell pathways are important in adaptive immunity and host defense. Th1 T cells are primarily involved in cell-mediated immunity while Th2 cells are involved in IgE antibody production and other allergic responses (Okada et al., 2010). Th1 cells have been shown to also be important in phagocyte-dependent inflammation and Th2 cells in phagocyte-independent inflammation, mediated through strong antibody responses and eosinophil accumulation (Romagnani, 2000). The differences between Th1 and Th2 are visualized below in Table 1. There are other Th subsets which have been found, including Th17, follicular helper T cells (Tfh), induced Tregs (iTreg) and regulatory type I (Tr1) cells (Luckheeram et al., 2012).

Table 1. Differences Between Th1 and Th2 Responses

	Th1	**Th2**
Cytokine Secretion	IL-2, IFN-g, TNF-beta	IL-4, IL-5, IL-6, IL-9, IL-13, IL-25
Inflammation	Phagocyte-dependent	Phagocyte-independent
Primary Function	Role in fighting off viral infections	Role in allergy responses, anti-helminth responses
Associated Autoimmune Diseases	Organ specific (Hashimoto's thyroiditis, MS, T1D, Crohn's, sarcoidosis, acute	Atopic Disorders, successful pregnancy, Omenn's Syndrome,

	kidney allograft rejection, some recurrent abortions)	Progressive Systemic Sclerosis, Cryptogenic Fibrosing Alveolitis
Differentiation Onset	IFN-g transcription (either TCR dependent or by IL-12 and IL-18)	CD28-dependent IL-4 production (reaches threshold becomes Th2)
Associated STAT	STAT4	STAT6
Associated transcription factor	T-Bet	GATA-3

Table 1: The differences between Th1 responses and Th2 responses are summarized here.

Vitamin D Receptor Pathway in the Immune Response

Vitamin D is a fat-soluble steroid that is essential for human biological processes, especially metabolism and calcium homeostasis. Vitamin D can be ingested in the form of diet or supplements, or can be converted in the skin from sunlight. Vitamin D is technically considered a hormone, not a vitamin, because it is readily synthesized in humans and animals that are exposed to adequate sunlight, and therefore not an essential part of dietary requirements. There are two major forms of vitamin D, cholecalciferol (vitamin D3) and ergocalciferol (vitamin D2), which can both be found in diet, however, cholecalciferol can also be synthesized from cholesterol when the skin is exposed to UVB radiation (at wavelengths of 290-315 nm). This produces a biologically inactive form, which is bound to vitamin D binding protein in the circulation and transported to the liver and kidney to be hydroxylated to produce the biologically active form, calcitriol (1,25(OH)2D). Cholecalciferol is first converted in the liver to the metabolite, calcifediol

(25-hydroxycholecalciferol), and ergocalciferol is first converted to the metabolite, 25-hydroxyergocalciferol, both of which are collectively known as 25(OH)D and used as biomarkers for vitamin D levels in the serum. Calcitriol regulates the concentration of calcium and phosphate in the blood by increasing reabsorption of calcium in the kidneys, dietary calcium from the GI tract and stimulating the release of calcium from bone (Kamen & Tangpricha, 2010).

The vitamin D nuclear receptor (VDR), also known as the calcitriol receptor, is found throughout the body in keratinocytes, macrophages and body tissues in both males and females, but it is also found in the human cycling endometrium in women (Proal, Albert, & Marshall, 2009). Vitamin D3 is produced through a pathway of steps beginning in the skin using UV light induced photolytic conversion of 7-dehydro- cholesterol, from which carbon 25 is hydroxylated in the liver and then enzymatically converted in the kidney. Vitamin D3 acts as ligand which binds to VDR, which then undergoes heterodimerization with the retinoid X receptor, binds to VDREs and recruits nuclear proteins into the transcriptional complex (Valdivielso & Fernandez, 2006).

The VDR is involved in the innate immune response through recognition of antimicrobial peptides (AmPs) and cathelicidin and 1,25-D activates the VDR to upregulate or down regulate at least 913 genes (Proal et al., 2009). The VDR might have a more widespread effect on hormonal balances than was originally anticipated because VDR dysregulation prevents the breakdown of calcitriol. When there are much higher levels of calcitriol, *in silico* data shows that it is capable of binding to other receptors (such as thyroid, glucocorticoid, and androgen), which then displaces their natural ligands and throws the body system into hormonal imbalance (Proal et al., 2009). This ultimately

leads to higher bacterial loads which might make it harder for the body to distinguish helpful gut bacteria from harmful antigens and pathogens.

Vitamin D Receptor Polymorphisms and SLE

Genetic variants in the VDR have been reported in several autoimmune diseases including SLE. These polymorphisms can lead to defects in numerous biological processes because the VDR is a nuclear transcription factor that is involved in gene activation, calcium metabolism, cell proliferation and immune function. The polymorphisms can change the protein sequence which alters some or all of the VDR's functions. Although there is not a definitive known reason why VDR gene polymorphisms are linked to T-cell mediated autoimmune diseases, it has been hypothesized that it's because vitamin D compounds are known to suppress T-cell activation by binding to the VDR. The use of vitamin D3 in the treatment of SLE has been considered because it can modulate inflammatory cytokine production (Vinh Quoc Luong & Nguyen, 2012). There are several other vitamin D related symptoms seen in SLE such as abnormal bone metabolism and disturbances in the calcium-parathyroid hormone (PTH)-vitamin D axis (Cutillas-Marco, Morales-Suárez-Varela, Marquina-Vila, & Grant, 2010), high prevalence of vitamin D2 deficiency in Brazilian patients with SLE and 85% of Spanish patients demonstrated suboptimal levels of vitamin D (Fragoso et al., 2012). In a study conducted in the Mediterranean region, about 95% of patients with SLE had very low serum 25OHD levels, which correlated with higher levels of PTH (Cutillas-

Marco et al., 2010). Expression of HLA-DR was significantly elevated in the CD4+ and CD8+ circulating T cells in patients with active SLE. This was also correlated with disease activity when compared to healthy controls (Vinh Quoc Luong & Nguyen, 2012). Genetic polymorphisms in the VDR have been shown to have an important role in T-cell mediated autoimmune diseases however the exact reason why is not known.

Estrogen Receptors

Estrogen receptors are found in the nucleus of cells where estrogens diffuse and bind to them, leading to a pathway which ultimately leads to either increased or decreased mRNA levels, and a physiological response (Deroo & Korach, 2006). There are two main forms of estrogen receptors, estrogen receptor alpha, which is the more well-known estrogen receptor that is involved in the classical estrogen effects, and estrogen receptor beta, whose affects are less well known. Estrogen receptor beta may have a minor role in the classical targets but has also been shown to have a role in the brain, cardiovascular system and colon, where it is expressed primarily in epithelial cells. Estrogen receptor beta is the most abundant estrogen receptor in the colon and complete absence of estrogen receptor beta expression is associated with disrupted tight junction formation and abnormal colonic architecture (Looijer-van Langen et al., 2011). The Looijer-van Langen et al study suggested a potential role for estrogen receptor beta signaling in the modulation of epithelial permeability and demonstrated reduced estrogen receptor beta mRNA in animal models of colitis and the colons of patients with irritable

bowel syndrome (IBS). Proinflammatory cytokines, such as TNF-alpha and IL-1beta, have been shown to downregulate estrogen receptors (Yang et al., 2018). They hypothesize that the reduction in estrogen receptor beta mRNA levels prior to the onset of colitis in mouse models could be related to increased levels of proinflammatory cytokines that are present prior to the onset of inflammation in these models. The estrogen receptor pathways have been shown to be important in not only autoimmune diseases, but also certain cancers, cardiovascular disease and obesity (Deroo & Korach, 2006). Estrogen receptors, through known agonists and antagonists, play important roles in cancer treatment and have been looked at in the context of treating autoimmune diseases.

Intestinal Epithelial Barrier/ Tight Junctions

The intestinal epithelial barrier covers a surface of about 400 square meters and requires approximately 40% of the body's calorie expenditure. It is comprised of a single layer of cells that line the gut lumen and act as a barrier and selective filter. It keeps toxins, foreign antigens and microorganisms out and allows translocation of helpful substances, such as dietary nutrients, electrolytes and water from the lumen to the circulation. The barrier consists of adherens junctions and tight junctions which function together to regulate cellular proliferation, polarization and differentiation (Groschwitz & Hogan, 2009). The intestinal barrier is the interface between host and microbial environment, preventing water and electrolyte loss while being selectively permeable to nutrients from the diet and other microbial products. The adaptive role of the barrier

seems to be to allow a symbiotic relationship with the intestinal microbiota without eliciting chronic inflammation while at the same time allowing for a defensive response against pathogens (Bischoff et al., 2014).

Tight junctions serve as selective, semi-permeable paracellular barriers which allows certain ions and solutes to cross through while preventing larger harmful substances (such as microorgansims) from passing through. Tight junctions have several integral membrane proteins including, occludins, claudins and junctional adhesion molecules which are specific for tight junctions. Normal expression of these membrane proteins and intact epithelial tight junctions are required for a healthy gut system. In diseases such as irritable bowel disease (IBD) and Crohn's disease, colon biopsies show reduced expression of certain claudins and occludins (Chiba, Osanai, Murata, Kojima, & Sawada, 2008). Intestinal barrier function, through modulation of tight junctions, might prove to have an important role in a host of autoimmune diseases.

CXCR3 Pathway and SLE

The chemokine receptor CXCR3 pathway, which is involved in regulating leukocyte trafficking, may be involved in the worsening of disease symptoms of autoimmune diseases by creating local amplification loops of inflammation in the target organs of the disease. This is relevant to SLE because high levels of CXCR3 were found

in skin lesions of patients with discoid lupus, where the increased expression is caused by UV light and leads to CXCR3+ lymphocytes and plasmacytoid DCs homing to the site of injury in the skin (Lacotte, Brun, Muller, & Dumortier, 2009). In another study, renal biopsies of patients with lupus nephritis showed an enrichment of CXCR3+ CD4+ T cells and also had higher levels in the urine than in the peripheral blood (Enghard et al., 2009). The CXCR3 receptor ligand complex is important in the chemotaxis of immune cells and angiogenesis. CXCR3 is expressed on activated T cells, NK cells, DCs, and B cells. It is preferentially expressed by Th1 polarized memory T cells.

Chapter II.

Materials and Methods

DNA was collected using AncestryDNA for each of the subjects' personal heritage and was not originally intended for this thesis project. Verbal consent was given and raw data files were downloaded from Ancestry.com and run through Promethease.com in accordance with Harvard's Internal Review Board (IRB18-0286). Raw DNA files were collected from a family of five; father, mother, and three daughters. The father has been diagnosed with systemic lupus erythematosus (Subject 1), the mother has been diagnosed with psoriasis (Subject 2), one of the daughters has been diagnosed with Hashimoto's disease (Subject 3), the other two daughters are autoimmune disease free (Subject 4 and Subject 5) (Table 2).

Table 2. Description of the Family Included in this Case Study

Subject ID	**Gender**	**Age**	**Relationship**	**Health Status**
Subject 1	Male	68	Father	SLE
Subject 2	Female	63	Mother	Psoriasis
Subject 3	Female	33	Daughter	Hashimoto's
Subject 4	Female	31	Daughter	Healthy
Subject 5	Female	27	Daughter	Healthy

Table 2: Description of the family studied in this case study. The father has been diagnosed with SLE, the mother has been diagnosed with psoriasis, the eldest daughter has been diagnosed with Hashimoto's thyroiditis, and the two younger daughters are autoimmune disease free.

AncestryDNA uses microarray-based autosomal DNA testing which provide an efficient means of identifying single nucleotide polymorphisms (SNPs) in DNA samples. "All DNA microarray methods for detecting sequence differences rely on the chemistry of DNA duplex formation. Under appropriate reaction conditions, duplexes that are perfectly complementary in their DNA sequence are strongly favored over duplexes that contain one or more mismatched bases. In a typical experiment, the efficiency of duplex formation is measured by labeling a DNA sample with a fluorophore and quantifying the fluorescent signal at thousands to millions of probes following a hybridization reaction. Sample DNA fragments that are perfectly complementary to the probe sequence will exhibit maximal fluorescent signals, whereas the presence of even a single base difference that reduces complementarity results in diminished signals" (Gresham, 2011). AncestryDNA looks at over 650,000 different SNPs, most of which have not been categorized in the context of their related genes and phenotypes.

The raw data files collected from AncestryDNA were run through Promethease.com, which is a website that provides a literature retrieval system for SNPs that are found in current scientific and medical literature. The website generates a personalized report that is searchable via SNP numbers or disease areas. These data files were combed through in order to find SNPs that were relevant to the diseases of interest through both the Promethease website as well as outside literature searches.

First, all of the SNPs that are currently correlated with the autoimmune diseases, SLE, psoriasis and Hashimoto's thyroiditis within the Promethease website were analyzed for each subject and graphed. Next, all the SNPs associated with other known autoimmune diseases were analyzed for each subject in order to see if there were any

common deleterious SNPs among the subjects with autoimmune diseases. Then an extensive literature search, using Harvard's Hollis library search and Pubmed, for autoimmune disease SNPs was performed and these SNPs were then entered into Promethease for each subject to see if they were captured in the AncestryDNA data set.

The literature search included other factors, besides solely genetic variants, that have been shown to be risk factors for autoimmune diseases. These risk factors, which are overviewed in the intro section include, the IL-21 pathway, vitamin D receptor polymorphisms, estrogen receptors, intestinal barrier functions, and the CXCR3 pathway were then looked at in the context of which SNPs could then be searched for through Promethease for each subject. This gave a broader look at the genetic variants that were affecting known SNPs as well as SNPs that themselves were known to be risk factors for autoimmune diseases.

Chapter III.

Results

A genetic study of a family of five members was carried out in order to determine if single nucleotide polymorphisms (SNPs) known to be associated with autoimmune diseases are the reason for the autoimmune disease incidence found in the family. The family consists of three members that have been diagnosed with different autoimmune diseases, SLE, Psoriasis and Hashimoto's Thyroiditis (see Table 2 in Materials and Methods above). The DNA was originally collected by Ancestry.com for the purpose of looking at genetic ancestry and then the raw data was collected and run through Promethease.com. In total over 650,000 SNPs were isolated and analyzed. A literature search for SNPs that are associated with autoimmune diseases was performed and analyzed for SNPs relevant to this biologically related family. The main goal was to have a clearer understanding of what factors are responsible for the autoimmune diseases in this family, a single SNP, a combination of SNPs, environmental factors, or environmental factors acting in concert with a combination of SNPs.

This case study involved five members of the same family, of which the father (Subject 1) has systemic lupus erythematous (SLE). Since SLE has a much higher predominance among women, with as much as 85-90% of patients being women (Budarf et al., 2011), the researcher chose to focus this thesis on the genetic factors that tend to lead to SLE, as well as the genetic variants that can be influenced by environmental factors. This genetic analysis was done in order to determine if there is clear genetic information that could help explain why the male subject has SLE while his wife (Subject

2) who has a separate autoimmune disease (psoriasis), and his three biological daughters (Subjects 3, 4, 5) of which only Subject 3 has a separate autoimmune disease (Hashimoto's thyroiditis), do not (see Table 2 in Materials and Methods).

Search and Identification of Disease-Associated MHC SNPs

There were well over one hundred MHC related SNPs captured in the data sets received from each subject, so the researcher searched through Promethease's repertoire to find ones that have been implicated in autoimmune diseases. Table 12 (appendix) shows a representative list of thirty seven of the over one hundred SNPs analyzed and which MHC gene and class they belong to. The most interesting MHC results were found for Subject 2, who has psoriasis, and has the deleterious alleles for HLA-B27 (G for SNP rs13202464, G for SNP rs4349859, and C for SNP rs3819299). The HLA-B27 haplotype was one of the first studied in the context of autoimmune diseases and has been implicated in the onset of several autoimmune diseases.

Identification of SNPs in MHC-II Associated Proteins

Another MHC II associated protein that has been implicated in autoimmune diseases is HLA-DM, also known as a nonclassical MHC II gene. Only one SNP

associated with HLA-DM was tested in our case study (SNP rs1063478) and each subject was homozygous for the normal allele (Table 12, appendix). Thus, although certain HLA-DM alleles may confer a high risk of autoimmunity based on reduced catalytic efficiency of peptide editing, that does not seem to be the case in any of the subjects tested here. Only one SNP associated with HLA-DO was tested in our case study (SNP rs2284191) and Subject 1 and Subject 5 were heterozygous for the potential risk allele (A), while Subjects 2, 3, and 4 were homozygous for the non-risk allele (G) (Table 12, appendix). Further analysis of this SNP will be needed in the context of autoimmune diseases.

Identification of SNPs Associated with TAP Protein

Transporter Associated with Antigen Processing (TAP) is a heterodimer made up of the proteins TAP1 and TAP2, which are encoded in the class II region of the HLA locus (6p21.32), and is a member of the ATP-binding cassette family (D. L. Zhou & Blum, 2002). If cells do not have functional TAP1 or TAP2 genes they will express very little surface MHC-I because peptides are not able to be imported into the ER. The single nucleotide polymorphism, rs1800454, encodes the TAP2 gene. Subject 2, who has psoriasis, is a carrier of a deleterious allele for this SNP. Subject 1 is homozygous for the normal allele, A. Subjects 3, 4, and 5 are heterozygous for the deleterious allele, G (Table 3). The SNP encoding TAP1, rs2071480, was not captured in this data set. In sum, these

findings show that TAP 2 SNP variants were found in this family, and the mother has two copies of the deleterious allele.

Table 3. TAP1 and TAP2 SNPs

SNP	**Gene**	**Subject 1**	**Subject 2**	**Subject 3**	**Subject 4**	**Subject 5**
rs2071480	TAP1	Not Tested	Not Tested	Not Tested	Not Tested	Not Tested
rs1800454	TAP2	A; A	G; G	A; G	A; G	A; G

Table 3: SNPs associated with components of the TAP heterodimer. Red letters denotes the harmful allele. Abbreviations: (A) adenine, (C) cytosine, (G) guanine, (T) thymine.

Immunoproteasome Involvement in Autoimmune Diseases

Another genetic factor that may be involved in the development of autoimmune diseases is a mutation in the immunoproteasome. The immunoproteasome is expressed in immune cells and is involved in proteolysis. There are two important subunits that are exchanged, Beta-1 and Beta-5. Beta-1 is replaced by the large multifunctional peptidase 2 (LMP2 aka proteasome subunit beta type 9 (PSMB9)) and Beta-5 is replaced by the large multifunctional peptidase 7 (LMP7 aka proteasome subunit beta type 8 (PSMB8)). The major SNP associated with LMP2 is rs17587 and the major SNP associated with LMP7 is rs2071543. In our study for SNP rs17587, Subject 1 was homozygous for the risk allele, A; Subject 2 was homozygous for the protective allele, G; and Subjects 3, 4, and 5 were heterozygous. The SNP for LMP7 was not tested in these subjects (Table 4). These findings showed that harmful alleles for the SNP associated with LMP2 were identified

in the subject with SLE. Further studies are required to determine if there is a role for SNP rs17587 in the development of SLE.

Table 4. Large Multifunctional Peptidases (LMPs) Altered in Subject with SLE

SNP	**Gene**	**Subject 1**	**Subject 2**	**Subject 3**	**Subject 4**	**Subject 5**
rs17587	LMP2	A; A	G; G	A; G	A; G	A; G
rs2071543	LMP7	Not tested	Not tested	Not tested	Not tested	Not tested

Table 4: SNPs associated with large multifunctional peptidases. Red denotes the harmful allele.

Abbreviations: (A) adenine, (C) cytosine, (G) guanine, (T) thymine.

Role of TNF Genes in Autoimmune Disease Development

Another set of genes located near the MHC genes on chromosome six are the tumor necrosis factor (TNF) genes, which are located in the class III region. TNF-alpha, a proinflammatory cytokine, has been implicated in the onset of SLE as well as various other autoimmune diseases. Serum TNF-alpha levels were significantly increased in SLE patients over controls with no autoimmune disease for all ancestral backgrounds tested (African-Americans, European Americans and Hispanic Americans) (Weckerle et al., 2012). There are several SNPs that are associated with TNF that confer a risk of SLE. Some of the more commonly studied ones are SNP rs3850641, which is a polymorphism in the TNFSF4 gene, SNP rs8110090, which is a polymorphism in the TGF-beta1 gene,

and SNP rs25882, which is a polymorphism in the CSF2 gene. In this case study, all subjects, including Subject 1, who has SLE, had the wild type form of rs3850641 and rs8110090. None of the subjects were tested for rs25882 (Table 5).

The TNF-alpha inducible protein 3 (TNFAIP3) gene is induced by TNF and encodes a zinc finger protein that inhibits NF-kappa B and TNF-mediated apoptosis. The deleterious allele (A) is associated with SLE, type 1 autoimmune hepatitis risk and rheumatoid arthritis risk (Adrianto et al., 2011). Subject 3 and Subject 4 have the harmful homozygous form of SNP rs6920220 (A; A) while Subject 1 and Subject 2 have the heterozygous form (A; G) (Table 5). Of the subjects in this case study that are either homozygous or heterozygous for the A risk allele, three of the four have some form of autoimmune disease. Of the two subjects who are homozygous for this allele only one has an autoimmune disease, this may lend credence to the hypothesis that environmental factors also need to be present in order to for autoimmune diseases to begin. The fact that Subject 3 and Subject 4 have the homozygous deleterious allele for TNFAIP3, while Subject 1 and Subject 2 do not, may suggest that risk polymorphisms could be shared between several autoimmune diseases. The risks may not be highly significant for any given population in a given study, because there is a wide breadth of other polymorphisms that could lead to the same outcome. In other words, each autoimmune disease might be caused by unique sets of polymorphisms in any given individual that work in synergy to cause the disease.

Table 5. SNPs Associated with TNF Genes

SNP	**Gene**	**Subject 1**	**Subject 2**	**Subject 3**	**Subject 4**	**Subject 5**

rs3850641	TNFSF4	C; C	C; C	C; C	C; C	C; C
rs8110090	TGF-β1	A; A	A; A	A; A	A; A	A; A
rs25882	CSF2	Not tested	Not tested	Not tested	Not tested	Not tested
rs6920220	TNFAIP3	A; G	A; G	A; A	A; A	G; G

Table 5: SNPs associated with TNF genes. Red letters denote the harmful allele. Abbreviations: (A) adenine, (C) cytosine, (G) guanine, (T) thymine.

Analysis of Common SLE Risk Loci

Suarez-Gestal et. al tested and confirmed eleven SLE risk loci that were previously described. Of these twelve loci, two SNPs were not tested in the five subjects (SNP rs2476601 in the PTPN22 gene and SNP rs2004640 in the IRF5 gene). Five of the SNPs were normal among all subjects (SNP rs7574865 in the STAT4 gene, SNP rs17435 in the MECP2 gene, SNP rs4963128 in the KIAA1542 gene, and SNP rs6445975 in the PXK gene, SNP rs17266594 in the BANK1 gene). One SNP was found to have both risk alleles in a subject other than Subject 1 (SNP rs13277113 in the C8orf13-BLK gene by Subject 3) but heterozygous for all other subjects. Two SNPs were found to have the risk alleles by Subject 1 and another subject (SNP rs2304256 in the TYK2 gene by Subject 1 and Subject 5, and SNP rs509749 in the LY9 gene by Subject 1 and Subject 2). Two SNPS were found to have the risk alleles in Subject 1 alone (SNP rs1143679 in the

ITGAM gene and SNP rs10798269 in the 1q25.1 gene). In total Subject 1, the subject who has been diagnosed with SLE, has the risk alleles for five out for the twelve loci (with two loci's SNPs not tested). Subject 1 has the risk alleles for the ITGAM gene, C8orf13-BLK gene, TYK2 gene, 1q25.1 and LY9 gene. These are visualized below in Table 6.

Table 6. Common SLE Risk Loci

Gene	**SNPI**	**Subject 1**	**Subject 2**	**Subject 3**	**Subject 4**	**Subject 5**
PTPN22	rs2476601	Not tested	Not tested	Not tested	Not tested	Not tested
IRF5	rs2004640	Not tested	Not tested	Not tested	Not tested	Not tested
ITGAM	rs1143679	A; A	G; G	A; G	A; G	A; G
STAT4	rs7574865	G; G	G; G	G; G	G; G	G; G
C8orf13-BLK	rs13277113	A; G	A; G	A; A	A; G	A; G
TYK2	rs2304256	C; C	A; C	A; C	C; C	C; C
MECP2	rs17435	A; A	A; A	A;A	A; A	A; A
1q25.1	rs10798269	A; A	G; G	A; G	A; G	A; G
PXK	rs6445975	T; T	T; T	T; T	T; T	T; T
BANK1	rs17266594	G; G	G; G	G; G	G; G	G; G
KIAA1542	rs4963128	G; G	A; G	G; G	G; G	G; G
LY9	rs509749	A; G	A; G	G; G	G; G	G; G

Table 7: Common SNPs and genes associated with SLE. Red letters denote the harmful alleles. Abbreviations: (A) adenine, (C) cytosine, (G) guanine, (T) thymine.

IL-21 Associated SNPs

IL-21 has been shown to be dysregulated in SLE and seems to have a genetic component. Sawalha et. al found a genetic association between SLE and two SNPs within in the IL-21 gene; rs907715 and rs2221903 (Sawalha et al., 2008). Subject 1 is homozygous for the deleterious A allele of rs907715, while Subject 2 is homozygous for the beneficial G allele and Subjects 3, 4, and 5 are heterozygous. Subjects 1, 3, and 5 are homozygous for the risk allele A for SNP rs2221903. Among the other SNPs that Subject 1 has that confers risk for SLE, he is homozygous for the deleterious allele for both SNPs associated with IL-21 (Table 7). Subject 1, who has SLE, has both risk alleles for two SNPs in the IL21 gene.

Table 7. IL21 Gene SNPs

SNP	**Gene**	**Subject 1**	**Subject 2**	**Subject 3**	**Subject 4**	**Subject 5**
rs907715	IL21	A; A	G; G	A; G	A; G	A; G
rs2221903	IL21	A; A	A; G	A; A	A; G	A; A

Table 7: SNPs associated with the IL21 gene. Red letters denote the harmful allele. Abbreviations: (A) adenine, (C) cytosine, (G) guanine, (T) thymine

Search and Identification of SNPs in Proinflammatory Pathways

In this case study there were fifteen SNPs that correlate with proinflammatory cytokines as per (Riva et al., 2009), two SNPs that correlate with IgE levels, as per

(Mehta et al., 2008), and seven SNPs that correlate with STAT6, as per searching through Promethease for SNPs related to STAT6. These findings are summarized in Table 14 (appendix). For most of the proinflammatory SNPs, the risk alleles are known, and are the ancestral allele, because mutations that decrease the rate of proinflammatory cytokines are beneficial in decreasing the amount of inflammation. For the IgE SNPs, the beneficial alleles, A for rs2427837 and C for rs2251746, are correlated with lower IgE levels, while the deleterious alleles, G for rs2427837 and T for rs2251746, are the ancestral alleles and the most common ones in the population. The STAT6 SNPs are less well studied and so the beneficial and/or deleterious alleles are not categorized. All five subjects have the ancestral allele for most of the SNPs analyzed. Subject 1 has both non-ancestral alleles for SNP rs6822844, in the IL2/IL21 gene and a copy of the beneficial allele for SNPs rs2427837 and rs2251746 in the gene associated with IgE.

Identification of Vitamin D Receptor Polymorphisms in SLE

Restriction fragment length polymorphisms (RFLPs) are a type of polymorphism that does not alter protein structure even though they occur in the exonic sequences of DNA. They are synonymous polymorphisms that may alter the sites where the restriction enzymes cut the DNA sequences (Valdivielso & Fernandez, 2006). There are a few RFLPs of the VDR that have been shown to have strong linkage to autoimmune diseases in numerous studies, these RFLPs are termed: Taq1, Bsml, EcoRV and ApaI.

Subject 1 has the BsmI polymorphism in the VDR SNP rs1544410 and the TaqI VDR polymorphism in the SNP rs731236. Subject 2 is homozygous for the ancestral alleles of both of these RFLPs and Subjects 2, 3, and 5 are heterozygous for both. All subjects are homozygous for the ancestral allele of ApaI and none of the subjects were tested for the FokI SNP (Table 8).

Table 8. VDR Polymorphisms

RFLP	**SNP**	**Subject 1**	**Subject 2**	**Subject 3**	**Subject 4**	**Subject 5**
BsmI	rs1544410	A; A	G; G	A; G	A; G	A; G
ApaI	rs7975232	A; A	A; A	A; A	A; A	A; A
TaqI	rs731236	C; C	T; T	C; T	C; T	C; T
FokI	rs2228570	Not tested	Not tested	Not tested	Not tested	Not tested

Table 8: SNPs associated with VDR polymorphisms. Red letters denote harmful alleles and green letters denote beneficial alleles. Abbreviations: (A) adenine, (C) cytosine, (G) guanine, (T) thymine

Identification of Estrogen Receptor Polymorphisms Associated with SLE

Estrogen receptor polymorphisms have been linked to SLE incidence. The estrogen receptor alpha polymorphisms, termed PvuII for SNP rs2234693 and XbaI for SNP rs9340799, were significantly associated with disease susceptibility (C. Wang et al., 2009). In this case study Subject 1 and Subject 5 have the risk allele for SNP rs2234693,

C. This allele is highly associated with malar rash and less severe disease manifestations, such as skin symptoms (A. Zhou et al., 2017), which is the main lupus symptom that Subject 1 experiences. The G allele of SNP rs9340799 is associated with greater photosensitivity (A. Zhou et al., 2017) however all subjects were homozygous for the A allele for this SNP. Polymorphisms for estrogen receptor alpha and estrogen receptor beta (of which the risk alleles are less well known) are visualized below in Table 9. Subject 1, the subject with SLE is heterozygous for a polymorphism in the estrogen receptor alpha and homozygous for the harmful allele in an estrogen beta receptor.

Table 9. Estrogen Receptor Polymorphisms

SNP	**Gene**	**Subject 1**	**Subject 2**	**Subject 3**	**Subject 4**	**Subject 5**
rs2234693	ER-alpha	C; T	T; T	T; T	T; T	C; T
rs9340799	ER-alpha	A; A	A; A	A; A	A; A	A; A
rs1256049	ER-beta	G; G	G; G	G; G	G; G	G; G
rs4986938	ER-beta	A; A	G; G	A; G	A; G	A; G
rs728524	ER-beta	A; A	A; A	A; A	A; A	A; A
rs1255998	ER-beta	C; C	C; C	C; C	C; C	C; C
rs1256030	ER-beta	C; C	T; T	C; T	C; T	C; T

Table 9: SNPs associated with estrogen receptors. Red letters denote harmful alleles. Abbreviations: (A) adenine, (C) cytosine, (G) guanine, (T) thymine

CXCR3 Pathway

There are a few SNPs encoding the CXCR3 gene that have been identified, including, rs1003951195, rs1004086764, rs1004470882, rs1005095693 and rs1005472899, however, none of these SNPs were tested in this case study (Table 10). One SNP that encodes the CXCR3 gene was tested in this case study but has not been widely studied in the context of autoimmune disease, but as the CXCR3 pathway most likely plays a role in disease progression it could be of relevance. This SNP is rs2280964 located on the X chromosome. The ancestral allele is C and the potential risk allele is T, Subject 1 and Subject 5 are homozygous for the potential risk allele, and Subjects 2, 3 and 4 are heterozygous (Table 10). This T allele of this SNP was shown to confer disease susceptibility in asthma patients and further in vitro testing showed that this allele was significantly associated with decreased CXCR3 gene expression and a decrease in chemotactic activity (J. W. Choi et al., 2008). In this case study, Subject 1 is homozygous for the risk allele and has SLE but Subject 5 is also homozygous for the risk allele and has no disease manifestations.

Table 10. CXCR3 Gene SNPs

SNP	**Gene**	**Subject 1**	**Subject 2**	**Subject 3**	**Subject 4**	**Subject 5**
rs1003951195	CXCR3	Not tested	Not tested	Not tested	Not tested	Not tested
rs1004086764	CXCR3	Not tested	Not tested	Not tested	Not tested	Not tested
rs1004470882	CXCR3	Not tested	Not tested	Not tested	Not tested	Not tested

rs1005095693	CXCR3	Not tested	Not tested	Not tested	Not tested	Not tested
rs1005472899	CXCR3	Not tested	Not tested	Not tested	Not tested	Not tested
rs2280964	CXCR3	T; T	C; T	C; T	C; T	T; T

Table 10: SNPs associated with the CXCR3 gene. Red letters denote harmful alleles. Abbreviations: (A) adenine, (C) cytosine, (G) guanine, (T) thymine

Summary of Findings for Subjects with Autoimmune Diseases

Subject 1 was diagnosed with SLE in his mid 20's. He has at least 26 SNPs with harmful haplotypes (Table 14, appendix). Some of these SNPs confer solely genetic susceptibility while others are associated with environmental risk factors. Subject 2 was diagnosed with psoriasis as a child, her symptoms flare up at certain times and are quiescent at others. She has 3 harmful SNPs associated with the HLA-B27 MHC haplotype which has been shown in numerous studies of different autoimmune diseases, the deleterious haplotype for MHC I antigen presentation via TAP (deficiency in TAP2), and the harmful haplotype for a SNP associated with psoriasis and psoriatic arthritis (Table 11). Subject 3 was diagnosed with Hashimoto's thyroiditis in her early 20's. The only known SNP associated with this disease was not captured in this data set. In sum, Promethease software was used to search through over 650,000 SNPs in a family case study and hundreds of autoimmune disease associated SNPs were found. These SNPs were narrowed down to look at the autoimmune diseases that occur within the family, SLE, psoriasis and Hashimoto's thyroiditis. The subject with SLE had dozens of SNPs

associated with his disease and the subject with psoriasis had five SNPs associated with her disease.

Table 11. Summary of Subject 2's Risk Allele SNPs

SNP	**Gene**	**Haplotype**	**Risk**
rs13202464	HLA-B27	A; G	Assoc. w/ several AID
rs4349859	HLA-B27	A; G	Assoc. w/ several AID
rs3819299	HLA-B27	A; C	Assoc. w/ several AID
rs1800454	TAP2	G; G	Impaired MHC I antigen processing
rs3212227	IL12B	A; A	Psoriasis and psoriatic arthritis

Table 11: Summary of SNPs associated with psoriasis and/or other autoimmune diseases for Subject 2. Red letters denote harmful alleles. Abbreviations: (A) adenine, (C) cytosine, (G) guanine, (T) thymine

Chapter IV.

Discussion

Autoimmune disease incidence in the US has been steadily rising while the cause of this upsurge is currently unknown. Autoimmune diseases are hard to diagnose, hard to treat, and yearly health care costs tied to treatment are upwards of $100 billion dollars in the US. As such, a considerable amount of research has gone in to determining the root cause of these diseases. Unfortunately, while there are certain biomarkers that determine which patients have which disease (such as autoantibody production), many patients can go years with symptoms before being diagnosed and the definitive cause of why the body suddenly turns on itself remains elusive.

Women have a much higher risk of developing autoimmune diseases than men. The National Institutes of Health (NIH) and the American Autoimmune Related Diseases Association (AARDA) estimate that anywhere between 25 million and 50 million people in the US are living with some form of autoimmune disease (sometimes more than one) and of these about 75-80% are women. The reason for the disparity in disease incidence between the sexes is currently unknown, but it is hypothesized to be a risk of genetic and environmental factors.

Although there are strong genetic components for many autoimmune diseases, genetics alone cannot solely determine whether someone will develop the disease. Twin studies have been conducted in several autoimmune diseases and they show varying levels of concordance, which shows that some autoimmune diseases are more closely tied to genetics than others. Some autoimmune diseases, such as celiac disease, show up to 75-85% concordance rates among monozygotic twins, while others, such as rheumatoid

arthritis, only show 15-30% concordance rates (Bogdanos et al., 2011). This implies that environmental factors must also be important in the onset of autoimmune disease.

This thesis study aimed to explore the genetic and environmental factors of autoimmune diseases by looking at a family case study. The family consists of a father, mother and their three biological offspring. The father and mother have both been diagnosed with different autoimmune diseases, the father has lupus and the mother has psoriasis, but only one of their three daughters has her own autoimmune disease, Hashimoto's thyroiditis (Table 2 in the Materials and Methods section). Raw DNA was collected and analyzed using Promethease.com, which provides a database of SNPs associated with diseases in medical literature. This study aimed to elucidate if specific genetic components could be found or if varying environmental factors that the subjects were exposed to could be the underlying cause. By combing through published literature and comparing hypothesized risk alleles of certain SNPs, a clearer understanding of the genetic components of each subjects autoimmune disease was obtained.

For this thesis, I first looked broadly at SNPs related to all autoimmune diseases to try and determine if I could figure out what was causing the disease incidence for each of the three subjects that are afflicted with autoimmune diseases. I then chose to mainly focus on the SNPs associated with lupus because this autoimmune disease predominantly occurs in women but the subject from this family that has been diagnosed is a male and none of his female daughters are inflicted. I wanted to see which genetic risk factors the father had and whether he passed these on to his daughters. If the daughters had a significant level of these deleterious alleles in SNPs correlated to lupus, this would

suggest that certain environmental risk factors were present in the father but not in the daughters.

The original hypothesis was that the likelihood of inheriting the genetic haplotypes that are associated with autoimmune diseases will be similar between males and females and that an environmental factor is to blame for the discrepancy in rates of incidence between the sexes. There seems to be a correlation between vitamin D deficiency and certain autoimmune diseases such as multiple sclerosis (Mahon et al., 2003), rheumatoid arthritis (Als et al., 1987), type 1 diabetes (Hyppönen, Läärä, Reunanen, Järvelin, & Virtanen, 2001) and other autoimmune/inflammatory rheumatic diseases (Sainaghi, Bellan, Antonini, Bellomo, & Pirisi, 2011). However, this doesn't explain why women have a higher rate of autoimmune disease, as intake of vitamin D (from UVB exposure, diet and supplements) should be similar between the sexes. I hypothesized that the difference in sex hormones and how they interact with the gut and microbiome are influencing the uptake of the fat-soluble vitamin D and the interaction between immune cells, which could account for the Th2 vs. Th1 response. Research has shown that estrogen receptor-ß signaling modulates epithelial barrier function (Looijer-van Langen et al., 2011) and so a fluctuation in estrogen (as seen during the menstrual cycle) could potentially affect the tight junctions of the gut epithelium. This could adversely affect the uptake of many compounds, including vitamin D. A recent study showed that in men and women, low vitamin D was associated with lower sex hormone binding globulin and higher free testosterone levels, and in women low vitamin D was associated with low estradiol and higher DHEA levels (Billups et al., 2016). It is also anecdotally seen that women with certain autoimmune diseases (such as SLE and IBD)

tend to have flare ups in the days prior to menstruation, although more stringent research needs to be done to corroborate this. Literature is conflicted on whether there is a gender difference in the microbiome distribution, however more studies are pointing to a decreased level of composition of Bacteroides species in women (Dominianni et al., 2015). This thesis study touched on these ideas and delved into the specific SNPs that are associated with autoimmune diseases and SNPs that are influenced by the environment.

Determining Factors for Autoimmune Disease Incidence Among Subjects

Subject 3 has Hashimoto's thyroiditis, an autoimmune disease where the body attacks the thyroid gland, and is most common in women (at a ratio of 7:1) (Ledesma & Lawson, 2018). Unfortunately, this autoimmune disease has been less well categorized in terms of SNP correlation than some of the other autoimmune diseases. The only SNP from the literature that showed any correlation was a SNP in the TGF-β1 gene, rs1800469, which was not tested in this case study. So the underlying cause of Subject 3's autoimmune disease could not be determined based on SNP genetic data present. More GWA studies will need to be performed with larger patient sizes to see if there are any other SNPs associated with this disease.

Subject 2 has psoriasis, an autoimmune disease categorized by a reaction of the skin to activated immune cells and cytokines (Guttman-Yassky & Krueger, 2017). The underlying cause of her pathology is probably due to impaired class I antigen processing. She is a carrier of the harmful HLA-B27 haplotype. The HLA-B27 haplotype was first

discovered in 1973 in the context of ankylosing spondylitis (AS) and has since been implicated in not only the onset but also the disease progression of a host of other diseases. This harmful haplotype is in a class I molecule and so may disrupt the ability of the class I molecule to present antigen to CD8+ T cells but it may also have unknown functions unrelated to antigen presentation (Sheehan, 2010). The HLA-B27 haplotype has been implicated in autoimmune disease onset and transgenic rats (HLA-B27/human β2-microglobulin-transgenic) have a greater Th17 phenotype with greater IL-17A and TNF-alpha production (Glatigny et al., 2012). This is in accordance with Subject's 2 psoriasis diagnosis, as a greater Th17 phenotype is seen in patients with psoriasis (Guttman-Yassky & Krueger, 2017).

Subject 2 is also a carrier of a mutated TAP2 gene which is also associated with impaired class I antigen processing. TAP (Transporter Associated with Antigen Processing) is a heterodimer made up of the proteins TAP1 and TAP2, which are encoded in the class II region of the HLA locus (6p21.32), and is a member of the ATP-binding cassette family (D. L. Zhou & Blum, 2002). If cells do not have functional TAP1 or TAP2 genes they will express very little surface MHC-I because peptides are not able to be imported into the ER. Interestingly, in antigen presenting cells, MHC-II presentation of cytoplasmic antigens derived from cells without TAP was enhanced, which could potentially suggest that other mechanisms can make up for impaired processes for MHC I presentation (Gadola, Moins-Teisserenc, Trowsdale[3], Gross, & Cerundolo, 2000). Although she does have an autoimmune disease (psoriasis) she does not present with the symptoms one would expect from a patient with deficient antigen processing. This mutation typically denotes a syndrome called Bare Lymphocyte

Syndrome (BLS). BLS has a few different forms which have only been described in handfuls of patients. The more severe form presents with severe recurrent infections (bacterial, fungal and parasitic) that causes patients to die early in life. The less severe form presents with recurrent bacterial infections and necrotizing granulomatous skin lesions. Some patients (only two described in this paper) have no symptoms present (Gadola et al., 2000). Since Subject 2, as well as the other two patients described, have this mutation and are asymptomatic this could be a real life example of MHC class II processing being able to make up for cells with deficient MHC class I processing via TAP deficiency. Subject 2 also has the harmful HLA-B27 haplotype which is also a defect in class I antigen presentation, so her body may have had to make up for this defect with an increased Th17 and/or Th2 response, leading to induction of a mild psoriasis phenotype. Psoriasis is thought to be an overreaction of Th17 cells which induce Th17 cytokines (IL-17, IL-26, IL-29 and TNF) and then activate the NFkB pathway and STAT1 in skin resident cells (Guttman-Yassky & Krueger, 2017).

Subject 1 has lupus, which has been more widely studied in the context of risk alleles in SNPs. He has the homozygous or heterozygous form of the risk alleles for a total of 26 SNPs associated with SLE incidence (see Table 14 in appendix for full summary). The major SNPs associated with SLE that he has are in the genes for ITGAM, TYK2, TNFSF4 (1q25.1 locus), LY9, IL21, IL12B, IL23R, IL6R, the VDR polymorphisms Bsml and TaqI, and the estrogen receptor alpha polymorphism PvuII. Based on strong associations between the known SNPs and SLE reported in the literature, these risk alleles likely contributed to the probability that he would develop lupus.

The risk allele for SNP rs1143679 in the Integrin Subunit Alpha M (ITGAM) gene is A. Subject 1 is homozygous for this allele while Subject 3, Subject 4 and Subject 5 are heterozygous for it. This allele is the most commonly studied SNP associated with SLE and was once thought to be different between males and females, but is now thought to be shared between men and women equally (J. W. Choi et al., 2008). This polymorphism at SNP rs1143679 is a non-synonymous variant (meaning it's a nucleotide mutation that alters the amino acid sequence) at exon 3 of the ITGAM gene. It causes the conversion of arginine at amino acid position 77 to a histadine. The protein encoded by this gene is the alpha chain of the integrin subunit which has a role in the regulation of leukocyte activation and adhesion and migration from the bloodstream via interactions with a range of structurally unrelated ligands (Tao et al., 2010). This non-synonymous polymorphism can change the binding affinity of alphaMBeta2. In SLE patients with active disease, the levels of alphaMbeta2 were increased on neutrophils and are likely involved in endothelial injury. This SNP is hypothesized to disturb ITGAM interaction with its ligands, however more functional evidence is needed to corroborate this hypothesis (Suarez-Gestal et al., 2009).

Signal transducer and activator of transcription 4 (STAT4) is a transcription factor that is required for the development of Th1 cells from naïve CD4+ T cells. Interleukin 12 (IL-12) is the hallmark cytokine activator of STAT4, which stimulates it to form a homodimer which binds to DNA in the nucleus and activates gene transcription (Kaplan, 2005). The SNP rs7574865 is the haplotype that marks STAT4, in this study all the subjects were all homozygous for the most common allele (G), the other allele (T) has been shown to confer a risk of SLE and RA (Padyukov et al., 2007). Mice deficient in

STAT4 (STAT4-KO mice) have been shown to be protected from T-cell mediated autoimmune diseases but not antibody mediated autoimmune diseases, like SLE (Kaplan, 2005).

The SNP rs13277113 maps to the interval between the two genes, *BLK* and *C8orf13*. *BLK* is a tyrosine kinase from the src family and *C8orf13* is a widely expressed gene of which the function is unknown. The homozygous AA allele was correlated with lower mRNA expression of BLK and higher mRNA expression of C8orf13 and the heterozygous A; G was associated with intermediate levels (Petri et al., 2008). Subject 3 is homozygous for the risk allele (A) while the rest of the subjects are heterozygous.

The risk allele for SNP rs2304256 in the TYK2 gene is C which has been shown to be correlated with an increased risk of SLE. In this case study Subject 1 and Subject 5 are homozygous for the C allele while Subject 2, Subject 3 and Subject 4 are heterozygous. The TYK2 gene encodes the enzyme non-receptor tyrosine-protein kinase 2, which is one of the four members of the Janus kinase protein family that is bound to cytokine receptors and becomes activated after ligand binding, the other members are Jak1, Jak2 and Jak3. Deficiency of TYK2 leads to defects of multiple cytokine pathways, including type 1 interferon, IL-6, IL-10, IL-22 and IL-23. Mutations can also lead to impaired Th1 differentiation and accelerated Th2 differentiation (Suarez-Gestal et al., 2009).

X-chromosome methyl CpG binding protein 2 (MECP2) has been shown to be a genetic loci for SLE development and was once hypothesized to be the reason females are more prone to this disease. This hypothesis was put into question by Suarez-Gestal et. al because they found that the MECP2 SNP is also associated with SLE in men, as well

as the fact MECP2 was recently shown to not be expressed on the inactivated X chromosome (Suarez-Gestal et al., 2009) . All of the subjects are homozygous for the normal ancestral allele of the MECP2 SNP rs17435.

The genetic loci 1q25.1 has been a known risk loci for SLE for many years but was only recently discovered to code for Tumor Necrosis Factor Ligand Superfamily Member 4 (TNSF4). TNFSF4 encodes a cytokine which is important in antigen presentation with T cells and in the adhesion of activated T cells to endothelial cells. The SNP rs10798269 has risk allele A, which Subject 1 is homozygous for and Subjects 3, 4 and 5 are heterozygous for.

The lymphocyte antigen 9 (LY9) SNP, rs509749, has a risk allele of A, which Subject 1 and Subject 2 are heterozygous for. This SNP is thought to be associated with the relative number of the major T cell subsets. Even one copy of the risk allele, A, is significantly associated with a decrease in the proportion of naïve CD4+ T cells and activated T cells and an increase in CD8+ memory T cells (D. S. C. Graham et al., 2008). SNP rs509749 is a non-synonymous mutation in exon 8 of LY9 at the position of amino acid 602, with the A allele coding for Met instead of Val. The A allele increases risk for SLE by increasing cytokine production and thereby enhancing the immune response. The SNP rs509749 is located within the consensus binding site for SAP/SH2D1alpa and so the mechanism for increased CD8+ memory T cells and decreased CD4+ naïve and activated T cells may be caused by the differential binding of SAP/SH2D1alpa to LY9 (Petri et al., 2008). SLAMF3 and SLAMF6 have been shown to promote Th17 differentiation by co-stimulation with the TCR in human T cells and their expression has

been shown to be increased on the surface of patients with active SLE (Chatterjee et al., 2012).

Comparison of Genetic and Environmental Risk Factors Among Subjects 1 and 5

However, having a significant amount of risk alleles of SNPs for SLE does not necessarily mean someone will develop lupus. Subject 5 has the most similar SNP allele configuration to Subject 1 (Table 15, appendix). She is a female in her late 20's who has never been diagnosed with an autoimmune disease and shows no outward symptoms of having one. She shares the majority of risk alleles with Subject 1, however she is heterozygous for some of the ones that he is homozygous for, such as in the IL10 gene. She is also homozygous for the risk alleles for HLA-DRB1*1501, which has shown to be significant in SLE disease incidence, as well as some other autoimmune diseases, such as juvenile rheumatoid arthritis and multiple sclerosis.

Comparing and contrasting Subject 1 and Subject 5 might be helpful in one of two ways, either it can help to delineate which SNPs are truly important in lupus disease incidence, or it can tell us that environmental factors may be an important factor in whether or not an individual develops lupus. The major SLE SNPs that Subject 1 is homozygous for and Subject 5 is heterozygous for are in LMP2, ITGAM, TNSFS4 (in locus 1q25.1), one SNP for IL21, IL6R, the VDR polymorphisms Bsml and TaqI, and estrogen receptor beta. Any of these genes might potentially be very important determiners of lupus incidence, however since Subject 1 is a male and Subject 5 is a

female (lupus affects women nine out of ten times in diagnosed patients), and Subject 5 is homozygous for the MHC risk alleles that Subject 1 is heterozygous for, it is likely that a factor other than genetic susceptibility comes in to play.

The major environmental factor that differs between Subject 1 (at the time of his diagnosis which was in his early 20s) and Subject 5 is that Subject 1 was a heavy smoker and Subject 5 has never smoked. This could be an important factor as a study showed that current smokers with the C; C or C; T genotype of the estrogen receptor alpha polymorphism PvuII, which Subject 1 and Subject 5 are both heterozygous for, have a significantly increased risk of developing SLE. In a generalized multifactor dimensionality reduction (GMDR) study that screened SLE associated SNP interactions with environmental risk factors, a significant risk was found between smokers that carry the C or C; T haplotype, compared to non-smokers with the T; T haplotype in SNP rs2234693 (A. Zhou et al., 2017). Subject 1, who carries one copy of the C allele, was a heavy smoker at the time of his lupus diagnosis and Subject 5, who also carries one copy of the C allele has never smoked and has never been diagnosed with lupus.

Subject 1 also had a subjectively worse diet at the time of diagnosis than Subject 5 currently has, which could factor in to some of the other topics that were touched on, such as vitamin deficiency and the role of the microbiome on epithelial barrier function.

Other Environmental Factors That May Play a Role in Autoimmune Disease Incidence

Regulation of tight junctions might prove to be a significant factor in autoimmune disease progression and/or onset. Estrogen receptor beta signaling modulates epithelial barrier function. Calcium homeostasis has shown to be important in regulation of tight junctions, with a reciprocal relationship in place. The tight junction proteins, claudins, regulate calcium transport by providing the mechanism of paracellular reabsorption of calcium in the renal tubule, however, fluctuations in calcium concentrations can also directly impact claudins and transcellular transport proteins at the molecular level (Bleich, Shan, & Himmerkus, 2012). The role of calcium homeostasis on tight junction protein expression has been studied mostly in context of tight junctions in the kidney, however some studies have also shown this to be true in tight junctions of the small intestine. In animal studies, CaBP-9 KO mice (mice that are deficient for the calcium binding protein, calbindin D9k) showed decreased tight junction gene expression in the duodenum, which shows that expression of paracellular tight junction genes is regulated by calcium binding proteins (An et al., 2013). Vitamin D has also been shown to be critical for the regulation of tight junction proteins because in patients with vitamin D deprivation the levels of the tight junction proteins, especially the claudin proteins, were affected (Stio, Retico, Annese, & Bonanomi, 2016). Dysregulation of the intestinal epithelial tight junctions may also be important in enhancing the severity of disease, not only in the onset (Marchiando, Graham, & Turner, 2010).

Disruption of the epithelial barrier may be a potential cause of autoimmune diseases, as increased permeability allows for larger food antigens and microbes from the intestinal tract to be released into the circulation. These antigens and microbes may be seen as harmless in their normal niche in the intestines, but in the circulation would be

seen as foreign and the body would send out cells, cytokines and chemokines to neutralize the invasion. This could lead to systemic inflammation from the cytokines cascading out of control. Chronic inflammation is a symptom/condition of many autoimmune diseases. If there is a gender specific difference in the integrity of the epithelial barrier, through variations controlled by sex hormones and estrogen receptors, this could help to explain why women are much more prone to autoimmune diseases.

Since estrogen beta receptor signaling has been shown to modulate epithelial barrier function, menstruation may affect the tight junctions of the small intestine. Estrogen production can be tied to autoimmune diseases in women in three ways. First, there is anecdotal evidence of women experiencing flareups of their autoimmune diseases during menstruation. Second, for many autoimmune diseases women experience worsening of their symptoms after menopause. And thirdly, in some cases (especially common in SLE and MS) women do not show symptoms of their disease while they are pregnant, especially during late pregnancy (C. Wang et al., 2009). In the cases when there is a decrease in estrogen in the body, as in during the menstruation phase of the menstrual cycle and after menopause, there is a worsening of symptoms. In the case where there is an increase in estrogen, as seen during gestation, there is a decrease in symptoms. In addition to this, animal models have shown that estrogen receptor positive cells in the small and large intestines vary according to the estrous cycle phase in mice (Kawano et al., 2004). Also, there is evidence that some of the factors modulating the intestinal epithelium are hereditary because increased intestinal permeability is seen in patients with Crohn's disease, but also in their healthy first degree relatives who do not have the disease (Looijer-van Langen et al., 2011).

The higher incidence of autoimmune disease progression in females vs males may partially be explained by these VDR polymorphisms. VDR is expressed in multiple cells of the body including keratinocytes, macrophages, pancreatic beta cells, reproductive tissues and placenta (Lee, Meyer, Benkusky, O'Brien, & Pike, 2018). It is possible that vitamin D levels affect the genes disproportionately in females due to an extra site of VDR in the cycling endometrium (Zarnani et al., 2010) or because of higher levels of estrogens in the body. There is a modulation of hormonal processes by cytokines, and a differing cytokine profile in males vs females with males tending to respond in a Th1 manner and females tending to respond in a Th2 manner (Fairweather et al., 2008). There is an increased Th2 cytokine profile seen in SLE patients. In animal models administration of estrogen enhances some autoimmune diseases while androgen therapy suppresses them (Beeson, 1994). Estrogen administration was also shown to have beneficial effects on mouse models of multiple sclerosis (EAE) and in treatment of patients, however the side effects were too numerous to warrant continuing treatment (Voskuhl et al., 2006).

Dysregulation of the VDR decreases the response of the innate immune system which then prevents the breakdown of the active vitamin D metabolite by CYP24A1. When 1, 25-D rises above normal range it binds to other receptors and displaces their native ligands, which has been shown in *in silico* data for the alpha/beta thyroid receptors, glucocorticoid receptors and androgen receptors (Proal et al., 2009). This disruption may then lead to increased levels of these hormones in the periphery which then further disrupts homeostasis.

Another environmental factor to consider is zinc levels. IBD patients commonly exhibit reduced plasma zinc levels. Zinc levels could have an impact on estrogen receptors because it has been shown to influence their expression and sensitivity and also an effect on the hormones themselves. In an animal model, rats fed a diet deficient in zinc, had significantly lower serum concentrations of luteinizing hormone, estradiol and testosterone however they had significantly higher levels of estrogen receptors (El-Tawil, 2008). Another dietary factor that has been shown to have an effect on the immune system is vitamin A, which can improve immunity by regulating the Th1/Th2 cytokine balance with retinoid active derivatives. A study of Crohn's disease showed that the presence of estrogen was necessary for bacteria to adhere to the mucosa of the colon and small intestines, by looking in post-menopausal women before and after estrogen therapy (El-Tawil, 2008).

Conclusion and Further Studies

While the results of this case study were inconclusive as to what the determining factors are for why women have a much higher incidence of autoimmune diseases, the research provided a synopsis of postulated hypotheses and insights as to why this might be the case. The higher incidence of autoimmune diseases in women is probably due to a combination of genetic and environmental causes, including differing sex hormone levels and their effects on estrogen receptors, vitamin deficiencies, the hygiene hypothesis, and epithelial barrier integrity. The combination of genetic and environmental risk factors allowed for the comparison and contrasting of subjects that have SLE and that do not.

This study provided new data and context on genetic variants that are involved in autoimmune diseases incidence, especially lupus and psoriasis. More studies will need to be completed with much higher numbers of participants in order to determine which SNP risk alleles are the most important for disease incidence and whether these are affected by environmental factors.

Appendix 1.

Supplemental Data

Table 12. MHC SNPs Identified Through Promethease Search

MHC Gene	**SNP**	**MHC Class**	**Subject 1**	**Subject 2**	**Subject 3**	**Subject 4**	**Subject 5**
HLA-A*0102	rs6902544	I	A; A	A; A	A; A	A; A	A; A
HLA-B	rs2523604	I	A; G	G; G	G; G	G; G	A; G
HLA-B	rs2523605	I	A; G	G; G	G; G	G; G	A; G
HLA-B27	rs13202464	I	A; A	A; G	A; A	A; A	A; A
HLA-B27	rs4349859	I	G; G	A; G	G; G	G; G	G; G
HLA-B27	rs3819299	I	A; A	A; C	A; A	A; A	A; A
HLA-B*5801	rs3117583	I	T; T	C; T	C; T	C; T	C; T
HLA-C	rs9264942	I	C; T	C; T	C; T	C; T	T; T
HLA-C	rs1049853	I	C; T	C; C	C; T	C; C	C; C
HLA-C	rs13191343	I	C; C	C; C	C; C	C; C	C; C
HLA-C*0602	rs4406273	I	G; G	G; G	G; G	G; G	G; G
HLA-DPB1	rs9277535	II	A; A	A; A	A; A	A: A	A; A
HLA-DPB1	rs9277378	II	A; A	A; A	A; A	A; A	A; A
HLA-DPB2	rs1883414	II	C; C	C; C	C; C	C; C	C; C
HLA-DQA1	rs1129740	II	A; G	G; G	A; G	G; G	G; G
HLA-DQA1*03	rs6457617	II	C; T	C; C	C; T	C; T	C; C
HLA-DQB1	rs9273363	II	C; C	C; C	C; C	C; C	C; C

HLA-DQB1	rs9275596	II	C; T	T; T	C; T	C; T	T; T
HLA-DQB2	rs7756516	II	T; T	C; T	C; T	C; T	C; T
HLA-DQB2	rs2051549	II	T; T	C; T	C; T	C; T	C; T
HLA-DQB2	rs2301271	II	C; C	C; T	C; T	C; T	C; T
HLA-DRA	rs7192	II	C; T	T; T	C; T	C; T	T; T
HLA-DRA	rs3129878	II	A; A	A; A	A; A	A; A	A; A
HLA-DRA	rs2227139	II	C; T	C; C	C; T	C; T	C; C
HLA-DRA	rs9268645	II	C; G	C; C	C; G	C; G	C; C
HLA-DRB1*0401	rs660895	II	A; G	A; A	A; G	A; G	A; A
HLA-DRB1*0401	rs6910071	II	A; G	A; A	A; G	A; G	A; A
HLA-DRB1*1501	rs3135388	II	C; T	C; T	C; T	C; T	T; T
HLA-DRB1*1501	rs3135391	II	C; T	C; T	C; T	C; T	T; T
HLA-DRB9	rs6903608	II	C; T	C; T	C; T	C; T	C; C
HLA-DMA	rs1063478	NC-II	C; C	C; C	C; C	C; C	C: C
HLA-DOA	rs2284191	NC-II	A; G	G; G	G; G	G; G	A; G
HLA-F	rs2523393	NC-I	C; C	T; T	C; T	C; T	C; T
HLA-G	rs4959039	NC-I	G; G	A; A	A; G	A; G	A; G
HLA-G	rs2249863	NC-I	A; A	C; C	A; C	A; C	A; C
HLA-G	rs9380142	NC-I	G; G	A; A	A; G	A; G	A; G

HLA-G	rs1063320	NC-I	G; G	C; C	C; G	C; G	C; G

Table 12: This is a partial list of the SNPs in the MHC that are associated with autoimmune diseases and were found by first searching through the literature and then looking for the SNPs in Promethease. The list comprises the SNP involved and the HLA gene associated with it, along with which class the gene is associated with, and each subject with their haplotype. The red letter denotes known deleterious alleles and the red background denotes the known harmful haplotype. Abbreviations: (A) adenine, (C) cytosine, (G) guanine, (T) thymine, (NC) non-classical.

Table 13. Genetic Variants Involved in Immunoregulation

SNP	**Gene**	**Subject 1**	**Subject 2**	**Subject 3**	**Subject 4**	**Subject 5**
rs6897932	*IL17R*	C; T	C; T	C; C	C; T	C; T
rs917997	*IL18RAP*	G; G	G; G	G; G	G; G	G; G
rs10045431	*IL12B*	C; C	C; C	C; C	C; C	C; C
rs7517847	*IL23R*	T; T	T; T	T; T	T; T	T; T
rs11209026	*IL23R*	G; G	G; G	G; G	G; G	G; G
rs11465804	*IL23R*	T; T	T; T	T; T	T; T	T; T
rs6822844	*IL2/IL21*	T; T	G; G	G; T	G; T	G; T
rs3024505	*IL10*	C; T	C; T	T; T	C; T	T; T
rs17810546	*IL12A*	A; G	A; A	A; G	A; G	A; G
rs13015714	*IL18R1*	T; T	T; T	T; T	T; T	T; T
rs2250417	*IL18*	A; G	A; G	A; G	A; G	A; G
rs7626795	*IL1RAP*	A; A	A; A	A; A	A; A	A; A
rs4129267	*IL6R*	C; C	T; T	C; T	C; T	C; T

rs6761276	*IL1F10*	C; T	C; T	C; T	C; T	C; T
rs12251307	*IL2RA*	C; T	C; C	C; C	C; C	C; T
rs2427837	*IgE*	A; G	G; G	A; G	A; G	A; G
rs2251746	*IgE*	C; T	T; T	C; T	C; T	C; T
rs703817	*STAT6*	A; G	A; G	G; G	G; G	A; A
rs167769	*STAT6*	C; T	C; C	C; C	C; C	C; T
rs3024974	*STAT6*	C; C	C; C	C; C	C; C	C; C
rs324015	*STAT6*	A; G	A; G	A; A	A; A	G; G
rs841718	*STAT6*	C; T	C; T	C; C	C; C	T; T
rs324011	*STAT6*	C; T	C; C	C; C	C; C	C; T
rs1059513	*STAT6*	A; A	A; A	A; A	A; A	A; A

Table 13: SNPs associated with proinflammatory cytokines. Red letters denote harmful alleles, while green letters denote beneficial alleles. Abbreviations: (A) adenine, (C) cytosine, (G) guanine, (T) thymine

Table 14. Summary of Subject 1's Risk Allele SNPs

SNP	**Gene**	**Haplotype**	**Risk**
rs3135388	HLA-DRB1*1501	C; T	Genetic MHC risk allele for SLE
rs3135391	HLA-DRB1*1501	C; T	Genetic MHC risk allele for SLE
rs2284191	HLA-DO	A; G	Impaired class II epitope selection
rs17587	LMP2	A; A	Impaired immunoproteasome

rs6920220	TNFAIP3	A; G	Impaired NFkB and TNF mediated apoptosis
rs1143679	ITGAM	A; A	Changed binding affinity of αMβ2
rs13277113	C8orf13-BLK	A; G	Intermediate levels of mRNA from C8orf13 and BLK
rs2304256	TYK2	C; C	Defects in IL-6, IL-10, IL-22, IL-23. Impaired Th1 diff.
rs10798269	1q25.1	A; A	TNFSF4 deficiency
rs509749	LY9	A; G	Increased memory CD8+ T cells, decreased naïve and activated CD4+ T cells
rs907715	IL21	A; A	Defects in B cell differentiation into plasma cells
rs2221903	IL21	A; A	Defects in B cell differentiation into plasma cells
rs6897932	*IL17R*	C; T	Increased inflammation
rs10045431	*IL12B*	C; C	Increased inflammation
rs11209026	*IL23R*	G; G	Increased inflammation
rs11465804	*IL23R*	T; T	Increased inflammation
rs3024505	*IL10*	C; T	Increased inflammation
rs17810546	*IL12A*	A; G	Increased inflammation
rs2250417	*IL18*	A; G	Increased inflammation
rs4129267	*IL6R*	C; C	Increased inflammation
rs12251307	*IL2RA*	C; T	Increased inflammation
rs1544410	Bsml VDR polym.	A; A	Impaired VDR functions

rs731236	TaqI VDR polym.	C; C	Impaired VDR functions
rs2234693	ER-alpha	C; T	PvuII estrogen receptor alpha polym. Increased SLE risk seen with environmental factors (smoking)
rs1256030	ER-beta	C; C	Impaired estrogen receptor
rs2280964	CXCR3	T; T	Impaired CXCR3 pathway

Table 14: Summary of SNPs associated with SLE for Subject 1. Red letters denote harmful alleles. Abbreviations: (A) adenine, (C) cytosine, (G) guanine, (T) thymine

Table 15. Comparison of Subject 1's Risk Alleles to Subject 5's Risk Alleles

SNP	**Gene**	**Subject 1**	**Subject 5**	**Risk**
rs3135388	HLA-DRB1*1501	C; T	T; T	Genetic MHC risk allele for SLE
rs3135391	HLA-DRB1*1501	C; T	T; T	Genetic MHC risk allele for SLE
rs2284191	HLA-DO	A; G	A; G	Impaired class II epitope selection
rs17587	LMP2	A; A	A; G	Impaired immunoproteasome
rs6920220	TNFAIP3	A; G	G; G	Impaired NFkB and TNF mediated apoptosis
rs1143679	ITGAM	A; A	A; G	Changed binding affinity of αMβ2

rs13277113	C8orf13-BLK	A; G	A; G	Intermediate levels of mRNA from C8orf13 and BLK
rs2304256	TYK2	C; C	C; C	Defects in IL-6, IL-10, IL-22, IL-23. Impaired Th1 diff.
rs10798269	1q25.1	A; A	A; G	TNFSF4 deficiency
rs509749	LY9	A; G	G; G	Increased memory CD8+ T cells, decreased naïve and activated CD4+ T cells
rs907715	IL21	A; A	A; G	Defects in B cell differentiation into plasma cells
rs2221903	IL21	A; A	A; A	Defects in B cell differentiation into plasma cells
rs6897932	*IL17R*	C; T	C; T	Increased inflammation
rs10045431	*IL12B*	C; C	C; C	Increased inflammation
rs11209026	*IL23R*	G; G	G; G	Increased inflammation
rs11465804	*IL23R*	T; T	T; T	Increased inflammation
rs3024505	*IL10*	C; T	T; T	Increased inflammation
rs17810546	*IL12A*	A; G	A; G	Increased inflammation
rs2250417	*IL18*	A; G	A; G	Increased inflammation
rs4129267	*IL6R*	C; C	C; T	Increased inflammation
rs12251307	*IL2RA*	C; T	C; T	Increased inflammation
rs1544410	Bsml VDR polym.	A; A	A; G	Impaired VDR functions
rs731236	TaqI VDR polym.	C; C	C; T	Impaired VDR functions

rs2234693	ER-alpha	C; T	C; T	PvuII estrogen receptor alpha polym. Increased SLE risk seen with environmental factors (smoking)
rs1256030	ER-beta	C; C	C; T	Impaired estrogen receptor
rs2280964	CXCR3	T; T	T; T	Impaired CXCR3 pathway

Table 15: Comparison of Subject 1 and Subject 5 in the context of SNPs associated with SLE. Red letters denote harmful alleles. Abbreviations: (A) adenine, (C) cytosine, (G) guanine, (T) thymine.

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