Distribution Agreement

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter now, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Christina Tejeda April 27, 2014

Investigation of Associations between Autoimmunity Associated Variants in PDCD-1 and Juvenile Idiopathic Arthritis Categories

by

Christina Tejeda

Sampath Prahalad

Adviser

Department of Biology

Sampath Prahalad

Adviser

Christopher Beck

Committee Member

Victor Corces

Committee Member

2014

Investigation of Associations between Autoimmunity Associated Variants in PDCD-1 and Juvenile Idiopathic Arthritis Categories

By

Christina Tejeda

Sampath Prahalad

Adviser

An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Biology

2014

Abstract

Investigation of Associations between Autoimmunity Associated Variants in PDCD-1 and Juvenile Idiopathic Arthritis Categories

By Christina Tejeda

Variants in the gene encoding Programmed Cell Death 1 (*PDCD-1*) have been associated with susceptibility to Systemic Lupus Erythematosus (SLE) and other autoimmune diseases. Given that clinically distinct autoimmune phenotypes share common genetic susceptibility factors, we sought to determine whether *PDCD-1* variants were associated with Juvenile Idiopathic Arthritis (JIA). 834 cases and 855 self-identified Caucasian controls had been recruited from the Pediatric Rheumatology Clinics at the University of Utah and Children's Healthcare of Atlanta. The cases and controls were genotyped for single nucleotide polymorphisms (SNPs) in the *PDCD-1* gene (rs10204525, rs7568402, rs7421861 and rs11568821) using TaqMan allelic discrimination assay. Variants were investigated for allelic association with JIA. Given that the phenotype of JIA is heterogeneous, composed of seven categories, stratified analysis was completed. Stratification by gender did not alter the results. Using a combined cohort of about 1700 subjects, we found no association between the *PDCD-1* variant and JIA as a whole. This is seen by JIA and SNP rs10204525 (p=0.13), rs7568402 (p=0.45), rs7421861 (p=0.63) and rs11568821 (p=0.13). However, we found a nominal association between enthesitis related JIA in our cohort and rs11568821 (OR=0.22, p=0.012) as well as an association between rs7568402 and systemic JIA (OR=0.53, p=0.0027). Unlike other autoimmunity-associated genes such as *PTPN22* and *TNFA* that are associated with JIA, *PDCD-1* does not appear to be associated with JIA, despite showing strong associations with other autoimmune phenotypes like SLE.

Investigation of Associations between Autoimmunity Associated Variants in PDCD-1 and Juvenile Idiopathic Arthritis Categories

By

Christina Tejeda

Sampath Prahalad

Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Biology

2014

Acknowledgements

Special thanks to Mina Rohani-Pichavant for mentoring me and providing countless assistance throughout this process. Thanks to the Genetic department, Alaine Broadaway and Michael Epstein, for their collaboration. Thanks to Milton Brown for his knowledge on genotyping and support. Thanks to the coordinators Lori Ponder and Kirsten Jenkins. Thanks to Gabriel Wang for his help in genotyping. Thanks to the Utah team, John Bohnsack, Kyle Berg, Breanna Caruso and Ann Rutherford. Also thanks to my honors committee Victor Corces, Christopher Beck and my principal investigator Sampath Prahalad.

Table of Contents

Index of Figures and Tables..i

Purpose..1

Introduction..1

Patient and Methods..14

Results...17

Discussion and Conclusion...22

References 26

Index of Figures and Tables

Figure 1: The cells of the immune system……………………….....................................2

Figure 2: Inflammation response to foreign molecules...3

Figure 3: HLA Region…………………………………...4

Figure 4: The involvement of genetic and environmental factors in disease......................7

Table 1: Frequency of JIA Categories in the population...10

Figure 5: PD-L1/PD-L2 Pathway...12

Table 2: Candidate SNPs in *PDCD-1* gene...13

Figure 6: Location of SNPs in the *PDCD-1* locus..14

Figure 7: Taqman assay during PCR...17

Figure 8: NanoDrop result of sample SEJ1-1363..19

Figure 9: PDCD-1 rs7421861 Allelic Discrimination Post-Run.....................................20

Figure 10: Allelic Discrimination Plot of DNA samples for the marker *PDCD-1* rs7421861...20

Table 3: Analyses of Variants and JIA for Cases and Controls.......................................21

Table 4: Analyses of Variants and JIA categories...22

I. Purpose

Previous studies have been conducted looking at different genic variants associated with Juvenile Idiopathic Arthritis (JIA). A meta-analysis by Kaalla et al. evaluated variants at the genes *MIF*, *TNF* and *PTPN22* and confirmed their existing association with JIA [1]. In this study we sought to test variants in a gene that had not been studied before for associations with JIA. Variants in the gene programmed cell death-1 (*PDCD-1*), which encodes Programmed Cell Death-1 (PD-1), have been found to be associated with systemic lupus erythematosus (SLE), type-1 diabetes and other autoimmune diseases. Given that clinically distinct autoimmune phenotypes share common genetic susceptibility factors, we proposed to determine whether selected *PDCD-1* variants are associated with Juvenile Idiopathic Arthritis. The purpose of this study is to investigate whether the variants in the gene encoding PD-1 are associated with susceptibility to JIA or JIA categories.

II. Introduction

Immune systems

The human immune system is composed of two systems: an innate and an adaptive system. The innate system is comprised of cells that are immediately ready to combat foreign antigens with no apparent specificity against them [2]. The innate system includes the epithelial barrier, phagocytic leukocytes, dendritic cells, natural killer cell and plasma proteins [2] (Figure 1). While the innate system's response is immediate, there is a 4-7 day delay before the adaptive immune system responds [2]. In addition, unlike the innate system, the adaptive immune system is antigen specific, thus has a high specificity [2]. The adaptive system is comprised of two types of lymphocytes: B-cells

and T-cells [2]. Lymphocytes have no functional activity until they encounter an antigen and are activated [2]. When activated by antigens, B-lymphocytes differentiate into plasma cells and make antibodies that attack the foreign bodies, while T-lymphocytes attack antigens directly [2]. There are two types of T-lymphocytes, one differentiates into cytotoxic T-lymphocytes and kills virus infected cells, an example being human immunodeficiency virus (HIV) [2]. The other differentiates into cells that later activate B cells and macrophages [2]. With each subsequent exposure to the same antigen, the adaptive system is able to react more quickly because it produces memory cells [2].

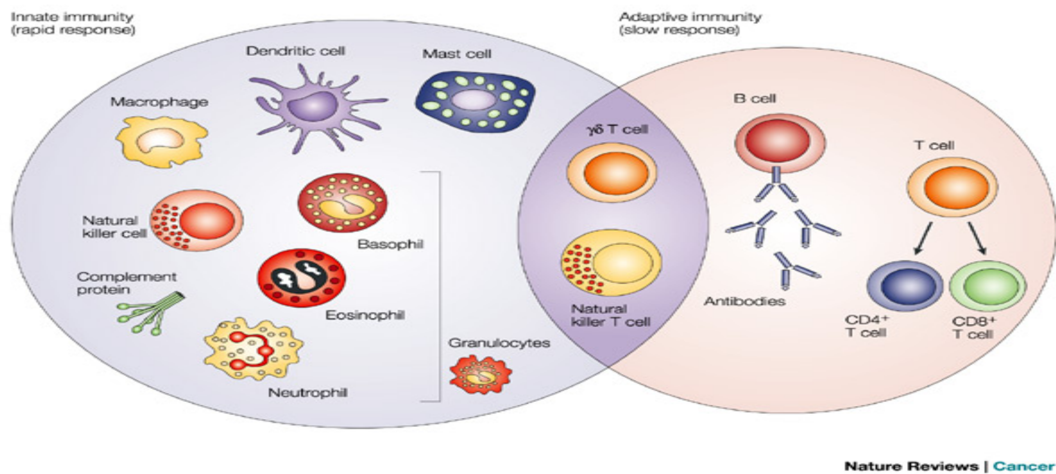

Figure 1: The cells of the Immune System [2]

Figure 2 shows that when the immune systems' macrophages encounter foreign antigens, they release cytokines and chemokines. The cyotkines cause the dilation of blood vessels, allowing for more fluid and thus protein to be able to reach the infected area [2]. The chemokines direct neutrophils to the site and also cause the blood vessel's endothelial cells to become adhesive so that cells are able to adhere to them [2]. The accumulation of fluid and immune cells causes redness, swelling, heat and pain, which in result is called inflammation [2].

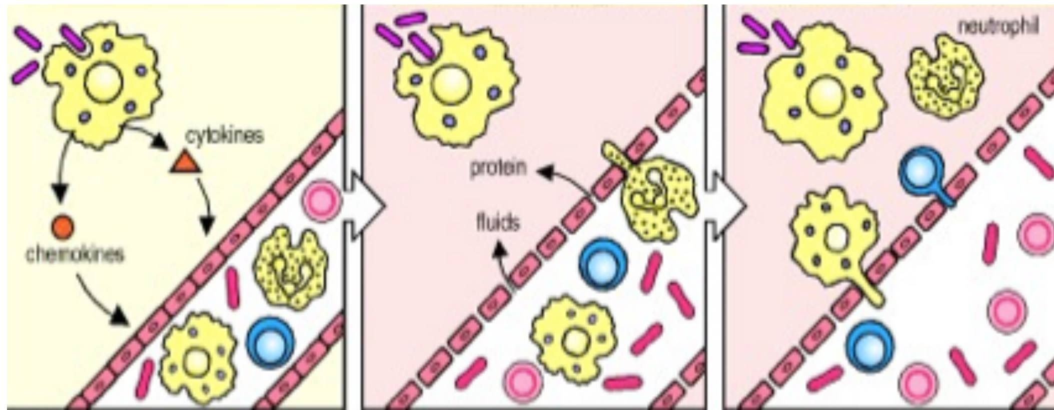

Figure 2: Inflammation response to foreign molecules [2]

The immune system response is important to understand because autoimmune diseases are associated with the innate and adaptive immune system and thus follow a similar mechanism.

Autoimmune diseases

Autoimmune diseases affect about 8% of the world population [3]. They are characterized by the host immune system attacking self-antigens because the immune system can't differentiate between healthy body tissues and foreign antigens [3]. Most autoimmune diseases are believed to be caused by mechanisms that are unclear but that result in inflammation and tissue damage [3]. Even though the mechanisms are unclear, autoimmune diseases are believed to be due to a combination of genetic and environmental factors [3]. In the following paragraphs, we will discuss more in detail about the different genetic and environmental contributors that contribute to the cause of autoimmune diseases.

There are hundreds of identified risk loci or regions that increase susceptibility for autoimmune diseases and many are shared between disorders [3]. Cotaspas et al. examined 107 autoimmune loci that were related to type-1 diabetes, rheumatoid arthritis,

SLE and multiple sclerosis (MS) and found 47 to be associated with more than one immune-mediated disease [3]. This again shows that many loci are related to more than one autoimmune disease. For example, the human leukocyte antigen (HLA) region has been shown to be a major susceptibility locus for many autoimmune diseases such as type-1 diabetes, rheumatoid arthritis and juvenile idiopathic arthritis [6]. The HLA region is found within the major histocompatibility complex (MHC) [5]. It is located on chromosome 6p21 and contains over 252 loci that are involved in the immune function [6]. Additionally, this region contains the highest density of polymorphisms in the human genome [5]. HLA has three classes of proteins, which are all present on the majority of the bodies' cell surfaces (Figure 3). Class I presents intracellular antigens, class II presents extracellular antigens and class III is involved in inflammation and other immune system responses [6]. The HLA region is important because it helps the immune system distinguish between self and foreign proteins.

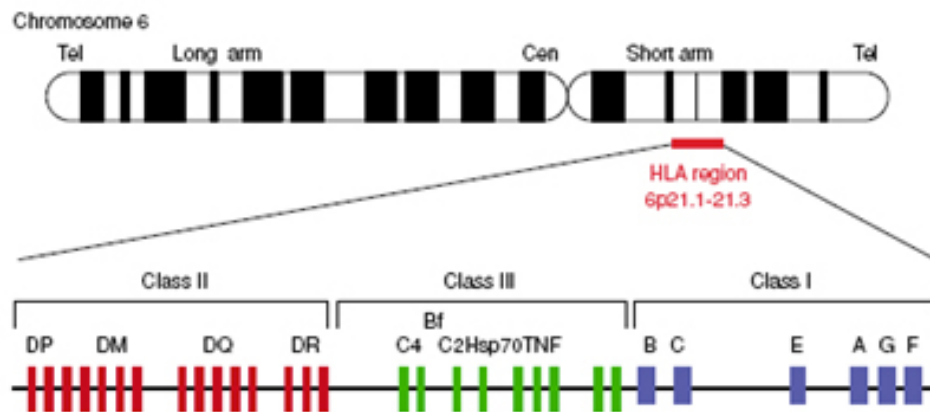

Figure 3: HLA Region [6]

Additionally, the genes *PTPN22* and *STAT4* have been associated with susceptibility to autoimmune diseases such as rheumatoid arthritis, type-1 diabetes, JIA and SLE [7,8]. The gene *PTPN22* encodes a lymphoid tyrosine phosphatase (LYP), which is involved in

the inhibition of T-cell activation [7]. *STAT4* encodes for a transcription factor that is expressed on macrophages, lymphocytes and dendritic cells [8]. It induces T-cell differentiation and also induces the pro-inflammatory cytokine interferon-ϒ [8].

De-oxyribose nucleic acid (DNA) methylation is another genetic risk factor that is associated with autoimmune diseases. DNA methylation is an epigenetic difference that has the ability to alter gene transcription and therefore, the ability to alter cell function [9]. DNA is normally tightly packed in nucleosomes, which are comprised of histones [9]. The tightly packed DNA is relatively inaccessible to transcription factors, thus is not transcribed from DNA into RNA [9]. DNA methylation modifies the histones, which changes the nucleosome structure, thus changing gene expression [9]. DNA methylation has also been linked to silencing genes and repressing expression of tumor suppressor genes. This is seen with the tumor suppressor gene *RASSF*, a Ras-family associated gene. DNA methylation of *RASSF* causes it to be repressed and ultimately causes diseases such as childhood leukemia and neuroblastoma [9,10].

Apart from genetic factors, autoimmune diseases are also associated with environmental risk factors, which include but are not limited to tobacco smoke, vitamin D deficiency, diet and microbial exposure. Tobacco smoke has more than 600 components, of which the majority is cytotoxic, mutagenic, carcinogenic and/or antigenic [3]. A meta-analysis of 13,000 cases confirmed an association between smoking and developing rheumatoid arthritis, showing that men who smoked had 2 times the risk and women who smoked tobacco had 1.3 times the risk of developing rheumatoid arthritis [3]. In addition, there is also an association between tobacco smoke and MS [3]. However, the mechanisms for both of these associations are largely unclear.

Another environmental factor is vitamin D, which is mainly obtained by the exposure to ultraviolet radiation in sunlight. Studies have shown an association between MS and low UV exposure [3]. Additionally lower vitamin D levels in sera have been seen in patients with rheumatoid arthritis and type-1 diabetes [3].

Currently, research has focused on diet influencing the human microbiome, the sum of bacterial communities and the role of microbes in the development of inflammatory arthritis [11]. On average, humans contain over about 1 kilogram of bacteria with about three million different bacterial genes [11]. As mucosal sites are exposed to a high amount of bacterial antigens, an individual's bacterial makeup is thought to be able to influence the initiation, progression and intensity of an autoimmune disease [11]. Several studies on rats have shown that rats reared in germ-free conditions were more susceptible to developing arthritis in the presence of mucosal microbes [9]. Also other studies have shown a relationship between the presence of certain bacterial genera and the development of arthritis [9]. These studies show that the presence of bacteria in mucosal regions may be sufficient to alter the host immune system and cause inflammation, a symptom of autoimmune diseases [11]. Though, it is still uncertain if the relationship between the microbiome and inflammation is one of cause and effect or if it is a secondary effect of inflammation [11].

Lastly, another environmental factor that has been associated with autoimmune diseases is microbial exposures, which has two hypotheses. One is that certain microbial exposures trigger the onset of disease. A meta-analysis of 19,000 cases and 16,000 controls showed that those with a history of infectious mononucleosis had an increased risk of developing MS [3]. There is also weak evidence showing an association between

Epstein-Barr virus and an increased risk of developing RA and SLE [3]. The second, known as the hygiene hypothesis, states that microbial exposure at an early age acts to prime the immune system, providing protection against developing immune disorders. The hygiene hypothesis suggests that western hygiene causes an increasing incidence of autoimmune disorders [3]. A study showed that those with more siblings and thus more exposure to microbes had a reduced risk of developing MS [3].

Although, environmental factors such as tobacco, vitamin D deficiency, diet and microbial exposures play a large role in autoimmune disease, many studies of autoimmune diseases do not collect environmental data. Figure 4 shows the complicated interplay between environmental and genetic factors. Genetic and environmental factors may be acting independently or interacting together to determine the risk of disease.

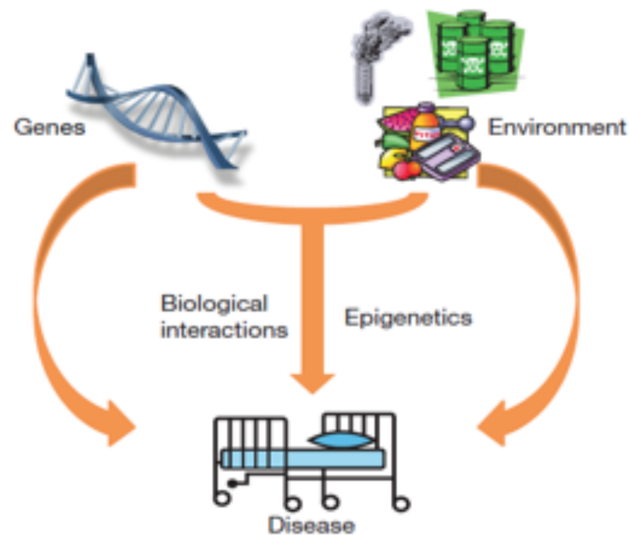

Figure 4: The involvement of genetic and environmental factors in disease [3]

Juvenile Arthritis

This project concentrated on one specific autoimmune disease: Juvenile Idiopathic Arthritis. JIA is the most common chronic rheumatic disease in children, having both autoimmune and inflammatory features [12,13]. Although, the cause of JIA is unknown,

like other autoimmune diseases, it is believed to result from both genetic and environmental factors [14].

Normally the synovial membrane, the inner membrane of the tissue that lines the joint, secretes synovial fluid that lubricates the joint. In JIA there is an autoimmune inflammation directed towards the synovial membrane. This results in inflammation that causes an excess amount of synovial fluid to be produced. The cells that respond to inflammation build up in the joint causing the joint to become stiff and swollen. Over time this inflammation can cause cartilage and bone damage.

JIA is not a single disease but a term that encompasses all forms of arthritis that occur before the age of 16 [15]. The seven different categories of JIA, classified by the International League of Association for Rheumatology (ILAR), are systemic JIA, oligoarticular JIA, rheumatoid factor (RF)-positive polyarticular JIA, RF-negative polyarticular JIA, enthesitis-related (ERA) JIA, psoriatic JIA and undifferentiated JIA [14]. All of these forms of JIA are characterized by synovial inflammation that can cause joint damage [14]. The categories are distinguished by their symptoms, number of joints affected, family history and laboratory tests.

Systemic JIA is diagnosed by the presence of arthritis that is accompanied with or preceded by a fever of at least two weeks in length [14]. It also needs at least one of the following: rash, lymph node enlargement, hepatomegaly (liver enlargement) and/or splenomegaly (spleen enlargement) and/or serositis (inflammation of lining of the lungs, heart and abdomen) [14]. Oligoarticular JIA is diagnosed as affecting four or fewer joints during the first 6 months of diagnosis [14]. It is typically an asymmetric arthritis that affects females more than males and usually onsets before 6 [14]. There are two

categories of oligoarticular JIA: persistent and extended [14]. The persistent type is confined to four or fewer joints after the first 6 months, while the extended type extends to more than four joints after the first 6 months [14]. In addition, children with oligoarthritis are often positive for antinuclear antibodies (ANA), which are antibodies that are directed towards nuclear antigens and serve as a marker of autoimmunity [16]. ANA increases a child's risk for developing uveitis, which is an anterior, chronic inflammatory eye disease that causes complications such as cataracts, glaucoma and vision loss [16]. Uveitis is the most common extra-articular complication of JIA, occurring in up to 38% of JIA patients [16].

RF-positive polyarticular JIA is diagnosed as arthritis affecting five or more joints during the first 6 months of disease with the presence of two IgM RF tests at least 3 months apart [14]. This disease is the same as adult RF and is most commonly seen in females [14]. Rheumatoid factors are antibodies produced by lymphocytes. RF-negative polyarticular JIA is diagnosed as arthritis affecting five or more joints during the first 6 months of symptoms with the absence of IgM RF [14]. It is more commonly seen in females and is characterized as symmetric arthritis with an early onset age. Like oligoarticular JIA, RF-negative JIA has a high prevalence of positive ANA [14].

ERA JIA is diagnosed as arthritis and enthesitis (inflammation and/or tenderness at the point of insertion tendons and ligaments into the bone), or arthritis or enthesitis with at least two of the following: HLA B27 positive, uveitis, onset in male over 6, history of spondylitis or Reiter's syndrome in a first degree relative [14]. HLA B27 is a protein on the surface of lymphocytes that helps in the differentiation between self and foreign. Psoriatic JIA is diagnosed by the presence of arthritis with a psoriatic rash. If no rash is

present then one or more of the following is required: first-degree family history of psoriasis, dactylitis or nail pitting [14]. Lastly, undifferentiated JIA is diagnosed when patients do not satisfy the criteria for a category or satisfy criteria for more than one [14]. The frequency at which each category is found in the population is seen in table 1, the most common category being oligoarticular JIA.

Table 1: Frequency of JIA Categories in the population [14]

Category	Frequency
Systemic	4-17%
Oligoarticular	27-56%
RF- Positive	2-7%
RF- Negative	11-28%
ERA	3-11%
Psoriatic	2-11%
Undifferentiated	11-21%

Treatment of JIA differs for each category but the overall goal is to prevent joint damage, to maintain the joint's function and to control the symptoms [17]. Treatment is a multidisciplinary approach through pharmacological interventions, psychological support and physical therapy [17]. The first pharmacological therapy consists of nonsteroidal anti-inflammatory drugs (NSAIDs), which reduce pain and inflammation but do not stop disease progression. If disease improvement is not achieved disease-modifying antirheumatic drugs (DMARDS) are used to delay disease progression [17]. Risks related to DMARDS are growth arrest and retardation [17].

The most common DMARDS used is methotrexate (MTX), an anti-inflammatory drug [17]. MTX risks are liver and/or renal dysfunction [17]. MTX is shown to provide improvement in 60-70% of patients [18]. MTX works by reducing the production of pro-inflammatory cytokines interleukin 1 (IL1), IL6 and TNF-a [19]. This results in the

increased gene expression of IL4 and IL10, anti-inflammatory cytokines [19]. MTX also inhibits thymidylate synthase and inhibits the transfer of single carbon units involved in the synthesis of thymidylate and purine deoxynucleosides [20]. This causes an increased release of adenosine into the blood [20]. The adenosine binds to adenosine surface receptors causing anti-inflammatory effects and causing increased amounts of cellular cAMP [19]. Increased cAMP levels leads to the inhibition of TNF and IL2 [19]. MTX also inhibits neutral metalloproteinase and collagenase, and thus stops the damage of the synovial tissue [19].

When patients do not respond adequately to MTX, anti-tumor necrosis factor (TNF) agents are used, such as etanercept (commonly known as Enbrel) and adalimumab (Humira) and infliximab (Remicade) [21]. TNF is a cytokine that is involved in the immune response, apoptosis and cell proliferation/differentiation [22]. Additionally, TNF and IL1 are believed to be the two major cytokines that are involved in joint inflammation [22]. Because anti-TNF agents are monoclonal antibodies made in the laboratory, the agents exhibit high specificity for TNF [23]. These drugs enter the joint and bind to the target TNF molecule, thus blocking communication pathways that create destructive immune cells [23].

Programmed Cell Death-1

The gene of interest, *PDCD-1*, belongs to the immunoglobulin superfamily, is located on chromosome 2q37 and has a molecular weight of 29,310 [24]. This gene encodes the inhibitory immunoreceptor PD-1, which is a receptor expressed on T-cells, B-cells and activated monocytes [25]. These cells play an essential role in cell-mediated immunity [26]. The programmed death ligand (PD-L) binds to the PD-1 receptor on the cells.

The role of the PD-1/PD-L pathway in the prevention of autoimmune diseases has been investigated extensively because PD-1 plays a role in the regulation of autoimmunity and also is essential for self-tolerance [27, 28]. Rui et al. studied mice that did not have the receptor PD-1 and found that all the mice developed spontaneous autoimmune diseases [28]. Activated T-cells express receptors that mediate inhibitory signals from antigen presenting cells (APC) [27]. Activation of a range of receptors on T and B immune cells and on endothelial and epithelial cells cause release of the two ligands PD-L1 and PD-L2 [27]. These ligands then inhibit activation of the immune response by binding to the PD-1 [27]. This in effect inhibits the differentiation of self-antigen specific inflammatory T-cells [28].

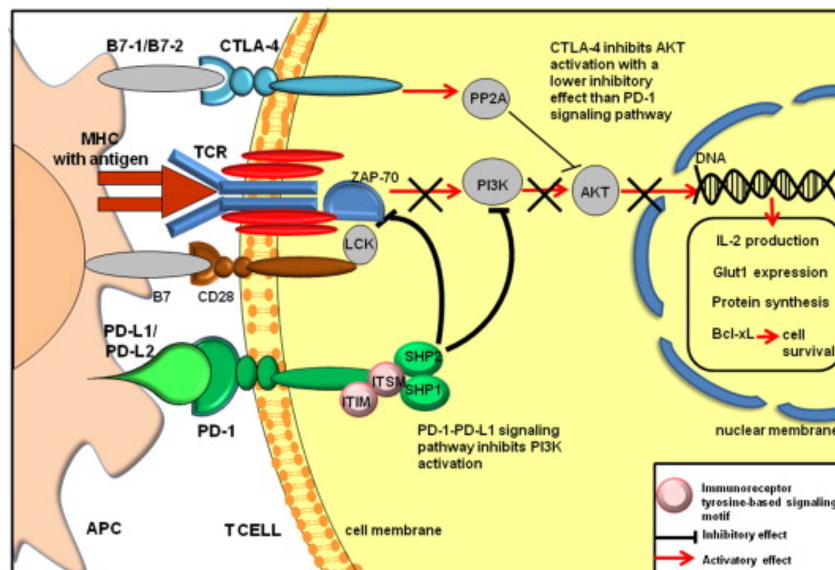

Figure 5: PD-L1/PD-L2 Pathway [29]

Figure 5 shows the complex interactions of PD-1 and other receptors and hormones. The activation of the T-cell is induced by the interaction between the TCR and the major histocompatibility complex (MHC) [29]. When PD-L1 or PD-L2 binds to PD-1 it causes the phosphorylation of ITIM and ITSM [28]. This phosphorylation then causes the

recruitment of SHP-1 and SHP-2, which block PI3K's phosphorylation of Akt, thus not allowing it to be activated [29]. As Akt is inhibited the T-cell is not differentiated.

PDCD-1 variations could potentially alter the function or expression of PD-1, which could result in autoimmunity. As found in multiple experiments, the functional *PDCD-1* variants are associated with several autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, and type-1 diabetes [30, 31, 32]. The association with these autoimmune diseases suggests that blockade of the PD-1/PD-L pathway could exaggerate or accelerate the development of autoimmune disease. This particular study observed if *PDCD-1* variants were associated with JIA.

As clinically distinct autoimmune phenotypes share common genetic susceptibility factors as shown with HLA, PTPN22 and STAT4 variants, our aim was to determine whether selected *PDCD-1* variants are associated with JIA and thus influence susceptibility to developing JIA. This study investigated four single nucleotide polymorphisms (SNPs) in the *PDCD-1* gene: rs10204525, rs7568402, rs7421861 and rs11568821. Table 2 shows the major allele (the original allele), the minor allele (the SNP) and shows the minor allele frequency (MAF) (the frequency that the least common allele occurs in a population) for each SNP. Rs11568821 has the lowest MAF of 0.048, while rs7568402 has the highest MAF of 0.376. Figure 6 shows the location of each SNP in the gene *PDCD-1*.

Table 2: Candidate SNPs in *PDCD-1* gene [33]

SNP	Major Allele	Minor Allele	Minor Allele Frequency
rs7568402	C	T	0.376
rs7421861	C	T	0.239
rs10204525	T	C	0.124
rs11568821	G	A	0.048

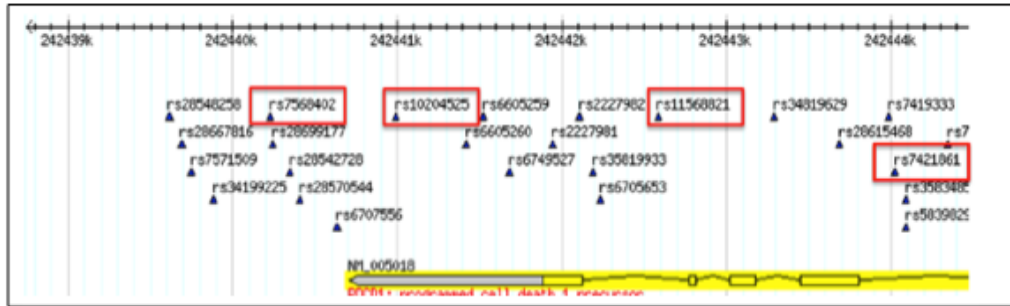

Figure 6: Location of SNPs in the *PDCD-1* locus [34]

It is important to study these SNPs because they are involved in other autoimmune diseases but have not yet been studied for correlation with JIA. A SNP is a naturally occurring single base pair mutation in the DNA sequence between individuals. On average any two individuals differ in 1 out of 1000 bases. The human genome carries million of SNPs, most of which have no overt effect on disease. While some SNPS are neutral and tolerated, others can be deleterious if they affect the protein or gene expression. Thus, SNPs have a potential to have functional consequences and result in either an increased or decreased risk for disease susceptibility. Emerging evidence suggests that several polymorphisms are involved in autoimmune disease development and/or progression [30].

III. Patient and Methods

Patients

Genotyping was completed on DNA from 1,935 subjects; however, the analysis was limited to the 1689 subjects of European ancestry, 834 cases and 855 controls. Children were recruited during routine visits at the clinic. DNA was obtained from cases previously enrolled at Pediatric Rheumatology Clinics at the University of Utah and from current patients at the Children's Physician Group at Children's Healthcare of Atlanta.

Caucasian controls were healthy volunteers from Utah who reported no history of autoimmunity at enrollment. A questionnaire was used to screen controls for autoimmune disorders and controls who reported an autoimmune disorder were excluded. Subjects were enrolled under protocols approved by the Institutional Review Boards (IRB) at the University of Utah and Emory University. Patients were diagnosed according to the ILAR criteria.

Methods

DNA extraction

DNA was extracted from whole EDTA blood samples following the Q Gentra Puregene Blood Kit (Qiagen). The DNA was normalized to a concentration of 5 μg/ml and transferred to 96 well plates for storage at -80°C.

Genotyping assay

This experiment investigated four SNPs that are in the *PDCD-1* gene; rs10204525, rs74568402, rs7421861 and rs11568821. These SNPS were studied using a fluorescent polymerase chain reaction (PCR) genotyping assay. Other genotyping assays include restriction fragment length polymorphisms assays (RFLP) that detects only variations that create or delete sites recognizable by restriction enzymes [35]. Another assay is a derived cleaved amplified polymorphic sequence assay (dCAPS), which detects mutations that create or delete a restriction enzyme site in the amplified product [36]. Both of these techniques require the SNP to alter a restriction site. However, the allelic discrimination assay is able to detect polymorphisms without the use of restriction enzymes by utilizing fluorescent probes complementary to the SNP sequence. Each TaqMan kit contains a pair of PCR primers to amplify the specific SNP and one

fluorescent probe for each allele in a two-allele SNP. Each probe also carries a quencher for the fluorescent dye. If a probe can bind to the amplified DNA i.e. contains the target allele, the quencher is removed by the Taq polymerase enzyme and the probe fluoresces. As more of the target recognized by each probe is amplified, the fluorescence increases with each cycle. A homozygous SNP will show only one dye fluorescence while heterozygotes will show fluorescence from both dyes.

Each candidate SNP was genotyped using commercially available TaqMan allelic discrimination kits. Each sample was assayed in a 10 µL reaction volume that contained 1X ABI Taqman Genotyping mix (proprietary mix of Taq polymerase, buffer, MgCl2 and enhancers), 0.9 µM of each primer, 0.2 µM of each probe and 10 ng DNA. To optimize pipetting accuracy, a mastermix containing all components except DNA was prepared. 8.0 µL of the mastermix and 2.0 µL of sample DNA were added to each well of a new 96 well optical reaction plate. Positive control samples for homozygous and heterozygous genotypes were included on each plate and for quality control at least one sample was assayed in duplicate on each plate. Homozygous genotypes are defined as the two alleles being the same and heterozygous genotypes are defined as two different alleles. The negative control well had 2.0 µL of water instead of DNA. The reaction plate was cycled in an ABI 7500 cycler using a two-step cycling protocol. A pre-run read of the plate measured the baseline fluorescence of the probes before cycling. The first step at 95°C for 10 min activates the polymerase and denatures the DNA (Figure 7). Step 2 at 95°C for 15 sec denatures the DNA then the extension step (step 3) at 60°C for 60 sec allows the primers to bind and the polymerase to synthesize the DNA and release the quenched fluorescent probes. Steps 2 and 3 are repeated 40 times. The post-run analysis

then reads the unquenched fluorescence of the two probes in each well. The ABI allelic discrimination software compares the pre-run and post-PCR fluorescence to determine which SNP alleles are present.

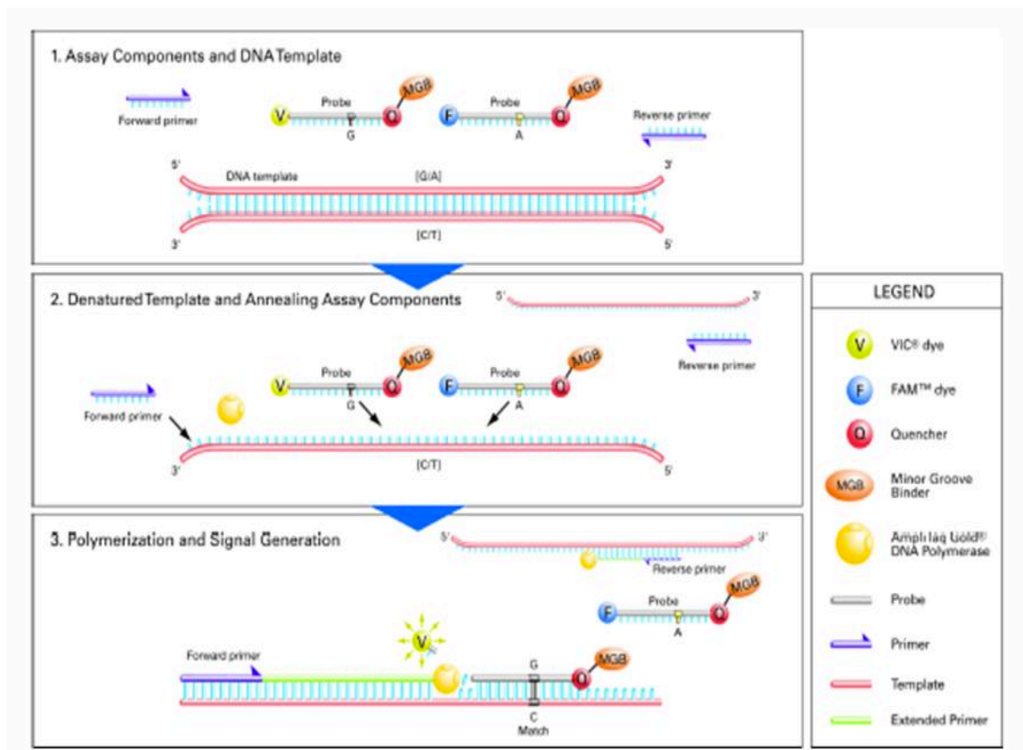

Figure 7: Taqman Assay during PCR [37]

Statistical Analysis

Prior to association analysis, we tested each variant to confirm that it was in Hardy-Weinberg equilibrium (HWE). We tested each SNP for additive association with JIA and JIA categories by using logistic regression, adjusting for gender. From the models, we calculated the allelic odds ratios (OR) and 95% confidence intervals (CI). We used permutations to adjust for multiple hypothesis testing. The permutation procedure we implemented allowed us to take into account effects of gender on JIA, as well as preserve linkage disequilibrium patterns of the SNPs. We performed 10,000 permutations under the null hypothesis of no association between genotype and JIA. We performed all

analyses using the statistical programming language R. Replicates were done on 9% of the samples.

IV. Results

Patient Results

Among our cohort of 834 cases and 855 controls of European ancestry, the median age of onset for the cases was 6.60 and 69.7% of the subjects were female. 7.1% of the cases were diagnosed with systemic JIA, 46.9% with oligoarticular JIA, 7.9% with RF positive polyarticular JIA, 25.6% with RF negative polyarticular JIA, 6.7% with ERA, and 5.9% with other categories. 59.5% of the control subjects from European ancestry were female, while 28 controls had unknown gender.

NanoDrop Results

The quality of all samples was tested using a NanoDrop Spectrophotometer. By looking at the OD 260/280 and 260/230 of the results we examined the DNA's quality. These two ratios describe the purity of the DNA samples. A 260/280 ratio of at least 1.8 shows that the DNA tested was pure. Thus all the samples used had at a 260/280 ratio of at least 1.8. If an OD ratio is substantially lower than 1.8, it could indicate the presence of protein contaminants in the sample. 260/230 is used as a secondary measure of DNA purity and a ratio above 2 is seen as pure for DNA. Figure 8 shows a nanodrop graph of sample SEJ1-1363. The 260/230 ratio is 1.86 and the 260/230 ratio is 2.07 showing that the DNA is pure and clean.

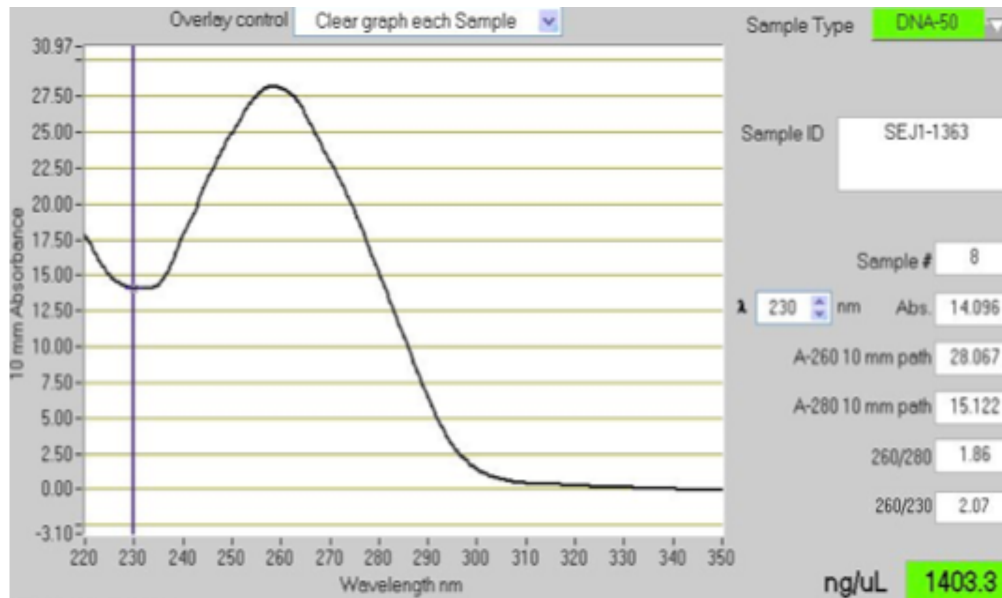

Figure 8: NanoDrop of sample SEJ1-1363

Genotyping Results

The real-time PCR was successful for 99% of the samples. The samples that failed were repeated, new samples were obtained from the patient or the DNA was re-extracted to give better yield and positive results in the PCR runs. Figure 9 shows a representative post-run allelic discrimination table for marker rs7421861. By looking at the highlighted row A1, it can be seen that each well is labeled with the corresponding sample number. It also shows allele X and allele Y, which correspond to the individual alleles (A or G) and show the corresponding fluorescent tags, VIC or FAM. The VIC and FAM tags were assigned to specific alleles when each SNP assay was set up on the cycler. The call column shows the samples' alleles to be homozygous AA, GG or heterozygous AG. The quality(%) column shows that the samples were at 100%, showing that the allele calls were unambiguous. This table also shows that well B2 contained the negative control; water and that well B12 failed and thus the call was undetermined. This sample was repeated.

Well	Sample Name	Allele X	Allele Y	Allele X Rn	Allele Y Rn	Call	Quality(%)
A1	64660	PDCD1_1861_A_VIC	PDCD1_1861_G_FAM	3.02014	1.93381	Both	100
A2	64671	PDCD1_1861_A_VIC	PDCD1_1861_G_FAM	4.40123	0.514227	PDCD1_1861_A_VIC	100
A3	63695	PDCD1_1861_A_VIC	PDCD1_1861_G_FAM	2.90254	1.53374	Both	100
A4	64998	PDCD1_1861_A_VIC	PDCD1_1861_G_FAM	3.03786	1.5428	Both	100
A5	65003	PDCD1_1861_A_VIC	PDCD1_1861_G_FAM	3.50262	2.19032	Both	100
A6	64975	PDCD1_1861_A_VIC	PDCD1_1861_G_FAM	3.74487	0.136887	PDCD1_1861_A_VIC	100
A7	64977	PDCD1_1861_A_VIC	PDCD1_1861_G_FAM	3.33511	1.62735	Both	100
A8	64954	PDCD1_1861_A_VIC	PDCD1_1861_G_FAM	3.11131	2.16797	Both	100
A9	64951	PDCD1_1861_A_VIC	PDCD1_1861_G_FAM	3.31165	2.23001	Both	100
A10	63747	PDCD1_1861_A_VIC	PDCD1_1861_G_FAM	2.1701	-0.0893132	PDCD1_1861_A_VIC	100
A11	53390	PDCD1_1861_A_VIC	PDCD1_1861_G_FAM	4.0549	2.5475	Both	100
A12	58083	PDCD1_1861_A_VIC	PDCD1_1861_G_FAM	3.68595	2.46207	Both	100
B1	63771	PDCD1_1861_A_VIC	PDCD1_1861_G_FAM	4.08686	-0.156776	PDCD1_1861_A_VIC	100
B2	WATER	PDCD1_1861_A_VIC	PDCD1_1861_G_FAM	0.420788	-0.0378793	NTC	
B3	64607	PDCD1_1861_A_VIC	PDCD1_1861_G_FAM	2.86119	0.87699	Undetermined	0
B4	65009	PDCD1_1861_A_VIC	PDCD1_1861_G_FAM	4.11937	-0.432101	PDCD1_1861_A_VIC	100

Figure 9: *PDCD-1* rs7421861 Allelic Discrimination Post-Run

The post-run of the ABI allelic discrimination software also produces an allelic discrimination plot, which shows the allele call as seen in Figure 10. This specific plot was of rs7421861 shows that 9 samples were homozygous GG (blue), 45 samples were heterozygous AG (green), and 41 samples were homozygous AA (red). Water was used as a control (grey).

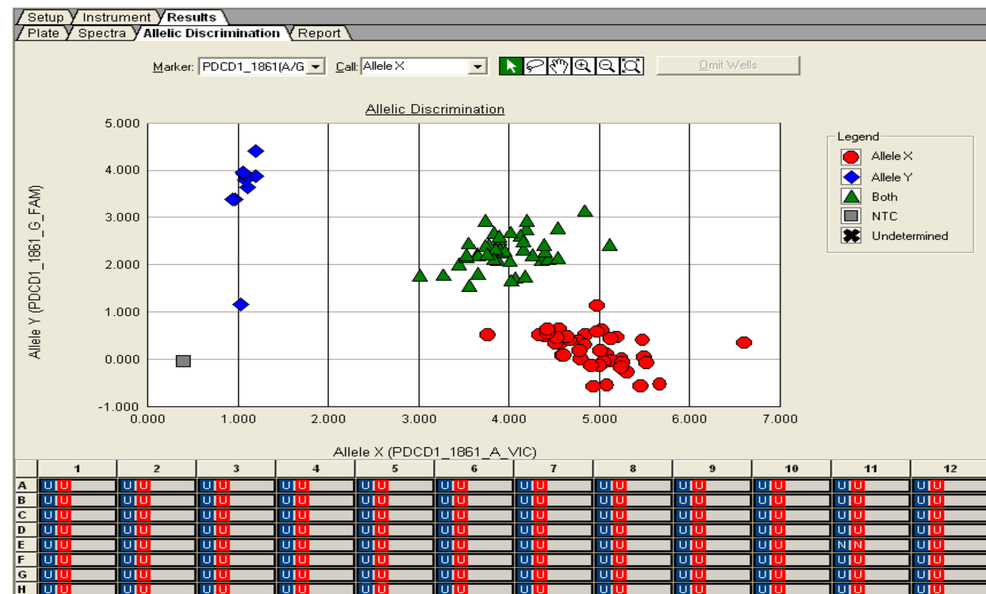

Figure 10: Allelic Discrimination Plot of DNA samples for the marker *PDCD-1* rs7421861

Statistical Analysis Results

All variants were in HWE in the controls (*p*>0.10) for all four SNPS. Using logistic regression, done in collaboration with our geneticist, we observed that *PDCD-1* variants showed no association with JIA as a whole. Table 3 shows that there was no association between any SNP and JIA as the *p*-values were over 0.005, meaning that they were not significant. Also the 95% confidence intervals include 1, showing that the probability of the variant being found in the cases and the controls is the same. After stratification by JIA categories, rs7568402 was nominally associated with systemic JIA in our cohort (OR=0.53, *p*=0.0027) (Table 4). After permutations this association remained significant with *p*=0.047. Also after stratification, rs11568821 was nominally associated with ERA JIA in our cohort (OR=0.22, *p*=0.012). There was no association between the SNPS rs10204525 and rs7421861 and the JIA categories. These results suggest that analysis of individual categories of JIA is important because they show nominal associations that would have been missed otherwise.

Table 3: Analyses of Variants and JIA for Cases and Controls

	Cases		**Controls**			
Variants	**# Cases**	**MAF**	**# Controls**	**MAF**	**OR (95% CI)**	**p-value**
rs7568402	815	0.43	845	0.44	0.95 (0.82-1.09)	0.45
rs10204525	830	0.13	855	0.12	1.12 (0.90-1.38)	0.31
rs11568821	810	0.095	845	0.11	0.84 (0.67-1.05)	0.13
rs7421861	819	0.33	852	0.33	1.04 (0.90-1.20)	0.63

Table 4: Analyses of Variants and JIA categories

	RS7568402			RS10204525			RS11568821			RS7421861		
Category	**# Cases**	**OR (95% CI)**	**p-value**	**# Cases**	**OR (95% CI)**	**p-value**	**# Cases**	**OR (95% CI)**	**p-value**	**# Cases**	**OR (95% CI)**	**p-value**
Systemic	58	**0.53 (0.35-0.80)**	**0.0027**	59	1.43 (0.82-2.39)	0.19	55	0.94 (0.48-1.71)	0.85	57	1.44 (0.99-2.10)	0.056
RF-Positive	64	0.88 (0.59-1.28)	0.5	63	1.40 (0.82-2.30)	0.19	62	1.26 (0.70-2.13)	0.42	65	1.12 (0.76-1.61)	0.56
RF-Negative	208	1.05 (0.84-1.31)	0.7	213	1.01 (0.72-1.41)	0.94	204	0.75 (0.51-1.08)	0.14	210	1.09 (0.87-1.36)	0.46
ERA	56	0.83 (0.55-1.23)	0.35	56	1.42 (0.79-2.41)	0.21	55	**0.22 (0.054-0.61)**	**0.012**	55	0.73 (0.46-1.12)	0.16
Oligoarti-cular	382	1.00 (0.84-1.20)	0.99	389	1.01 (0.77-1.31)	0.96	378	0.96 (0.72-1.26)	0.76	382	1.06 (0.89-1.28)	0.5

V. Discussion and Conclusion

This study was a backward direction retrospective case-control study that examined JIA and its association with SNPS in the gene *PDCD-1*. JIA is caused by the complex interaction of genes and the environment. Due to JIA's genetic complexity, extensive research has been conducted on the genetic component, while fewer experiments have been conducted on the environmental factors that influence JIA.

JIA genetics are complicated because it has incomplete penetrance (JIA does not necessarily develop even with a predisposing allele), polygenic inheritance (JIA might require the presence of multiple mutations in multiple genes) and genetic heterogeneity (mutations in different genes might each result in JIA) [38]. In addition, JIA is a heterogenic disease meaning that different factors contribute to the pathogenesis and cause [14]. Due to the focus on the genetic component, immense research has been

conducted on the HLA region because of the vast amount of immune response genes found in this region. In addition, about 100 different non-HLA candidate loci have been investigated for associations with JIA, although only a few such as *STAT4, TNFAIP3, CCR5,* and *PTPN22* have been found to have an association [1]. As genes involved in the immune system, like *PDCD-1,* have been found to be linked with autoimmune diseases we sought to test if this is the same between JIA and *PDCD-1*.

The SNPS rs10204525, rs7568402, rs7421861 and rs11568821 were chosen for this study because they have been involved in other autoimmune diseases, for example rs7568402 was found to be implicated in SLE. The likelihood of detecting associations may have been improved by examining more SNPs, though scanning the whole *PDCD*-1 gene for allelic associations would be very time consuming. Fortunately linkage disequilibrium (LD) helps with this problem as it allows for only a portion on the alleles to be examined. In population genetics, the linkage disequilibrium (LD) states that alleles that are in close proximity will descend from a single ancestral chromosome. Thus with LD we can suspect similar results to be found for adjacent SNPs. Or in other words, LD measures the degree that two loci are not independent of each other. Similarly to HWE, it is affected by natural selection, genetic drift, inbreeding, mutation and gene flow [39]. Gene flow between two populations with different frequencies of the alleles at both loci can cause high LD [39]. Importantly, even if linkage disequilibrium is detected it does not ensure linkage [39]. Figure 8 shows that a large number of the SNPS are overlapping, thus two close SNPS would essentially give the same results

In addition, the SNPs studied were in different locations around and in the gene. SNP rs7568402 is in the region preceding the gene and thus may be involved in the

regulatory sequence, as regulatory sequences can be as far as 2 million bases away from the gene-encoding region. SNP rs10204525 is in the promoter region and SNPs rs11568821 and rs7421861 are in the intronic region. The SNPs in the intronic region can still play a role in regulation and can also effect splicing.

Interestingly, the effect a SNP has on the amino acid sequence differs. Three mRNA nucleotides together make up a codon, which corresponds to an amino acid. Codon degeneracy allows for multiple codons to code for the same amino acid. Thus in some cases even though a SNP changes a nucleotide the codon may code for the same amino acid, while in other cases it might change the amino acid. Though as none of the SNPs we studied were involved in the exonic region, none were coded and thus do not produce or change the amino acids.

By studying these SNPS we found that the *PDCD-1* variants showed no association with JIA as a whole. After stratification by JIA categories, rs11568821 was nominally associated with ERA JIA in our cohort (OR=0.22, p=0.012) and rs7568402 was associated with systemic JIA in our cohort as it withstood correction by permutation (OR=0.53, p=0.0027). Odd ratios greater than 1 show that the cases are more likely exposed than the controls, OR equal to 1 show the null hypothesis, no association, and OR less than 1 show that the cases are less exposed than the controls. As the two associated OR values are less than 1 it shows that these SNPS are protective, meaning that patients with these SNPS are less likely to develop systemic and ERA JIA respectively (Table 4).

It is interesting that this study confirmed an association with systemic JIA as this specific category largely differs from the other JIA categories. Firstly, when we compare

systemic JIA to other categories, systemic is associated with macrophage activation syndrome and innate pro-inflammatory cytokines, while other categories are associated with the adaptive immune system systemic. Additionally, other autoimmune diseases have a strong association to MHC Class II, while systemic JIA has a weak association [40]. An example of this importance is that a study showed that the MHC loci contributed to most of the genetic predisposition to oligoarticular JIA, the most common JIA category [41]. In addition, systemic JIA does not respond to therapeutic agents and does not have a sex bias or peak onset age like the other categories do [40,41]. These immense differences show that this is a truly significant association and evaluation in a larger cohort would be helpful. The difference between systemic and the other categories could also account for the instance that certain genes are associated with different categories, as PTPN22 is associated with the polyarticular and oligoarticular categories of JIA but not systemic [1].

We are confident about our results as the protocol was done meticulously. Most case-control studies are usually limited by a small cohort, which could cause the study to be underpowered to detect association with modest OR [7]. Also many other studies do not correct for multiple comparisons so it is difficult to confirm if their findings are not due to false positives [7]. This study did not share these limitations as we tested 1689 samples, thus cohort size was in fact large and performed about 10,000 permutations to confirm our findings. In addition, population stratification can be a problem with many case control studies. In our study the cases were from both the University of Utah and Children's Healthcare of Atlanta, while the controls were just from the University of Utah. We think it is unlikely to have affected our results, since the Utah population has

been shown to be representative of the Northern European population in the United States.

For future proceedings, it also may be interesting to examine the expression of the PD-1 and other proteins using techniques such as FACS (fluorescent activated cell sorting) and flow cytometry of immune cells. Flow cytometry would permit examination of the PD-L1/PD-L2 pathway in more detail specifically in relation to how their function differed between JIA categories that showed nominal association and those that did not. This approach would require fresh or fixed immune cells from each subject for flow analysis. Unfortunately, these types of samples were not available for this study. Also, it may be interesting to perform a power analysis to test if the size of our cohort was in fact large enough to able to confirm an association.

In summary, *PDCD-1* was found not to be associated with JIA as a whole, although there may be associations with JIA categories. This may be due to the fact that *PDCD-1* may be specific for certain autoimmune diseases and is not involved in JIA.

VI. References

1. Kaalla, M. et al., "Meta-analysis confirms association between TNFA-G238A variant and JIA and between PTPN22-C1858T variant and oligoarticular, RF-polyarticular and RF-positive polyarticular JIA"

2. Janeway C., Travers P., Walport M, et al., "Immunobiology: The Immune System in Health and Disease" Garland Science (2001) 5

3. Ellis J., Kemp A., Ponsonby A. "Gene-environment interaction in autoimmune disease" Expert Reviews in Molecular Medicine. (2014) 16(4):1-23.

4. Goronzy JJ, Weyand CM. "The innate and adaptive immune systems" Cecil Medicine. (2007) 23(42).

5. Gough S., Simmonds M. "The HLA region and autoimmune disease: associations and mechanisms of action" Current Genomics (2007) 8:453-465.

6. Chistiakov D., Savost'anoc K., Barnaov A. "Genetic background of juvenile idiopathic arthritis" Autoimmunity (2014)

7. Prahalad, S., Glass, D., "A comprehensive review of the genetics of juvenile idiopathic arthritis" Pediatric Rheumatology (2008) 6(11)

8. Han, S., Prahalad, S., "The genetics of juvenile idiopathic arthritis: what is new in 2010?" Curr Rheuamtol Rep (2010) (12):87-93

9. Woo P., Colber R. "An overview of genetics of paediatric rheumatic diseases" Best Practice and Research Clinical Rheumatology (2009) 23:589-597.

10. Djos A., Martinsson T., Kogner P., Caren H. "The RASSF gene family members RASSF5, RASSF6 and RASSF7 show frequent DNA methylation in neuroblastoma" Molecular Cancer (2012) 11(40)

11. Brusca S., Abramson S., Scher J. "Microbiome and mucosal inflammation as extra-articular triggers for rheumatoid arthritis and autoimmunity" (2013) 26:101-107.

12. Neff et al., The Plant Journal (1998) 14(3): 387-392.

13. Cobb J., HInks A., Thomson W. "The genetics of juvenile idiopathic arthritis: current understandings and future prospects" Rheumatology (2013)

14. Ravelli A, Martini A. "Juvenile idiopathic arthritis" The Lancet (2007) 369(9563):767-778.

15. Magni-Manzoni S, Malattia C, Lanni S, Ravelli A. "Advances and challenges in imaging juvenile idiopathic arthritis" Nature Reviews (2012)

16. Angeles-Han S., Yeh S., Vogler L. "Updates on the risk markers and outcomes of severe juvenile idiopathic arthritis-associated uveitis" Int J Clin Rheumtol (2013) 8(10)

17. Hamilton J., Capell H. "The treatment of juvenile arthritis" Expert Opin. Pharmacother. (2001) 2(7):1085-1092.

18. Lomholt J., Thastum M., Herlin T. "Pain experience in children with juvenile idiopathic arthritis treated with anti-TNF agents compared to non-biologic standard treatment" Pediartric Rheumatology (2013) 11:21

19. Talukder M., Rahman S. "Use of methotrexate in the treatment of juvenile idiopathic arthritis"

20. Ramanan A., Whitworth P., Baildam E. "Use of methotrexate in juvenile idiopathic arthritis" (2003) Arch Dis Child 88:197-200.

21. Russo R., Katsicas M. “Clinical Remission in Patients with Systemic Juvenile Idiopathic Arthritis Treated with Anti-Tumor Necrosis Factor Agents” Journal of Rheumatology (2009) 36(5):1078-1082

22. Darnay B., Aggarwal B. “Signal transduction by tumour necrosis factor and tumour necrosis factor related ligands and their receptors” (1999) Ann Rheum Dis 58:2-13.

23. “The science behind the success of anti-TNF therapy” Arthritis Research UK (2010)

24. Wilman B, Prokunina L, Padyukov L, et al., “Association of the PD-1.3A allele of the PDCD-1 gene in patients with rheumatoid arthritis negative for rheumatoid factor and the shared epitope.” Arthritis & Rheumatism (2004) 50(6):1770-1773.

25. Cheng X., Veverka V., et al., “Structure and interactions of the human programmed cell death 1 receptor” Journal of Biology Chemistry (2013) 288:11771-11785.

26. Goronzy JJ, Weyand CM. “The innate and adaptive immune systems” Cecil Medicine. (2007) 23(42).

27. Sharpe A, Wherry J, Ahmed R, Freeman G. “The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection.” Nature Immunology. (2007) (8):239-245

28. Rui Y., Honjo T., Chikuma S. “Programmed cell death 1 inhibits inflammatory helper T-cell development through controlling the innate immune response” PNAS (2013) 110(40):16073-16078

29. Gianchecchi E., Delfino D., Fierabracci A. “Recent insights into the role of the PD-1/PD-L1 pathway in immunological tolerance and autoimmunity” Autoimmunity Reviews (2013) 12:1091-1100.

30. Ming Z. J., Hui H., Miao M., et al., “Polymorphisms in PDCD-1 gene are not associated with aplastic anemia in Chinese Han population” Rheumatology International Clinical (2011)

31. Kristjandottir H., Saevarsdottir S., et al., “Association of three systemic lupus erythematosus susceptibility factors, PD-1.3A, C4AQ0, and low levels of mannan-binding lectin, with autoimmune manifestations in icelandic multicase systemic lupus erythematosus families” Arthritis and Rheumatology (2008) 58(12):3865-2872.

32. “dbSNP, short genetic variations” NCBI http://hapmap.ncbi.nlm.nih.gov/cgi-perl/gbrowse/hapmap24_B36/#search

33. “International HapMap Project” NCBI http://hapmap.ncbi.nlm.nih.gov/cgi-perl/gbrowse/hapmap24_B36/#search

34. Gestal M., Vidal I., et al., "Analysis of the functional relevance of a putative regulatory SNP of PDCD-1, PD1.3, associated with systemic lupus erythematosus" Genes and Immunity (2008) 9:309-315

35. Botstein et al., Am. J. Hum. Genet. 32: 314-331, 1980.

36. Neff et al., The Plant Journal (1998) 14(3): 387-392.

37. "Taqman assays" http://www.dnavision.com/taqman-genotyping-assays.php

38. Prahalad, S., "Genetics of juvenile idiopathic arthritis: an update" Current Opinion in Rheumatology (2004) 16(5):588-594.

39. Slatkin, M., "Linkage disequilibrium--understanding the evolutionary past and mapping the medical future" Nature Review Genetic (2008) 9(6):477-485.

40. Mellins, E., Macaubas C., Grom, A., "Pathogenesis of systemic juvenile idiopathic arthritis: some answers, more questions" Nature Reviews Rheumatology (2011) (7):416-426

41. Ramanan, A., Grom A., "Does systemic-onset juvenile idiopathic arthritis belong under juvenile idiopathic arthritis" Rheumatology (2005) 44(11):1350-1353