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Role of Single Nucleotide Polymorphisms (SNPs) in PTPN2/22 and Mycobacterium Avium Subspecies Paratuberculosis (MAP) in Rheumatoid Arthritis and Crohn's Disease

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ROLE OF SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS) IN *PTPN2/22* AND *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* (MAP) IN RHEUMATOID ARTHRITIS AND CROHN'S DISEASE

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ABSTRACT

Both genetic pre-disposition and potential environmental triggers are shared between Rheumatoid arthritis (RA) and Crohn's disease (CD). We hypothesized that single nucleotide polymorphisms (SNPs) in the negative T-cell regulators *Protein Tyrosine Phosphatase Non-receptor type 2* and *22* (*PTPN2/22)* lead to a dysregulated immune response as seen in RA and CD. To test the hypothesis, peripheral leukocytes samples from 204 consented subjects were TaqMan genotyped for 9 SNPs in *PTPN2*/22. The SNPs effect on *PTPN2*/22 and *IFN-y* expression was determined using RT-PCR. Blood samples were analyzed for the *Mycobacterium avium* subspecies *paratuberculosis* (MAP) *IS900* gene by nPCR. T-cell proliferation and response to phytohematoagglutonin (PHA) mitogen and MAP cell lysate were determined by BrdU proliferation assay. Out of 9 SNPs, SNP alleles of *PTPN2*:*rs478582* occurred in 79% RA compared to 60% control (*p*-values ≤ 0.05). SNP alleles of *PTPN22:rs2476601* occurred in 29% RA compared to 6% control (*p*-values ≤ 0.05). For the haplotype combination of *PTPN2:rs478582*/*PTPN22rs2476601*, 21.4% RA had both SNPs (C-A) compared to 2.4% control (*p*-values ≤ 0.05). *PTPN2/22* expression in RA was decreased by an average of 1.2 fold. *PTPN2:rs478582* upregulated *IFN-y* in RA by an average of 1.5 fold. Combined *PTPN2:rs478582*/*PTPN22:rs2476601* increased T-cell proliferation by an average of 2.7 fold when treated with PHA. MAP DNA was detected in 34% RA compared to 8% controls (*p*-values ≤ 0.05), where samples with *PTPN2:rs478582* and/or *PTPN22:rs2476601* were more MAP positive. *PTPN2:rs478582/PTPN22:rs2476601* together with MAP infection significantly increased T-cell response and *IFN-y* expression in RA samples. The same experimental approach was followed on blood samples from CD patients. Both *PTPN2:rs478582/PTPN22:rs2476601*

affected *PTPN2*/22 and *IFN-y* expression along with T-cell proliferation significantly more than in RA. MAP DNA was detected in 64% of CD. This is the first study to report the correlation between SNPs in *PTPN2/22, IFN-y* expression and MAP in autoimmune disease.

This is dedicated to all my family and friends who have supported me throughout my entire life.

Without you all, I would be nothing. Thank you.

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LIST OF ACRONYMS/ABBREVIATIONS

AIEC: Adherent-Invasive *Escherichia coli*

Anti-CCP: Anti-Cyclic Citrullinated Proteins

ATG12: Autophagy-related protein 12

ATG16L1: Autophagy-related protein 16-1

ATG5: Autophagy-related protein 5

ATG7: Autophagy-related protein 7

BAX: Bcl-2-Associated X protein

BCL-2: B-cell Lymphoma Two Protein Family

BLAST: Basic Local Alignment Search Tool

BrdU: Bromodeoxyuridine

CCR6: Chemokine Receptor 6

CD: Crohn's Disease

CD40: Cluster of Differentiation 40

CFU: Colony Forming Units

CSLM: Confocal Scanning Laser Microscopy

CTLA4: Cytotoxic T-Lymphocyte-Associated Protein 4

DMARDs: Disease-Modifying Anti-Rheumatic Drugs

EDTA: Ethylenediaminetetraacetic Acid

ESR: Erythrocyte Sedimentation Rate

FISH: Fluorescent in situ Hybridization

GAD65: Glutamic Acid Decarboxylase 65 kDa

GWAS: Genome-Wide Association Studies

HLA: Human Leukocyte Antigen

HLA-DRB1: HLA class II histocompatibility antigen, DRB1 beta chain

HSP65: Heat Shock Protein 65 kDa

IBD: Inflammatory Bowel Disease

ICAM-1: Intercellular Adhesion Molecule 1

IFN-α: Interferon Alpha

IFN-β: Interferon Beta

IFN-γ: Interferon Gamma

IL-1: Interleukin 1

IL-17: Interleukin 17

IL-2: Interleukin 2

IL-2: Interleukin 2

IL-23: Interleukin 23

IL23R: Interleukin-23 Receptor

IL-6: Interleukin 6

IL-8: Interleukin 8

IRF5: Interferon Regulatory Factor 5

IRGM: Immunity-Related GTPase family M protein

IS900: Insertion Sequence 900

JAK-STAT: Janus Kinases-Signal Transducer and Activator of Transcription protein

JNK: c-Jun N-terminal Kinases

K. pneumonaie: *Klebsiella pneumoniae*

L. monocytogenes: *Listeria monocytogenes*

LB Broth: Luria Broth

M. avium: *Mycobacterium avium subspecies avium*

M. fortuitum: *Mycobacterium fortuitum subspecies fortuitum*

M. smegmatis: *Mycobacterium smegmatis*

M. tuberculosis: *Mycobacterium tuberculosis*

M. xenopi: *Mycobacterium xenopi*

MAC: *Mycobacterium avium complex*

MAP: *Mycobacterium avium* subspecies *paratuberculosis*

MCP1: Monocyte Chemoattractant Protein 1

Multiplex PCR: Multiplex Polymerase Chain Reaction

NOD2: Nucleotide-binding Oligomerization Domain-containing protein 2

nPCR: Nested PCR

NSAIDs: Non-Steroid Anti-Inflammatory Drugs

OR: Odds Ratio

P. gingivalis: *Porphyromonas ginivalis*

P. mirablis: *Proteus mirablis*

PBS: Phosphate Buffer Saline

PFA: Paraformaldehyde

PHA: Phytohematoagglutunin

PPD-Like: Purified Protein Derivative-Like

PTP1B: Protein-Tyrosine Phosphatase 1B

PTPN2: Protein Tyrosine Phosphatase Non-receptor type 2

PTPN22: Protein Tyrosine Phosphatase Non-receptor type 22

PTPs: Protein Tyrosine Phosphatases

RA: Rheumatoid Arthritis

RF: Rheumatoid Factor

RPMI: Roswell Park Memorial Institute Medium

RT-PCR: Real Time PCR

S.aureus: *Staphylococcus aureus*

SDS: Sodium Dodecyl Sulfate

SNPs: Single Nucleotide Polymorphisms

STAT1: Signal Transducer and Activator of Transcription protein 1

STAT3: Signal Transducer and Activator of Transcription protein 3

T1D: Type 1 Diabetes

TE: Tris-EDTA

TLR: Toll-Like Receptor

TNF-α: Tumor Necrosis Factor Alpha

TRAF1: TNF Receptor-Associated Factor 1

UC: Ulcerative Colitis

UCF4: University of Central Florida 4

CHAPTER ONE: INTRODUCTION

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Autoimmune Diseases

Crohn's Disease (CD)

Crohn's disease (CD) is an inflammatory bowel disease (IBD) that is characterized by transmural inflammation of the intestinal wall, which may occur at different sites of the gastrointestinal tract [1]. IBD prevalence is rapidly increasing at an alarming rate. In a recent epidemiologic study in the State of Florida, United States, it was estimated that the prevalence of CD is 222 per 100,000 persons [2]. The prevalence of CD was higher among people ages 30–80 years old, non-Hispanic Whites and females [2]. The literature is enriched with reports suggesting that CD is caused by multiple factors including genetic anomalies, environmental factors, and immune system malfunctions. The latter has significant impact on the pathophysiology of the disease including gut microbiota [3]. Genome-wide association studies (GWAS) have shown that several single nucleotide polymorphisms (SNPs) in specific genes may cause an increase in the susceptibility to developing CD [9]. These genes include *NOD2*, *ATG16L1*, *IL23R*, *IRGM*, *CCR6*, *PTPN2*, and *PTPN22* [9]. Environmental factors that have been associated with CD include pathogenic *Escherichia coli* strains, *Mycobacterium avium* subspecies *paratuberculosis* (MAP), and others [5]. Naser et al. has shown that MAP was found in the blood and breast milk of patients with CD [6][7]. Excessive secretion of pro-inflammatory cytokines and aberrant T-cell differentiation have also exacerbated CD, resulting in loss of tolerance, and intestinal dysbiosis [8].

Rheumatoid Arthritis (RA)

Rheumatoid arthritis (RA) is a deliberating autoimmune disease that affects synovial joints of individuals, where an increase of inflammation that leads to cartilage damage and bone erosion occurs [9]. The prevalence of RA in the United States alone is estimated to be at 1.36 million adults, where it continues to rise each year [10]. Diagnosis of RA begins with examination of the effected joints on the body and then with a serological blood test that examine autoantibodies such as rheumatoid factor (RF) and anti-cyclic citrullinated proteins (anti-CCP) [9][11].

As with other autoimmune diseases, such as type 1 diabetes (T1D) and CD, RA is multifactorial autoimmune diseases that has both genetic predisposition and environmental triggers. When examining GWAS, there are many SNPs that have been associated with RA including *HLA* genes, such has *HLA-DRB1* and non-*HLA* genes, such as *CD40, PTPN2,* and *PTPN22* [12][13][14]. Environmental elements along with these genetic mutations have also been examined in RA. Both chemical pressures, such as smoking tobacco or drinking alcohol, and biological pressures, such as viral and bacterial infections, have been linked to RA [15][16]. When examining biological triggers for RA, some of the viruses and bacterial species that have been associated with this disease include Rubella virus, *Porphyromonas gingivalis,* and *Mycobacterium* species [16]. Both the genetic factors and the environmental triggers together in RA patients will lead to an excess production of pro-inflammatory cytokines, such as TNF-α, IL-6, and IFN-γ [17]. This excess amount of pro-inflammatory cytokines will lead to the intense inflammation and overall destruction of the joints in RA patients [17].

Common Genes Associated with Autoimmune Diseases

GWAS have identified many genes to be involved in the development of autoimmune diseases such as T1D, CD, and RA. Most often, the mutation is due to a SNP resulting in immune system impairment and ultimately increased susceptibility to disease. The potential role of some of these genetic mutations have been examined in previous studies, but the mechanisms by which these mutated genes play a role in autoimmune diseases is still unclear and requires further research.

Recently, two possible candidate genes that are involved with T1D, CD, and RA have been examined. These genes are *PTPN2* (*protein tyrosine phosphatase non-receptor type 2*) and *PTPN22* (*protein tyrosine phosphatase non-receptor type 22*) [1][4][12][13][18][19][20][21]. *PTPN2/22* genes both encode for protein tyrosine phosphatases (PTPs) signaling molecules that modulate and regulate a variety of cellular processes such as cell growth, differentiation, mitotic cycle, oncogenic transformation, and survival [22][23]. Studies have shown that PTPs in general are key regulators of signaling transduction. Most cells of the immune system show high expression of tyrosine phosphorylation and express more PTP genes than other tissues in the body. In fact, a distinct phenotype exists among PTP-knockout mice having deficient or hyperactive immune status with severe abnormalities in hematopoiesis. This suggests a crucial role of PTP in maintaining a balanced immune system [23]. Predisposing variants in these genes can potentially lead to a less efficient suppression of inflammatory response due to a reduced amount of negative regulation, which may contribute to autoimmune diseases.

Genetic Variations of *PTPN2*/*22*

PTPN2 Role in Autoimmune Diseases

The *PTPN2* gene is located on chromosome 18 and is a member of the PTP family, which dephosphorylates receptor protein tyrosine residues and regulates many signaling pathways and processes [20]. The protein has two major isoforms—one in the endoplasmic reticulum (48 kD) and the other in the nucleus (45 kD) [20]. PTPN2 is produced by alternative splicing and share a highly conserved PTP catalytic domain but different C-terminus [20]. *PTPN2* expression plays an important role in regulating signal transduction and it is of pivotal importance to the pathogenesis of many autoimmune diseases.

The involvement of the *PTPN2* gene in autoimmune diseases is complex due to its ubiquitous expression which may play a role in tissue cell apoptosis [24]. This modulation occurs after exposure to type I (IFN-α and IFN-β) and type II interferon (IFN-γ), which leads to destruction of a variety of tissue cells including beta cells, intestinal tissues, and synovial joints [24][25][26]. Moreover, studies indicated that local IFN production interacts with *PTPN2* expression and induces a malfunctioning pro-apoptotic activity of Bim, a BH3-only protein [24][25][26]. Bim is a member of the B-cell lymphoma two protein family (Bcl-2) that mediates apoptosis by activating Bax and Bak. This ultimately results in an increase of cell death via JNK activation and intrinsic apoptotic pathways [24]. PTPN2 is a negative regulator of the JAK-STAT signaling pathway, which is activated downstream by IFN receptors [24][25][26]. Studies have shown that the *PTPN2* gene knockdown exacerbates type I and II IFN-induced cell death by inducing BAX translocation to the mitochondria after subsequent exposure to type I and II IFNs [24][25][26]. This occurs because when *PTPN2* is mutated or knockdown, there is less of a negative regulation

of apoptotic processes, thus, increasing the signaling of the destruction of cells. Along with these reactions, there is an increase of Bim phosphorylation, which is regulated by JNK1 that also induces apoptosis of the tissue cells.

Recently, the role of *PTPN2* in chemokine producing cells, such as pancreatic beta cells, have shown to control endocrine function and insulin secretion. In a study by Xi et al. the deficiency in *PTPN2* expression by knockout affected beta cell function in mice [27]. The reduced insulin secretion was associated with a decreased insulin content and glucose sensing, which showed that STAT3 could be a relevant target for the PTPN2 phosphatase regulation in the pancreas [27]. PTPN2 regulates insulin signaling by inactivating its receptor through de-phosphorylation of the insulin receptor β-chain in conjunction with the PTP1B phosphatase. This regulates gluconeogenesis in the liver by attenuating STAT3 signaling, which decreases glucose levels [20][28]. A deficiency of *PTPN2* expression will lead to a cytokine-induced beta cell apoptosis of the pancreatic cells after inducing the mitochondrial apoptotic pathway along with impacting glucose homeostasis/utilization [24][28]. With these two systems out of control, T1D could occur in patients who have a mutation in the *PTPN2* gene. This evidence shows that adequate *PTPN2* expression is required for STAT dimer regulation during chemokine production in various cell types.

With the help of CD4+ helper T cells, CD8+ cytotoxic T-cells are the primary mediators of cell destruction via secretory (perforin/granzyme) or Fas mediated pathways. Wiede et al. showed that a variant in *PTPN2* (rs1893217) in mice greatly increases T cell receptor signaling, which can lead to reduced self-antigen tolerance due to decreased negative regulation [29]. With this

occurring, the response after self-antigen presentation could cause destruction of a variety of cell types [29]. Moreover, the risk variant rs1893217 in the *PTPN2* gene is associated with a reduction in the receptor signaling of IL-2, which alters expression of FOXP3+ T regulatory cells (Tregs) in autoimmune disease patients [20]. Tregs are a group of T-cells that modulate the immune system homeostasis by maintaining tolerance to self-antigens. They also prevent autoimmune diseases by acting as suppressors to the immune response. This dysregulation of FOXP3+ Tregs leads to both T-cells and B-cells being unregulated due to FOXP3+ Treg cells suppressing their activation [20]. With these altered FOXP3+ Tregs, over reactivity of both T-cells and B-cells could cause self-antigens to be recognized as foreign [20]. It explains how genetic variations in *PTPN2* could lead to the development of autoimmunity due to the deregulation of Tregs homeostasis [20].

When examining the effect of *PTPN2* in cell regulation, the epithelial barrier of CD patients is a good model to look into. It is a fact that epithelial barrier dysfunction coincides with immune response dysregulation in CD, where *PTPN2* regulates intestinal epithelial barrier function and is activated by IFN-γ which is up regulated by TNF-α in intestinal epithelial cells (IEC)[25][30][31]. IFN-γ is an effector cytokine for Th-1 and potentially Th17-propagated immune responses [25][31]. Scharl et al. showed that PTPN2 gets activated by IFN-γ and in turn, it limits the pro-inflammatory cytokine-induced signaling and barrier defects [3]. IFN-γ plays a role in CD pathogenesis and is noted to increase the permeability of intestinal epithelial barrier [3]. IFN-γ is involved in tissue destruction and possibly, in reduction of barrier functions as a result of reconfigured tight junctions [3]. PTPN2 usually protects the barrier by reducing its permeability and prevent induction of pore forming protein claudin-2 [3]. Claudin-2 is part of a

family of proteins that regulates paracellular permeability and functions as sealer-like in tight junctions [3]. Expressions or localization changes in claudins result in increased barrier permeability [3]. Recent study showed that claudin-2 upregulation in CD increased number of tight junction strand breakages [25]. *PTPN2* expression plays a role in the regulation of inflammatory response, as loss of it leads to a severe IFN-γ signaling cascade, leading to problems in the intestinal epithelial barrier function [3][25]. PTPN2 has an important role in cytokine signaling of immune cells by inactivating STAT1 and STAT3; where the loss of *PTPN2* gene expression enhances STAT phosphorylation [3]. This evidence shows the importance in how a mutation altering function of the *PTPN2* gene could lead to deleterious effects and may explain the pathogenesis of associated diseases.

Loss of *PTPN2* expression is associated with increased expression and secretion of pro-inflammatory cytokines [1][3][31][32]. As previously stated, there is an aberrant T-cell differentiation and tissue cell destruction in autoimmune diseases, which *PTPN2* seems to play a role in. It is very important to regulate T helper (Th)-cell differentiation into effector T-cell populations to maintain tolerance toward self-antigens. There is a potential role of the PTPN2 protein in regulating differentiation of CD4+ Th-cells into its subset population. A loss of the PTPN2 protein in T-cells results in a disease promoting state. Loss of *PTPN2* in T-cell compartments leads to enhanced induction of Th1 and Th17 cells while having an impaired induction of regulatory T-cells [31]. In several mouse models as shown by Spalinger et al., increased inflammation occurred as a result of high numbers of Th1 and Th17 cells due to the loss of the PTPN2 protein function, where higher amounts of pro-inflammatory cytokine production was examined [31].

PTPN2 also plays a role in autophagosome formation in tissue cells. Autophagy is an essential process for maintaining cell homeostasis, survival, and modulating inflammation. Studies have shown that knockdown of *PTPN2* caused impaired autophagosome formation and dysfunctional autophagy resulting in response to TNF-α and IFN-γ [19][32][33][34]. Moreover, silencing *PTPN2* in vitro exacerbates intestinal epithelial barrier dysfunction when exposed to IFN-γ [33]. Impairment in this gene shows that the pathway that leads to the perpetual tissue inflammation is associated with autoimmune diseases. Loss of *PTPN2* expression can also lead to an increase in cytokine-induced mTOR phosphorylation, which leads to a decrease in autophagy [19][32][33][34]. It was reported that *PTPN2* deficiency leads to a reduction of expression of autophagy genes that include: *beclin 1, ATG7, ATG5, ATG12* conjugates, and *ATG16L1* [20][32][33][34]. Consequently, this leads to low amounts of autophagy proteins that create an abnormal autophagosome in the intestinal cells [25].

PTPN2 expression is very important in immune regulation as can be noted with *PTPN2* deficient mice that suffer severe inflammation and die swiftly after birth. A balance between inflammatory and regulatory T-cells should be maintained for optimal tolerance and protection against pathogens. The mutation in *PTPN2* could not only cause T1D, CD, or RA, but could also have comorbidity with each other due to the presence of this mutation in both disease states. With this unregulated immune system due to the loss of PTPN2 function, cytokines that play a role in inflammation are substantially increased, and T-cells/B-cells begin to react to self-antigens. These changes will affect major tissue areas of the body, such as pancreatic beta cells, intestinal

tissues, or synovial joints, of these genetically susceptible patients and lead to T1D, CD, and/or RA.

PTPN22 Role in Autoimmune Diseases

The *PTPN22* gene is located on chromosome 1p13, which is a member of the PTPs that negatively regulate T-cell activation [20]. The encoded protein is a lymphoid specific intracellular phosphatase that associates with the molecular adapter protein CBL [20]. *PTPN22* has alternatively spliced transcript variants encoding several distinct isoforms [20]. It is located in the cytoplasm, and consists of an N-terminal phosphatase domain and a long non-catalytic C terminal with several proline rich motifs [20]. PTPN22 dephosphorylates kinases Lck, Fyn, and ZAP70, which are all involved in T-cell signaling [20]. A SNP mutation (rs2476601) in *PTPN22* is associated with autoimmune diseases [4][18][35]. Variants within these genes lead to the development of an abnormal immune response [4][18][35]. The *PTPN22* rs2476601 SNP causes a single substitution of arginine for tryptophan in the encoded protein (R620W) leading to problems in T-cell receptor and B-cell receptor signaling [36]. This may ultimately result in an unbalanced establishment of tolerance in both T-cells and B-cells [36].

In B-cells, *PTPN22* SNPs prevent the removal of developing auto-reactive B-cells [36]. Menard et al. showed that new mature naive B-cells from carriers of this variant had higher frequencies of auto-reactive clones as opposed to non-carriers [36]. This demonstrates defective central and peripheral B-cell tolerance checkpoints leading to the development of the previously mentioned auto-reactive B-cells. To be noted, there are essentially two methods to removing autoreactive B-cells. First, a central tolerance checkpoint is done to remove most of the developing B-cells

expressing polyreactive antibodies in the bone marrow [37]. Second, a peripheral tolerance checkpoint is done in order to counter select autoreactive new B-cells before entering compartments designed for mature naive B-cell [37]. This shows that a single risk allele would have a dominant effect of changing auto-reactive B-cell counter-selection before onset of any autoimmunity. Menard et al. also performed gene array experiments on mature naive B-cells with the risk variant and found an upregulation of genes such as *CD40, TRAF1*, and *IRF5* [36]. These genes encode proteins promoting B-cell activation and are susceptibility genes of many deregulated immune diseases [36]. They concluded that the association of the *PTPN22* gene with autoimmunity is due to impaired removal of auto-reactive B-cells and the upregulation of the genes mentioned above [36].

In T-cells, PTPN22 is directly involved in threshold setting for T-cell receptor signaling [38]. Recent studies on *PTPN22* knockout mice suggested that the increase risk of developing autoimmune diseases could occur through alterations of the periphery Treg cells while *PTPN22* knockout increases the thymic selection of Treg cells [39]. Both Wu et al. and Zheng et al. reported a "gain-of-function" model of Treg cell selection, where even though *PTPN22* knockout did have reduced TCR signaling, they did not have an impairment of their ability to negatively select autoreactive T-cells in the thymus [40][41]. Overall, this shows that the *PTPN22* SNP does not necessarily affect Treg cells, but could possibly affect other T-cells once they leave out of the thymus or even have other effects on the immune system. With the "loss-of-function" model, it shows that if *PTPN22* is knocked out or mutated, then there is a loss of self-tolerance earlier on in the T-cell life, which can then be activated by self-antigens. This also leads to a higher amounts of T-cell activity and pro-inflammatory cytokine production due to loss of negative

regulation. Even though the role of *PTPN22* mutation is still debatable, both models ("gain-of-function") and ("loss-of-function") can still play a role in the development of autoimmune diseases.

PTPN22 expression could potentially influence immuno-receptors, which could explain how it contributes to the development of diseases. Immuno-receptor signaling is governed by Src and Syk kinases, which are substrates of the PTPN22 protein [42]. A function of PTPN22 is to downregulate T-cell signaling by interacting with its negative regulatory kinase, C-terminal Src tyrosine kinase or Csk. A mutation of the *PTPN22* gene ends up encoding products with different Csk binding affinities [42]. The R620W (rs2476601) substitution in PTPN22 decreases the ability of the phosphatase to bind to the SH3 domain of Csk, thus, showing how *PTPN22* expression is associated with T-cell signaling pathways [42]. This can be used as a marker for disease progression by the noted appearance of autoantibodies and increased pro-inflammatory cytokine levels when examining autoimmune diseases [20][35].

When examining the *PTPN22* rs2476601 SNP in CD, for example, the alteration in *PTPN22* expression levels and its dysfunction can have deleterious effects depending on the mechanism involved [4]. Normally, the intestinal immune system is usually tightly controlled by an existing balance of pro-inflammatory and anti-inflammatory cytokines. Patients suffering from IBD have a disturbed balance with more pro-inflammatory cytokines present. CD have a reduced expression of *PTPN22* in intestinal tissues [32][34]. Spalinger et al. showed that *PTPN22* expression regulates intracellular signaling as induced by IFN-γ in human monocytes [32]. Studies have shown that knocking down the *PTPN22* gene alters the activation of inflammatory

signal transducers and increases the secretion of Th17-related inflammatory mediators [32]. By this mechanism, genetic variants may induce pathogenesis of CD by prompting Th17 vs Th1 differentiation [32]. Spalinger et al. also found that the loss of *PTPN22* protein function results in increased p38-MAPK but reduces STAT1 and STAT3 signaling [32]. This leads to increase levels of IL-6 and IL-17 secretion, and decrease expression and secretion of T-bet, ICAM-1, MCP-1, IL-2, IL-8, and IL-12p40 [32]. The reduced *PTPN22* levels contribute to increased levels of IL-6 found in CD [32]. Also, p38 activation and IL-6 secretion by antigen presenting cells play a huge role in differentiation of CD4+ T cells into Th17 cells, which induces CD pathogenesis [32]. The mechanism behind how *PTPN22* genetic variants are associated with CD is an example on what happens in other autoimmune pathogenesis as well.

PTPN22 plays an important role in cytokine secretion balance, which is crucial for activation and regulation of the immune system [32]. Mutations of *PTPN22* not only will lead to cytokine imbalance, but it can also lead to T-cells and B-cells losing their ability to recognize self-antigens from foreign antigens. These imbalances can lead to the destruction of tissues, which can lead to autoimmune diseases like T1D, CD, and RA.

PTPN2/22 and Environmental Triggers in Autoimmune Diseases

SNPs and Environmental Triggers Associated with Autoimmune Diseases

Although autoimmune diseases have always been established to be associated with genetic mutations in specific immunoregulatory genes, recent literature has shown that certain environmental triggers (chemical, bacterial, or viral) have also been investigated to be involved in the pathophysiology of these diseases. The interplay between genetic mutations and

environmental triggers is a topic of research in autoimmune etiology that is beginning to become more relevant in the field [43][44][45][46][47][48]. For CD patients, SNPs found in *NOD2* have shown to increase susceptibility to bacterial infections [44][45]. The prevalence of an increase of SNPs found in *HLA* genes together with smoking tobacco has been found more so in RA patients than other autoimmune diseases as well [46][47]. Overall, the literature shows that when SNPs are involved in these autoimmune diseases, there appears to be some connection to an environmental trigger that goes with the pathophysiology of the diseases. This proposes the hypothesis that genetic factors and an environmental trigger need to be present together to produce an autoimmune response.

SNPs in PTPN2/22 and Infections in Autoimmune Disease

When examining SNPs in *PTPN2/22* along with environmental triggers in autoimmune disease, it is shown that these two factors could be related to each other [24][25][48][49]. SNPs in *PTPN2/22* have shown to possibly increase the susceptibility and the impact of viral and bacterial infections in T1D, CD, and RA [24][25][48][49]. This is due to the dysregulation of the immune system, where the viral and bacterial infections exacerbate inflammation [24][25][48][49]. For example, loss of function or poor activity of PTPN2/22 has shown to increase type 1 and type 2 IFN production, which leads to higher amounts of apoptosis in beta cells, intestinal tissues, and synovial joints [24][25][48][49]. With a viral or bacterial infection in these genetically susceptible individuals, this should significantly increase IFN production, thus further advancing the apoptotic processes in the specific tissue areas [24][25][48][49].

When examining T1D, CD, and RA, it is shown that infections with *Mycobacterium* species could be associated with the pathophysiology of these diseases [6][7][47]. However, correlation between SNPs in *PTPN2/22* and mycobacterial infection have not been fully established. In this dissertation, exploration of SNPs in *PTPN2/22* along with mycobacterial infection is investigated in autoimmune disease patient samples. We hypothesized that SNPs in *PTPN2/22* lead to a dysregulated immune response, susceptibility to environmental triggers, and continued apoptosis as seen in chronic inflammation in autoimmune disease patients.

Our hypothesis is based on our earlier finding as shown in Figure 1 that since SNPs in *PTPN2/22* are proposed to cause an increase of pro-inflammatory cytokines, there will be a higher activation of both CD4+ helper T-cells and CD8+ cytotoxic T-cells [20][29][32][36][38][40][41]. With this higher levels of pro-inflammatory cytokines, there will be a higher increase of cellular death of macrophages from CD8+ cytotoxic T-cells and a higher amount of macrophage activation from CD4+ helper T-cells [50][51][52][53]. Since MAP can avoid phagosome-lysosome fusion and can survive in the macrophages, higher amounts of macrophage activation from overactive CD4+ helper T-cells will lead to a higher chance of MAP to thrive in the macrophages [54][55][56]. Moreover, overactive CD8+ cytotoxic T-cells will increase overall cell death in MAP infected macrophages, thus releasing the MAP bacterium that survive the apoptosis or necrosis process in the macrophages [54][55][56]. Overall, chronic inflammation and destruction of tissue cells in autoimmune diseases could potentially occur due to the increase of overactive T-cells and increased tissue apoptosis by both SNPs in *PTPN2/22* and the constant cycle of MAP infecting macrophages (Figure 1).

The second part of this dissertation will be focused on confirmation that MAP is found more readily in CD patients than in other IBD groups, such as ulcerative colitis (UC). There have been many different pathogenic bacterial infections that have been associated with IBD including not only MAP, but also *Klebsiella pneumoniae* (*K. pneumoniae*) and pathogenic adherent-invasive *Escherichia coli* (AIEC) [5][6][7][57][58][59]. The hypothesis proposed is that MAP is found in the CD patients more so than the other bacterial pathogens examined, where MAP should not be found in the UC patients. To elucidate this hypothesis, a development of a multiplex polymerase chain reaction (multiplex PCR) and a fluorescent *in situ* hybridization (FISH) visualization method was designed to examine the presence of MAP, *K. pneumoniae*, and AIEC together in one clinical sample.

Figures

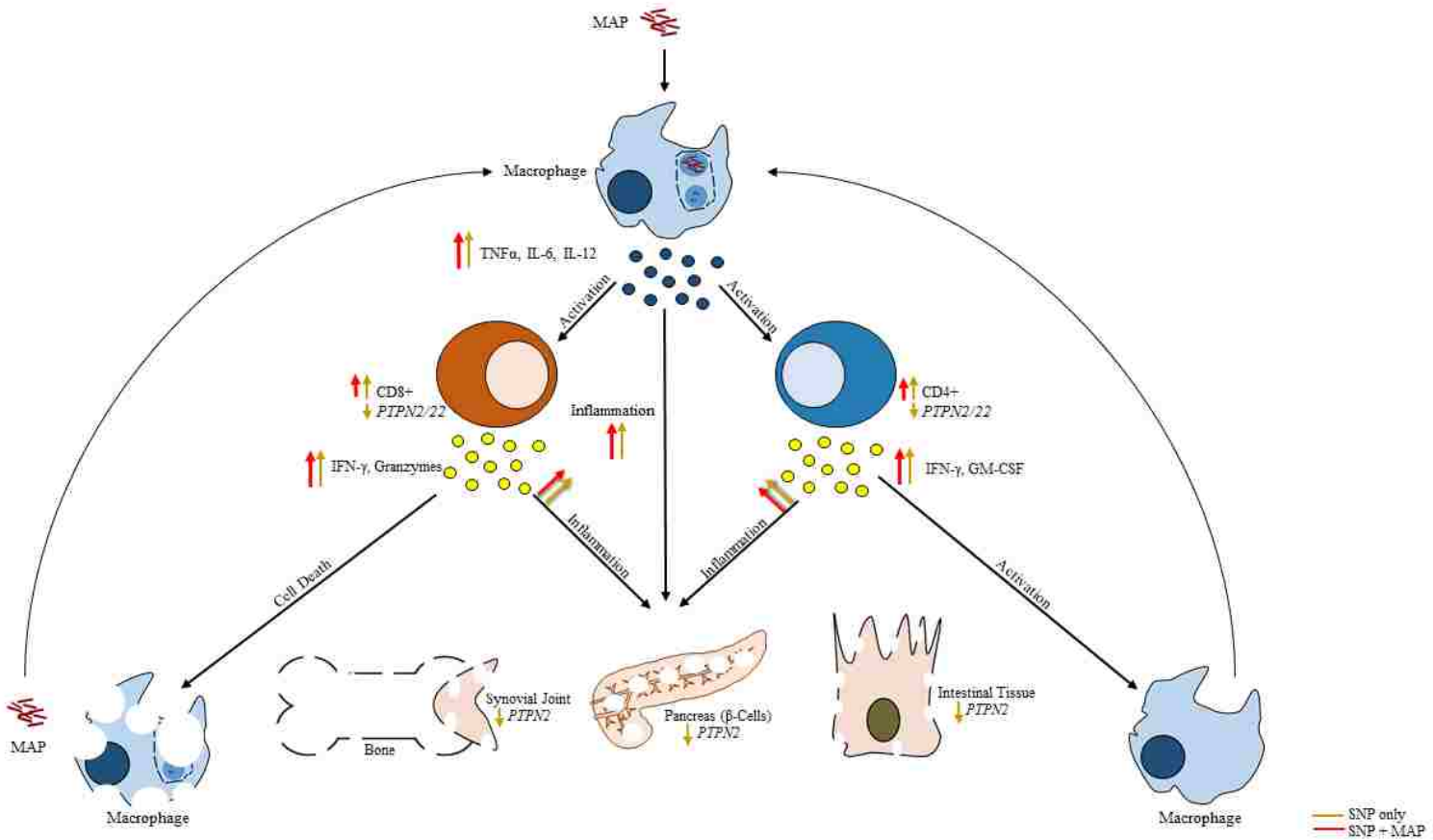

Figure 1. Single Nucleotide Polymorphisms (SNPs) in *Protein Tyrosine Phosphatase Non-Receptor Type 2* and *22* (*PTPN2/22*) and *Mycobacterium avium* subspecies *paratuberculosis* (MAP) Interaction in Autoimmune Disease

The proposed hypothesis suggests that when SNPs in *PTPN2/22* is present along with a MAP infection, chronic inflammation occurs leading to autoimmune diseases such as type 1 diabetes (T1D), Crohn's disease (CD), and rheumatoid arthritis (RA). SNPs in *PTPN2/22* will make CD8 + cytotoxic and CD4+ helper T-cells overactive, thus producing high amounts of pro-inflammatory cytokines. These cytokines will activate more macrophages and induce more cell destruction in infected macrophages. MAP infection exacerbates the disease since MAP survives in the macrophage and able to re-infect new macrophages, thus causing more pro-inflammatory cytokines production, more inflammation and worsening the disease.

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CHAPTER TWO: POLYMORPHISMS IN *PROTEIN TYROSINE PHOSPHATASE NON-RECEPTOR TYPE 2 AND 22* (*PTPN2/22*) ARE LINKED TO HYPER-PROLIFERATIVE T-CELLS AND SUSCEPTIBILITY TO *MYCOBACTERIA* IN RHEUMATOID ARTHRITIS

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Introduction

In RA, several SNPs have been reported in *HLA class 2 histocompatibility antigen, DRB1 beta chain* (*HLA-DRB1*), *PTPN22, cytotoxic T-lymphocyte-associated protein 4* (*CTLA4*), and *cluster of differentiation 40* (*CD40*) [1][2][3]. SNPs in these genes alter or stimulate the activation and regulation of major components of the immune system (T-cells, B-cells, macrophages, etc.) and osteoclasts which could lead to immune-dysregulation [1][3][4]. Consequently, this leads to accumulation of immune cells in and around synovial joints and excessive production of anti-CCP, RF, and various pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-1, and IL-6 [1][3][4]. Specifically, SNPs in immune regulatory genes such as *PTPN2/22* could potentially cause these problems in RA. We agree that the prevalence of SNPs in *PTPN2/22* may vary and we support the possibility that the effect on gene expression may be significant which ultimately may void their functions as negative regulators (Figure 2). Consequently, T-cells remain constantly active, leading to hypersecretion of pro-inflammatory cytokines and inflammation along with tissue damage [5][6].

RA is an idiopathic autoimmune disease with suspected genetic predisposition and environmental triggers association. Due to intense inflammation, hyperplasia of the joints occurs along with cartilage and bone destruction, which leads to extreme pain and deformity of the extremities [1][3][7]. RA symptoms include joint swelling and pain of three or more joints, morning stiffness lasting 30 min and subcutaneous rheumatoid nodules [8]. Anti-CCP along with RF have also been useful to diagnose RA, more so than erythrocyte sedimentation rate (ESR) and C-reactive protein serum levels [7][9][10][11][12]. Although, anti-CCP seems to be more specific, but less sensitive than RF in RA diagnosis. Overall, ~30% of patients with RA are negative for anti-CCP [9][10][11]. RF, on the other hand, seems to have lower specificity but higher sensitivity compared to anti-CCP test. Overall, ~30–40% of patients with RA are negative for rheumatoid factor [9][10][11]. The limitation in early and accurate diagnosis of RA affects many patients who are left with continued pain and debilitating suffering. It is imperative that new and improved methods of testing for RA (i.e., genetic testing or identification of potential environmental antigens) is discovered to not only better diagnose RA, but to also find better treatments for the disease as well.

Treatment of inflammatory diseases such as RA and CD includes non-steroid anti-inflammatory drugs (NSAIDs), glucocorticoids, and disease-modifying anti-rheumatic drugs (DMARDs) [3][8][13]. NSAIDs and glucocorticoids are used for RA patients to help reduce overall pain and stiffness [3][8][13]. However, these medications have a wide-variety of long-term side effects such as ulceration, osteoporosis, hypertension, weight gain, etc., thus NSAIDs and glucocorticoids need to be paired with other medications to reduce the side effects [3][8][14]. DMARDs includes synthetic products such as methotrexate, sulfasalazine and

hydroxychloroquine and includes biologics such as adalimumab/infliximab (anti-TNF-α), tocilizumab (anti-IL-6 receptor), abatacept (T-cell co-stimulator), and rituximab (B-cell deactivator) [3][8]. Using multi-therapy or mono-therapy of different DMARDs is controversial and is continued to be argued among clinicians due to conflicting side effects of each medication. Problems with both synthetic and biological DMARDs continue to be the high risk of developing sides effects including GI intolerance, hypersensitivity to the medication, production of antibodies against the medication, and increasing the risk of developing opportunistic infections such as *Mycobacterium tuberculosis* infection [3][15][16]. DMARDs and synthetic DMARDs are re-classified as DMAIDs when used in inflammatory bowel treatment such as CD [17]. Infliximab is most commonly prescribed medication for both RA and CD [17][18]. RA and CD patients share the same treatments, thus it is possible that both RA and CD pathogenesis share common factors involved in disease pathogenesis [17][18][19][20][21]

Environmental triggers involved in RA include cigarette smoking, air pollutants, and bacteria including *Porphyromonas ginivalis* (*P. gingivalis*) and *Proteus mirablis* (*P. mirablis*) [3][22][23]. Molecular mimicry between a haemolysin protein sequence (ESRRAL) produced by *P. mirablis* and a RA susceptibility sequence (EQRRAA) was reported, thus showing possible connections to genetic pre-disposition and an environmental trigger synergistic threat [24]. Most recently, MAP has been associated with other autoimmune diseases including CD, T1D, and possibly in RA [6][25][26][27]. The association of MAP with these inflammatory diseases was based on shared genetic predisposition and molecular mimicry with environmental antigens [6][25][26][27]. MAP infection in a genetically predisposition patient should trigger, exacerbate and possibly dysregulate the immune system by stimulating the production of pro-inflammatory

cytokines and, through molecular mimicry, production of autoantibodies [6][25][26][27]. This is the first study designed to explore the effect of an environmental trigger, such as MAP, and SNPs in *PTPN2/22* on gene expression and the consequent effect on T-cells reactivity and inflammation. We hypothesize that SNPs in *PTPN2/22* and, along with MAP infection, causes hyper-proliferative T-cells and overexpression of *IFN-γ*, leading to possible inflammation in RA patients.

Materials and Methods

Clinical Samples

Three 4.0-mL K_2-EDTA coded blood tubes were obtained from 132 consented RA and healthy control subjects that were acquired from the University of Central Florida Health Center. The study was approved by the University of Central Florida Institutional Review Board #IRB00001138. Each subject completed and signed a written consent form before samples were collected. The average age of healthy controls was 30.7 ± 13.4 with a gender ratio of 41.9% male and 58.1% female subjects. The average age of RA patients was 49.9 ± 13.7 with a gender ratio of 11.4% male and 88.6% female subjects. Many factors including the higher prevalence of RA in older females than in males (3:1 ratio) found in other studies, the preference of a female rheumatologists by female RA patients, and the selection of rheumatologists around the area has been noted and considered in this study [28][29]. Table 1 lists age, gender and other demographic information and current medications for all RA subjects participating in this study. One tube of blood sample was processed for detection of MAP *IS900* DNA. Another tube of blood sample was processed for *PTPN2/22* genotyping and gene expression experiments, whereas the third tube of blood sample was utilized in T-cell proliferation study.

Table 1. Demographics, Treatment History, and Results of MAP and Allele Frequency of *rs478582/rs2476601* in RA Samples Used in Study

SAMPLE CODE	DIAGNOSIS	GENDER	AGE	MEDICATIONS CURRENTLY TAKEN	MAP STATUS	*PTPN2: rs478582**	*PTPN22: rs2476601***
MAP-1000	RA	F	60	Hydroxychloroquine	-	CC	GG
MAP-1001	RA	M	75	Methotrexate, Prednisone	-	TT	GG
MAP-1003	RA	F	68	Humira®, Methotrexate, Prednisone	-	TC	GG
MAP-1004	RA	F	37	Methotrexate	-	TC	GG
MAP-1002	RA	F	62	Methotrexate	+	TC	GA
MAP-1005	RA	M	30	Methotrexate, Prednisone	-	TC	GG
MAP-1006	RA	F	55	Methotrexate	-	CC	GG
MAP-1007	RA	F	59	Methotrexate, Hydroxychloroquine	-	TC	GG
MAP-1008	RA	F	68	Methotrexate	-	TC	GG
MAP-1009	RA	F	33	Methotrexate, Prednisone	-	TC	GG
MAP-1010	RA	F	62	Methotrexate, Prednisone, Humira®, Sulfasalazine	-	CC	GG
MAP-1011	RA	F	45	Humira®	+	TC	GA
MAP-1012	RA	F	76	Hydroxychloroquine	+	CC	GG
MAP-1013	RA	F	52	Enbrel®, Methotrexate	+	CC	GG
MAP-1014	RA	F	43	Methotrexate	+	TC	GG
MAP-1015	RA	F	47	Enbrel®, Methotrexate	+	TC	GG
MAP-1016	RA	M	48	Methotrexate, Prednisone	+	TC	GG
MAP-1017	RA	F	22	Methotrexate, Simponi®	-	CC	GA

SAMPLE CODE	DIAGNOSIS	GENDER	AGE	MEDICATIONS CURRENTLY TAKEN	MAP STATUS	*PTPN2: rs478582**	*PTPN22: rs2476601***
MAP-1019	RA	F	52	Enbrel®	-	TC	GG
MAP-1020	RA	F	60	Orencia®, Methotrexate	-	CC	GA
MAP-1021	RA	F	57	Methotrexate, Simponi®	-	TC	GG
MAP-1023	RA	F	51	Methotrexate, Prednisone	-	CC	GG
MAP-1024	RA	F	62	Methotrexate, Humira®	-	CC	GA
MAP-1022	RA	F	62	Hydroxychloroquine, Methotrexate	-	CC	GG
MAP-1025	RA	F	49	None	-	CC	GG
MAP-1026	RA	F	64	None	-	TC	GG
MAP-1027	RA+IBD	F	56	Prednisone, Xeljanz®	+	CC	GG
MAP-1028	RA+IBD	F	61	Methotrexate, Humira®	-	CC	GA
MAP-1029	RA	F	25	Orencia®, Prednisone	-	CC	GG
MAP-1300	RA	F	39	Orencia®, Methotrexate	-	TC	GA
MAP-1031	RA	F	58	Enbrel®, Leflunomide	+	TT	GG
MAP-1032	RA	F	30	Humira®	-	TC	GG
MAP-1033	RA	F	56	Hydroxychloroquine	+	TC	GG
MAP-1034	RA	F	43	Humira®, Hydroxychloroquine	-	TC	GG
MAP-1035	RA	F	28	Humira®, Methotrexate, Hydroxychloroquine	-	TT	GG
MAP-1036	RA	F	49	Methotrexate, Hydroxychloroquine	+	CC	GA
MAP-1037	RA	F	53	Enbrel®	-	TT	GA
MAP-1039	RA	F	56	Hydroxychloroquine	-	CC	GA
MAP-1040	RA	F	56	Enbrel®, Methotrexate	-	TT	GG
MAP-1041	RA	F	30	Humira®, Prednisone, Leflunomide	-	TC	GG
MAP-1042	RA	F	44	Methotrexate	+	CC	GG

SAMPLE CODE	DIAGNOSIS	GENDER	AGE	MEDICATIONS CURRENTLY TAKEN	MAP STATUS	*PTPN2: rs478582**	*PTPN22: rs2476601***
MAP-1043	RA+UC+T1D	F	28	Sulfasalazine, Budesonide	+	TT	GA
MAP-1044	RA	F	39	Hydroxychloroquine	+	TC	GA
MAP-1046	RA	F	54	Hydroxychloroquine	-	CC	GA
MAP-1047	RA	F	65	None	-	TC	GG
MAP-1048	RA	M	65	Methotrexate	+	TT	GA
MAP-1049	RA	F	59	Stelara®	-	TC	GA
MAP-1050	RA	F	73	Humira®	+	TC	GG
MAP-1051	RA	F	34	Prednisone	-	TC	GG
MAP-1052	RA	F	20	Hydroxychloroquine	-	CC	GG
MAP-1053	RA	F	63	Cimzia®, Methotrexate, Predenisone	-	CC	GA
MAP-1054	RA	F	36	Methotrexate	+	TC	GA
MAP-1057	RA	F	51	Methotrexate, Predenisone	-	TC	AA
MAP-1055	RA	F	63	Methotrexate, Hydroxychloroquine, Predenisone	+	TT	GG
MAP-1056	RA	F	47	None	+	TT	GG
MAP-1058	RA	M	42	Methotrexate, Humira®	-	TT	GG
MAP-1059	RA	F	51	Humira®	-	TT	GG
MAP-1060	RA	M	47	Prednisone	+	TC	GG
MAP-1061	RA	F	52	Hydroxychloroquine	+	TC	GG
MAP-1062	RA+T1D	F	50	None	-	TT	GG
MAP-1063	RA+SLE	F	29	Orenseia®, Methotrexate, Predenisone	-	TC	GG
MAP-1064	RA+UC	F	40	None	-	CC	GG
MAP-1065	RA	F	42	Methotrexate, Humira®	-	TT	GA
MAP-1066	RA	F	65	None	-	TC	GG

SAMPLE CODE	DIAGNOSIS	GENDER	AGE	MEDICATIONS CURRENTLY TAKEN	MAP STATUS	*PTPN2: rs478582**	*PTPN22: rs2476601***
MAP-1068	RA+CD	F	28	Humira®	-	CC	GG
MAP-1069	RA	M	56	Enbrel®, Methotrexate	-	TC	GG
MAP-1070	RA	F	48	Enbrel®	-	TT	GA
MAP-1067	RA	M	70	Methotrexate, Cimzia®	+	TT	GG
MAP-1071	RA	F	32	Methotrexate	+	TC	GG
MAP-1072	RA	F	58	None	+	TC	GG

RA: Rheumatoid Arthritis
IBD: Inflammatory Bowel Disease
UC: Ulcerative Colitis
T1D: Type 1 Diabetes
SLE: Systemic Lupus Erythematosus
CD: Crohn's Disease
*: TT = Homozygous Major Allele/No SNP
TC = Heterozygous Allele
CC = Homozygous Minor Allele
**: GG = Homozygous Major Allele/ No SNP
GA = Heterozygous Allele
AA = Homozygous Minor Allele

Detection of MAP IS900 DNA in Peripheral Leukocytes

Blood sample tubes designated for MAP *IS900* detection were centrifuged at 3,000 RPMs for 10 min at room temperature. A 1.0 mL sample of plasma was transferred to sterile 1.5 mL microcentrifuge tube and was stored at −20°C for further analysis. Buffy coat layer containing peripheral leukocytes were also transferred into new sterile 1.5 mL microcentrifuge tube containing double volume of red cell lysis buffer (ammonium chloride solution, G-Biosciences®). Tubes were then incubated by rocking on a gentle shaker for 10 min, which then were centrifuged at 5,000 RPMs for 5 min at room temperature. The supernatant was removed and purified buffy coat pellets were stored in Tris-EDTA (TE) buffer and subjected to genomic DNA extraction using a modified DNAzol® extraction protocol as follows. Fresh or thawed buffy coat pellets suspended in 1.0 mL DNAzol® reagent was mixed with 400 μL of 100% isopropanol. Tubes were then incubated for 15 min at room temperature followed by centrifugation at 8,000 RPMs for 6 min. The supernatant was discarded and DNA pellets were washed once with 500 μL DNAzol® reagent and centrifuged at 8,000 RPMs for 5 min. Genomic DNA pellets were washed again with 1.0 mL of 75% ethanol and centrifuged at 8,000 RPMs for 5 min. DNA pellets were then dried in a speedvac for 5 min. Dried DNA pellets were dissolved in 20 μL molecular biological grade sterile H_2O and stored at −20°C for analysis by nest polymerase chain reaction (nPCR). Detection of MAP *IS900* DNA was done following our nPCR protocol and nucleotide primers as described previously [30]. The presence of a 298 bp band on a 2% agarose gel was indicative of presence of MAP in patient sample. Positive MAP DNA control originated from our UCF4, a culture of clinical strain isolated from CD patient.

Negative control tube for each PCR step contained all PCR ingredients except DNA template was used.

PTPN2/22 Genotyping

Genotyping of *PTPN2/22* for 9 SNPs were performed on DNA from peripheral blood. Genotyping was done at the University of Florida Pharmacotherapy and Translational Research Department (Gainesville, FL) using the TaqMan™ SNP Genotyping Assays (Applied Biosystems™). We investigated 4 SNPs specific to *PTPN2* including *rs1893217, rs2542151, rs7234029,* and *rs478582* along with 5 SNPs specific for *PTPN22* including *rs2476601, rs2488457, rs33996649, rs34209542,* and *rs2476599*. Table 2 summarizes SNPs allele mutations and amino acid mutations used in this study. Briefly, 1 mL blood was stored at −20°C until all samples were collected. DNA extractions were performed on whole blood samples using QIAamp® DNA Blood Mini Kit (Qiagen™) following the manufacturer's protocol. Similarly, TaqMan™ genotyping assays for *PTPN2/22* were performed on DNA samples following manufacturer protocol (Applied Biosystems™). Briefly, reaction mixtures consisted of 2x TaqMan™ Master Mix and 20x Assay Working Stock (primers with VIC and FAM fluorophore attachment) were transferred into a 384-well microtiter plate. DNA samples and controls were then added to the plate which then was subjected to RT-PCR using Applied Biosystems™ QuantStudio™ RT-PCR System. The protocol consisted of 95°C for 10 min for 1 cycle, 92°C for 15 s and 58°C for 1 min for 50 cycles. The plate was read for VIC and FAM fluorophores for each sample at 551 and 517 nm, respectively. Fluorescence of VIC or FAM alone determined that the sample had allele 1 or allele 2, while VIC and FAM together determined that the sample is heterozygous for each allele.

Table 2. List of SNPs in *PTPN2/22* Examined in this Study

GENE	RS#	MUTATION	LOCATION	MUTATION PHENOTYPE
PTPN2	rs2542151	T>G	5.5 kb Upstream (Espino-Paisan et al., 2011)	High Susceptibility to CD, UC, T1D, T2D, RA, and Juvenile Idiopathic Arthritis
	rs1893217	T>C	Intron 7 (Espino-Paisan et al., 2011)	High Susceptibility to CD, T1D, MS, RA, and Celiac Disease
	rs7234029	A>G	Intronic Section (Zhang et al., 2014)	High Susceptibility to CD, UC, RA, and Juvenile Idiopathic Arthritis
	rs478582	T>C	Intron 3 (Espino-Paisan et al., 2011)	High Susceptibility to T1D, MS, RA, and Celiac Disease
PTPN22	rs2476601	G>A	R620W (Qu et al., 2005)	High Susceptibility to CD, T1D, MS, RA, SLE, and Celiac Disease
	rs2488457	C>G	Promoter Region (Fan et al., 2015)	High Susceptibility to UC, T1D, RA, SLE, and Juvenile Idiopathic Arthritis
	rs33996649	C>T	R263Q (Rodriguez et al., 2011)	High Susceptibility to CD, UC, and RA
	rs34209542	A>G	Intronic Section (Skinningsrud et al., 2008)	High Susceptibility to T1D, RA, and Juvenile Idiopathic Arthritis
	rs2476599	G>A	Intron 19 (Taniyama et al., 2010)	High Susceptibility to RA

PTPN2/22 and IFN-γ Gene Expression

A total of 1 mL of fresh whole blood was transferred into 2.0 mL RNA-ase free microcentrifuge tube and was immediately processed for RNA extraction. RNA was isolated from peripheral

leukocytes and then used to synthesis cDNA for determining gene expression of *PTPN2/22* and *IFN-γ* via RT-PCR. RNA extraction was performed following the TRIzol® Reagent (Invitrogen) manufacturer's instruction. Briefly,1.0 mL of whole blood was transferred into 2.0 mL RNase free microcentrifuge tubes and centrifuged at 3,000 RPMs for 15 min. Plasma was discarded and buffy coat layer containing peripheral leukocytes were transferred to new RNA-ase free microcentrifuge tubes with double volume of red cell lysis buffer (ammonium chloride solution, G-Biosciences®). Tubes were incubated by rocking on gentle shaker for 10 min which then was centrifuged at 5,000 RPMs for 5 min at room temperature. Supernatant was then removed and peripheral leukocyte pellets were suspended in 1.0 mL of TRIzol®. Tubes were then incubated by rocking on a gentle shaker for 15 min. A volume of 0.2 mL of chloroform was then added to each tube. The mixture was then incubated at room temperature for 3 min. Tubes were then centrifuged at 11,400 RPMs for 15 min at 4°C. The colorless, upper aqueous phase containing RNA was transferred into new 2.0 mL RNA-ase free microcentrifuge tubes. A volume of 0.5 mL of 100% isopropanol was added followed by incubation at room temperature for 10 min. Tubes were then centrifuged at 11,400 RPMs for 10 min at 4°C. RNA pellets were washed in 1 mL of 75% ethanol and then centrifuged at 8,700 RPMs for 5 min at 4°C. RNA pellets were air-dried for 15–30 min and then suspended in 20 μL of RNase free H2O and heated at 60°C for 10 min.

cDNA synthesis was performed following the iScript™ Reverse Transcription (Bio-Rad®) manufacturer's instruction. Briefly, 600 ng of RNA from each sample was added to PCR reaction tubes containing 0.2 mL PCR reaction, 4 μL of iScript™ Reverse Transcription (Bio-Rad®), and up to 20 uL RNase free H_2O. Tubes were then placed in a thermal cycler (MyGene™ Series

Pelteir Thermal Cycler) and ran for 5 min at 25°C, 20 min at 46°C, and 1 min at 95°C. Final concentration of cDNA for each sample was 30 ng/μL.

RT-PCR reactions in a 96-well microamp plate consisted of 1 μL of cDNA (30 ng), 10 μL of Fast SYBR Green Mastermix (Thermofisher Scientific®), 1 μL of either *PTPN2, PTPN22,* or *IFN-γ* PrimePCR SYBER Green Assay mix (Bio-Rad®). and 8 μL of molecular biological grade sterile H_2O. Oligonucleotide primers for *18s* RNA gene (forward primer: 5′-GTA ACC CGT TGA ACC CCA TT-3′; reverse primer: 5′-CCA TCC AAT CGG TAG TAG CG-3′) were used as a control and to obtain baseline CT readings. RT-PCR reaction was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems®). Relative mRNA expression levels were calculated using ΔCT (Sample RT-PCR CT reading–18s CT baseline) and using the equation ($2^{-\Delta CT} \times 1{,}000$).

Isolation of Peripheral Lymphocytes and Proliferation Assay

Isolation of peripheral lymphocytes was performed using Lymphoprep™ reagent (Axis-Shield®) as described previously [31]. A stock of 2X freezing media containing 10.0 mL of 25% human serum albumin (Gemini®), 10.0 mL of sterile RPMI-1640 (Sigma-Aldrich®), and 5.0 mL DMSO was made for the use of preserving lymphocytes for storage at −80°C. Isolated lymphocytes were transferred into 1.0 mL cryogenic vials (Nalgene®) with double the amount of 2x freezing media added to samples and stored at −80°C for future use. Lymphocytes were thawed and washed with cRPMI, which contained 10% sterile heat-inactivated FBS (Sigma-Aldrich®) and 1% sterile antibiotic/antimycotic solution (Sigma-Aldrich®) added to RPMI-1640 before T-cell isolation. T-cell isolation from lymphocyte samples were done using EasySep™

Human T-cell Isolation Kit (Stemcell™) following manufacturer's instruction. Briefly, isolated lymphocytes were transferred into a 2.0 mL round-bottom microcentrifuge tubes. The Isolation Cocktail mixture was added at 50 µL/mL to sample tubes and was incubated at room temperature for 5 min. The RapidSpheres™ mixture was added to the tubes at 40 µL/mL and were placed in the EasySep™ magnet for 3 min. Isolated T-cells were poured from the tubes in the magnet to new 2.0 mL microcentrifuge tubes. T-cells were then counted using trypan blue solution (0.4%, Sigma®) cell viability assay.

T-cell proliferation assay was done using bromodeoxyuridine (BrdU) labeling proliferation ELISA kit (Roche Molecular Biochemicals®) as described previously [31]. Phytohematoagglutunin (PHA) was used to evaluate T-cell response. Purified Protein Derivative-like (PPD-like) from MAP was prepared by purification of supernatant from sonicates of protein extract obtained from clinical strain UCF4 culture pellet. It was used to determine T-cell response and prior exposure to MAP antigens. Briefly, 1×10^5 of isolated T-cells were transferred in triplicates onto a 96-well culture plate and were incubated in either RPMI only, PHA (10 µg/mL, Sigma-Aldrich®) or PPD-like (5 µg/mL) along with respected patients' plasma for 72 h at 37°C and 5% CO_2. T-cells were then labeled with BrdU and incubated for 24 h at 37°C and 5% CO_2. Cell proliferation was measured through Roche BrdU proliferation ELISA kit as described previously [31]. Relative T-cell proliferation levels of samples were compared to blanks (RPMI only) and controls (isolated T-cells in RPMI only) by examining fold change in absorbance reading of each well at 450 nm.

Statistical Analysis

Samples were analyzed for significance using unpaired, two-tailed *t*-tests; unpaired, two-tailed *z*-score; and odds ratio. GraphPad Prism 7 was used for statistical analysis and creation of graphs. *P*-values ≤ 0.05 were considered significant. Relative mRNA gene expression was determined by the use of ΔCT of the gene of interest found in each sample and the equation $2^{-\Delta CT} \times 1{,}000$ [32].

Results

Mycobacterium avium subspecies paratuberculosis IS900 DNA Detected in RA Frequency of SNP Alleles in PTPN2/22 in RA

Purified DNA from peripheral leukocytes of 118 subjects (70 RA and 48 healthy controls) was analyzed by nPCR using oligonucleotide primers specific to MAP *IS900.* MAP DNA was detected in blood samples from RA subject as illustrated in Figure 3A. The 298 bp PCR product purified from representative gels was sequenced and BLAST analysis confirmed the identity of MAP, which has previously been used to confirm if patient samples are considered having the MAP infection [25][[26][30]. As shown in Figure 3B, out of 70 blood samples from RA subjects, 24 (34.3%) were positive for *MAPbacteremia* compared to only 4 out 48 (8.3%) healthy controls (*p*-value ≤ 0.05). The odds ratio (OR) value was determined to be 5.74 (95% CI: 1.84–17.9; *p*-value ≤ 0.05), where the presence of MAP DNA is most likely to occur in RA patients. MAP bacteria has been successfully re-cultured from at least one RA buffy coat sample (MAP-1015, see Table 1) via BD Bactec™ MGIT™ Para-TB medium (Becton, Dickinson and Company). The cultured sample was confirmed to be MAP positive by way of nPCR as previously mentioned. Culturing of MAP bacteria from other RA patient samples is still ongoing.

Frequency of SNP Alleles in PTPN2/22 in RA

TaqMan™ genotyping was done on purified DNA from 132 subjects (70 RA and 62 healthy controls). DNA from each subject was analyzed for 4 SNPs specific to *PTPN2* (*rs1893217, rs2542151, rs7234029, rs478582*) and 5 SNPs specific to *PTPN22* (*rs2476601, rs2488457, rs33996649, rs34209542, rs2476599*). Data referred to as homozygous major allele is considered normal/no SNP, while heterozygous allele and homozygous minor allele were considered abnormal and designated as SNP positive. As shown in Figure 4, Out of 4 SNPs specific to *PTPN2, rs478582* was significant in RA since heterozygous (TC) or minor (CC) alleles were detected in 55/70 (78.6%) RA samples compared to 36/60 (60.0%) healthy controls (p-value ≤ 0.05, Figure 4A). Specifically, 22/70 (31.4%) minor (CC) alleles were detected in RA compared to 9/60 (15.0%) healthy controls (p-value ≤ 0.05), whereas heterozygous (TC) alleles were detected in 33/70 (47.1%) RA compared to 28/60 (46.7%) healthy controls. Out of 5 SNPs specific to *PTPN22, rs2476601* was significant in RA since heterozygous (GA) or minor (AA) alleles were detected in 20/70 (28.6%) RA samples compared to only 4/62 (6.45%) healthy controls (p-values ≤ 0.05, Figure 4B). Specifically, heterozygous alleles (GA) were detected in 19/70 (27.1%) RA compared to 4/62 (6.45%) healthy controls (p-value ≤ 0.05). There was rare minor (AA) alleles detected in all samples. The OR value for the significance of *PTPN2:rs478582* was 2.28 (95% CI: 1.05–4.93; p-value ≤ 0.05) whereas OR value for *PTPN22:rs2476601* was 5.90 (95% CI: 1.89–18.4; p-value ≤ 0.05).

For determination of haplotype combinations, we examined the significant SNPs *PTPN2:rs478582* and *PTPN22:rs2476601* allele combinations to confirm the allele distribution among samples (Figure 4C). Examination of the following haplotype combinations were

determined in the samples, where *PTPN2:rs478582* and *PTPN22:rs2476601* allele types were combined respectively: T-G, C-G, T-A, and C-A. T-G haplotype (major/major) was found more significantly in healthy controls (21/59 = 35.6%) than in RA samples (10/70 = 14.3%, *p*-value ≤ 0.05). C-G haplotype (heterozygous or minor/major) was found in 40/70 (57.1%) RA samples compared to healthy 34/59 (57.6%). T-A haplotype (major/heterozygous or minor) was found more in RA samples (5/70 = 7.14%), compared to healthy controls (2/59 = 3.39%), while C-A (heterozygous or minor/heterozygous or minor) was found significantly more in RA samples (15/70 = 21.4%) than in healthy controls (2/59 = 2.39%, *p*-value ≤ 0.05). When examining the haplotypes in more detail, CC-GA haplotype was found more significantly (*p*-value ≤ 0.05) in RA patients (8/70 = 11.4%) than in healthy controls (1/59 = 1.69%).

Effect of PTPN2:rs478582 and PTPN22:rs2476601 on PTPN2/22 Expression

Gene expression of *PTPN2/22* in 37 RA and 31 healthy controls were reported. The overall relative mRNA expression of *PTPN2* was lower in RA compared to healthy controls (8.22 ± 5.33 and 10.3 ± 6.95, respectively). Similarly, relative mRNA expression of *PTPN22* was also lower in RA compared to healthy controls (2.55 ± 1.74, and 3.24 ± 1.84, respectively). Examination of relative mRNA expression of *PTPN2/22* in relationship with samples with either *PTPN2:rs478582* or *PTPN22:rs2476601* was examined as seen in Table 3.

Table 3. Effect of *PTPN2:rs478582* and *PTPN22:rs2476601* on *PTPN2/22* Expression

DIAGNOSIS	*PTPN2* EXPRESSION OF SAMPLES WITH *PTPN2:RS478582* ($2^{(-\Delta CT)}$ X 1000)				*PTPN22* EXPRESSION OF SAMPLES WITH *PTPN22:RS2476601* ($2^{(-\Delta CT)}$ X 1000)			
	TT	**TC**	**CC**	**TC + CC**	**GG**	**GA**	**AA**	**GA + AA**
RA	7.38 ± 4.91 (N=13)	7.42 ± 4.01 (N=15)	10.7 ± 7.33 (N=9)	8.67 ± 5.59 (N=24)	2.41 ± 1.98 (N=24)	2.77 ± 1.28 (N=12)	3.16 (N=1)	2.79 ± 1.23 (N=13)
HEALTHY	9.49 ± 5.13 (N=8)	10.3 ± 7.01 (N=18)	11.9 ± 9.73 (N=5)	10.6 ± 7.47 (N=23)	3.24 ± 1.91 (N=27)	3.40 ± 1.19 (N=4)	NA	3.40 ± 1.19 (N=4)

The effect of *PTPN2:rs478582* on gene expression was evaluated. The average relative mRNA expression in RA with heterozygous (TC) or minor (CC) allele was 8.67 ± 5.59 (N = 24) compared to 10.6 ± 7.47 (N = 23) in healthy controls with similar SNPs and lower than healthy controls without SNPs (TT) (9.49 ± 5.13; N = 8). Specifically, the average relative mRNA expression in RA with heterozygous (TC) allele was 7.42 ± 4.01 (N = 15) which is much lower than healthy control with the heterozygous (TC) allele (10.3 ± 7.01; N = 18) and normal (TT) healthy controls (9.49 ± 5.13; N = 8). The effect of minor (CC) allele on *PTPN2* expression in RA was 10.7 ± 7.33 (N = 9) and lower than healthy controls with minor (CC) allele (11.9 ± 9.73; N = 5). The effect of *PTPN2:rs478582* on mRNA expression in each subject group was not significant. Among healthy controls, the average relative mRNA expression in samples with heterozygous (TC) or minor (CC) allele was 10.6 ± 7.47 (N = 23) compared to 9.49 ± 5.13 (N =

8) in normal (TT) samples. The average relative mRNA expression with only heterozygous (TC) allele was 10.3 ± 7.01(N = 18), whereas samples with minor (CC) allele had 11.9 ± 9.73 (N = 5) compared to normal (TT) healthy controls (9.49 ± 5.13 N = 8). Among RA samples, the average relative mRNA expression in samples with the heterozygous (TC) or minor (CC) allele was 8.67 ± 5.59 (N = 24) compared to 7.38 ± 4.91 (N = 13) in normal (TT) samples. The average relative mRNA expression samples with only heterozygous (TC) allele was 7.42 ± 4.0 (N = 15), whereas samples with the minor (CC) allele had 10.7 ± 7.33 (N = 9) compared to RA normal (TT) samples (7.38 ± 4.91; N = 13). The overall average relative mRNA expression in all samples with heterozygous (TC) or minor allele (CC) was 9.63 ± 6.58 (N = 47) compared to 8.19 ± 4.98 (N = 21) in samples without any SNP. Specifically, the average relative mRNA expression in all samples with only heterozygous (TC) allele was 8.99 ± 5.94 (N = 33) and with only the minor (CC) allele was 11.2 ± 7.91 (N = 14) compared to the samples without any SNP (8.19 ± 4.98; N = 21).

Correlation analyses were also performed to determine if *PTPN22:rs2476601* alters *PTPN22* expression. The average relative mRNA expression in RA with heterozygous (GA) or minor allele (AA) was 2.79 ± 1.23 (N = 13) compared to 3.40 ± 1.19 (N = 4) in healthy controls with similar SNP and normal (GG) healthy control (3.24 ± 1.91, N = 27). Specifically, the average relative mRNA expression in RA with only heterozygous (GA) allele was 2.77 ± 1.28 (N = 12) compared to 3.40 ± 1.19 (N = 4) in healthy controls with similar SNP and normal (GG) healthy controls (3.24 ± 1.91; N = 27). There was rare occurrence of minor (AA) allele in all samples. Among each group, there was not any significance. Among healthy controls, the average relative mRNA expression in samples with heterozygous (GA) or minor (AA) allele was 3.40 ± 1.19 (N

= 4) compared to 3.24 ± 1.91 (N = 27) normal (GG) samples. The average relative mRNA expression with only heterozygous (GA) allele was 3.40 ± 1.19 (N = 4), where there were no samples with minor (AA) allele. Among RA samples, the average relative mRNA expression in samples with the heterozygous (GA) or minor (AA) allele was 2.79 ± 1.23 (N = 13) compared to 2.41 ± 1.98 (N = 24) in normal (GG) samples. The average relative mRNA expression samples with heterozygous (GA) allele only was 2.77 ± 1.28 (N = 12) compared to 3.16 in minor (AA) allele. There was no significant difference in the overall average relative mRNA expression in all samples with heterozygous (GA) or minor allele (AA) and samples without any SNP. Specifically, the average relative mRNA expression in all samples with only heterozygous (GA) allele was 2.92 ± 1.25 (N = 16) and with only the minor (AA) allele was 3.16 (N = 1) compared to the samples without any SNP (2.85 ± 1.97, N = 51).

Effect of PTPN2:rs478582 and PTPN22:rs2476601 on T-cell Response

To evaluate the effect of *PTPN2:rs478582* and/or *PTPN22:rs2476601* on T-cell function, we treated purified T-cells from RA (N = 25) and healthy controls (N = 15) with PHA and MAP PPD-like and measured T-cell proliferative response (Figure 5). T-cells were isolated and purified from clinical samples, which tested positive for heterozygous and/or homozygous minor alleles for *PTPN2:rs478582* and/or *PTPN22:rs2476601*.

Effect of *PTPN2:rs478582* on T-cell Response

Unlike T-cells from RA subjects, there was an increase in T-cell proliferation response between healthy controls with heterozygous (TC) allele (2.1± 0.3-fold increase, N = 5) and those without SNP (TT) (1.7 ± 0.5-fold increase, N = 5) when induced with PHA (Figure 5A). On the contrary,

there was 2.0 ± 0.4-fold increase (N = 5) in T-cells response in RA samples with minor (CC) allele compared to a 1.8 ± 0.3-fold increase (N = 5) in RA normal (TT) T-cells. There was no difference in T-cell response in healthy controls with (N = 3) and without (N = 5) minor allele. The effect of heterozygous (TC) allele on T-cell proliferation response from healthy controls treated with MAP PPD-like (Figure 5D) resulted in a 1.9 ± 0.3-fold increase (N = 5) compared to only 1.4 ± 0.2-fold increase (N = 5) in normal (TT) T-cells from healthy control (p-value ≤ 0.05). T-cells from healthy controls with minor (CC) allele responded to MAP PPD-like with 1.6 ± 0.2-fold increase (N = 3) compared to 1.4 ± 0.2 (N = 5) in normal T-cells from healthy controls. RA samples with heterozygous (TC) allele had a significantly higher T-cell proliferation response fold increase to MAP PPD-like (1.9 ± 0.2, N = 5) compared to healthy controls with normal (TT) (1.4 ± 0.2, N = 5, p-value ≤ 0.05).

Effect of *PTPN22:rs2476601* on T-cell Response

The effect of heterozygous (GA) allele on T-cell proliferation response from healthy controls treated with PHA (Figure 5B) resulted in a 2.0-fold increase (N = 1) compared to only 1.7 ± 0.5-fold increase (N = 5) in normal (GG) T-cells from healthy controls. Similarly, T-cells from RA subjects with heterozygous (GA) allele responded with a 2.2 ± 0.1 (N = 5) fold increase compared to a 1.8 ± 0.3-fold increase (N = 5) in normal (GG) T-cells from RA subjects (p-value ≤ 0.05). There were no patient samples with just the minor (AA) allele to do T-cell proliferation. The effect of heterozygous (GA) allele on T-cell proliferation response from healthy controls treated with MAP PPD-like (Figure 5E) resulted in a 2.2 (N = 1) fold increase compared to only 1.4 ± 0.2 (N = 5) fold increase in normal (GG) T-cells from healthy controls. T-cells from RA samples with heterozygous (GA) allele responded lower (2.3 ± 0.2-fold increase, N = 5) to MAP

PPD-like than normal T-cells (3.4 ± 1.8-fold increase, N = 5). RA samples with normal (GG) T-cells had a significantly higher response to MAP PPD-like (3.4 ± 1.8, N = 5) compared to healthy controls with normal (GG) (1.4 ± 0.2, N = 5, *p*-value ≤ 0.05). RA samples with heterozygous (GA) allele had a significantly higher T-cell proliferation response to MAP PPD-like (2.3 ± 0.2, N = 5) compared to healthy controls with normal (GG) (1.4 ± 0.2, N = 5, *p*-value ≤ 0.05).

Effect of Combined *PTPN2:rs478582* and *PTPN22:rs2476601* on T-cell Response

Response of T-cells from RA samples with both SNPs treated with PHA was 2.7 ± 2.2-fold increase (N = 5) compared to a 1.8 ± 0.3-fold increase (N = 5) in T-cells from RA samples without SNP (Figure 5C). There was no difference in T-cells response against PHA in samples from healthy controls with (N = 1) and without combined SNPs (N = 5). T-cells from RA samples with both SNPs responded to MAP PPD-like with a 7.4 ± 6.7-fold increase (N = 5) compared to a 3.4 ± 1.8-fold increase (N = 5) in normal RA samples (Figure 5F). Similarly, T-cells from healthy controls with combined SNPs resulted in 1.7-fold increase (N = 1) when treated with MAP PPD-like compared to only 1.4 ± 0.2-fold increase (N = 5) in T-cells from healthy control without SNPs.

Effect of PTPN2:rs478582 and PTPN22:rs2476601 on IFN-γ Expression

The effect of *PTPN2:rs478582* and *PTPN22:rs2476601* on *IFN-γ* expression was determined on 35 RA and 24 healthy controls (Figure 6). The average relative mRNA expression of *IFN-γ* in all samples with *PTPN2:rs478582* heterozygous (TC) or minor (CC) allele, regardless of disease, was 0.39 ± 0.31 (N = 38) compared to 0.28 ± 0.16 (N = 21) in normal (TT) samples.

Specifically, the average relative mRNA expression of *IFN-γ* in all samples with *PTPN2:rs478582* minor (CC) allele was 0.48 ± 0.39 (N = 12). In RA samples, the average relative mRNA expression of *IFN-γ* in samples with *PTPN2:rs478582* heterozygous (TC) or minor (CC) allele was 0.33 ± 0.32 (N = 22), compared to 0.22 ± 0.16 in 13 normal (TT) RA samples (Figure 6A). Surprisingly, the effect of the *PTPN2:rs478582* minor (CC) allele on *IFN-γ* expression in RA samples was more significant (0.43 ± 0.41; N = 8). However, the average relative mRNA expression of *IFN-γ* in healthy control samples with and without *PTPN2:rs478582* heterozygous (TC) or minor (CC) allele was similar [0.47 ± 0.28 (N = 16), 0.39 ± 0.12 (N = 8), respectively (Figure 6A)]. As observed in RA samples, the effect of *PTPN2:rs478582* minor (CC) allele on *IFN-γ* expression in healthy controls was elevated (0.58 ± 0.39; N = 4). Correlation analyses were also performed to determine if *PTPN22:rs2476601* alters *IFN-γ* expression (Figure 6B). In healthy controls, the average relative mRNA expression of *IFN-γ* in samples with the heterozygous (GA) or minor allele (AA) for *PTPN22:rs2476601* was 0.67 ± 0.28 compared to 0.40 ± 0.21 in normal (GG) samples (p-value ≤ 0.05). There was no significant effect for *PTPN22:rs2476601* heterozygous (GA) or minor allele (AA) on *IFN-γ* expression in RA samples.

Effect of PTPN22:rs478582 and PTPN22:rs2476601 on Susceptibility to MAP Expression

Correlation analyses were performed to determine if *PTPN2:rs479592* in RA may affected susceptibility to MAP infection (Figure 7A). Out of 55 RA samples with either heterozygous (TC) or minor (CC) allele for *PTPN2:rs478582*, 18/55 (32.7%) were positive for *MAPbacteremia* compared to only 2/31 (6.5%; p-value ≤ 0.05) in healthy controls. The OR value was 7.05 (95% CI: 1.51–32.9). Specifically, MAP presence in RA samples with only

heterozygous (TC) allele was 13/33 (39.3%) compared to none in 23 healthy controls samples (p-value ≤ 0.05).

Similarly, correlation analyses were performed to determine if *PTPN22:rs2476601* in RA may affected susceptibility to MAP infection (Figure 7B). Out of 20 RA samples with either the heterozygous or minor allele for *PTPN22:rs2476601,* 7/20 (35.0%) had *MAPbacteremia* compared to none in healthy controls. OR value of significance was 5.00 (95% CI: 0.23–106.1). Specifically, MAP presence in RA samples with heterozygous allele was 7/19 (36.8%) compared to none in healthy controls. MAP was absent in all samples with minor allele.

We also investigated the *PTPN2/22* expression in MAP positive samples. Overall, samples with *MAPbacteremia* had an average relative *PTPN2* mRNA expression of 10.0 ± 6.31 (N = 15) compared to 9.00 ± 6.16 (N = 52) in MAP-free samples, regardless of disease. In RA samples with MAP, the average relative mRNA expression of *PTPN2* was 9.53 ± 5.42 (N = 12) compared to 7.59 ± 5.28 (N = 25) in MAP-free samples. Only three healthy controls samples were positive for MAP and they had average relative mRNA expression of *PTPN2* 11.9 ± 10.57 compared to 10.3 ± 6.71 (N = 27) in MAP-free samples. There was no change in *PTPN22* expression in samples with or without MAP.

Effect of Combined PTPN2:rs478582, PTPN22:rs2476601, and MAP on PTPN2/22 Expression

The correlation of *PTPN2/22* expression in samples with either *PTPN2:rs478582* or *PTPN22:rs2476601* that had MAP presence was examined as seen in Tables 4, 5. The overall relative mRNA expression of *PTPN2* was lower in RA compared to healthy controls (8.22 ± 5.33

and 10.3 ± 6.95, respectively). The effect of *PTPN2:rs478582* on *PTPN2* gene expression in RA with heterozygous (TC) or minor (CC) allele was 8.67 ± 5.59 (N = 24) compared to 10.6 ± 7.47 (N = 23) in healthy controls with similar SNPs and lower than healthy controls without SNPs (TT) (9.49 ± 5.13; N = 8). In MAP positive RA samples with *PTPN2:rs478582*, the average relative mRNA expression of *PTPN2* was 9.49 ± 6.15 compared to 6.01 ± 4.70 (N = 8) in normal (TT) MAP-free samples. Only one healthy control sample was positive for MAP and had heterozygous (TC) allele had an average relative mRNA expression in *PTPN2* of 24.1 compared to 8.36 ± 4.42 (N = 4) in healthy controls without MAP presence and without the SNP.

Table 4. Effect of Combined *PTPN2:rs478582* and MAP Presence on *PTPN2* Expression

DIAGNOSIS	*PTPN2* EXPRESSION OF SAMPLES WITH *PTPN2:RS478582* AND MAP NEGATIVE ($2^{(-\Delta CT)}$ X 1000)				*PTPN2* EXPRESSION OF SAMPLES WITH *PTPN2:RS478582* AND MAP POSITIVE ($2^{(-\Delta CT)}$ X 1000)			
	TT	**TC**	**CC**	**TC + CC**	**TT**	**TC**	**CC**	**TC + CC**
RA	6.0 ± 4.7 (N=8)	7.78 ± 4.67 (N=10)	9.14 ± 6.84 (N=7)	8.33 ± 5.5 (N=17)	9.59 ± 4.89 (N=5)	6.73 ± 2.57 (N=5)	16.4 ± 8.17 (N=2)	9.49 ± 6.15 (N=7)
HEALTHY	10.7 ± 5.44 (N=6)	10.3 ± 7.0 (N=18)	11.9 ± 9.73 (N=5)	10.6 ± 7.47 (N=23)	5.86 ± 0.75 (N=2)	NA	24.1 (N=1)	24.1 (N=1)

Table 5. Effect of Combined *PTPN22:rs2476601* and MAP Presence on *PTPN22* Expression

DIAGNOSIS	*PTPN22* EXPRESSION OF SAMPLES WITH *PTPN22:RS2476601* AND MAP NEGATIVE ($2^{(-\Delta CT)}$ X 1000)				*PTPN22* EXPRESSION OF SAMPLES WITH *PTPN22:RS2476601* AND MAP POSITIVE ($2^{(-\Delta CT)}$ X 1000)			
	GG	**GA**	**AA**	**GA + AA**	**GG**	**GA**	**AA**	**GA + AA**
RA	2.27 ± 1.72 (N=16)	2.51 ± 1.57 (N=7)	3.16 (N=1)	2.59 ± 1.47 (N=8)	2.8 ± 2.49 (N=8)	3.12 ± 0.7 (N=5)	NA	3.12 ± 0.7 (N=5)
HEALTHY	3.22 ± 1.94 (N=26)	3.4 ± 1.19 (N=4)	NA	3.4 ± 1.19 (N=4)	2.83 ± 1.95 (N=3)	NA	NA	NA

Similarly, relative mRNA expression of *PTPN22* was also lower in RA compared to healthy controls (2.55 ± 1.74, and 3.24 ± 1.84, respectively). The average relative mRNA expression in RA with heterozygous (GA) or minor allele (AA) was 2.79 ± 1.23 (N = 13) compared to 3.40 ± 1.19 (N = 4) in healthy controls with similar SNP and normal (GG) healthy control (3.24 ± 1.91, N = 27). Overall, samples with MAP presence and the *PTPN22:rs2476601* heterozygous (GA) or minor (AA) allele had an average relative mRNA expression of 3.12 ± 0.70 (N = 5) compared to 2.85 ± 1.89 (N = 41) in normal MAP free samples. In MAP positive RA samples with *PTPN22:rs2476601*, the average relative mRNA expression of *PTPN22* was 3.12 ± 0.70 (N = 5) compared to normal MAP-free (2.24 ± 1.67; N = 17). None of healthy control samples has both MAP presence and *PTPN22:rs2476601*.

Only 3 RA samples had *PTPN2:rs478582*, *PTPN22:rs2476601* and were positive for MAP. *PTPN2/22* expression and T-cell response were not significantly different from early observation.

Effect of Combined PTPN2:rs478582, PTPN22:rs2476601, and MAP on IFN-γ Expression

Overall, there was no significant difference in *IFN-γ* mRNA expression in 59 samples with or without MAP presence. The average relative mRNA expression in *IFN-γ* was 0.35 ± 0.26 in samples with MAP present, while samples without MAP presence (N = 45) was 0.35 ± 0.28. Similar data was observed when gene expression was evaluated in RA and healthy control samples.

Correlation analyses were performed to determine if MAP presence with *PTPN2:rs478582* heterozygous (TC) or minor (CC) allele changes gene expression of *IFN-γ* (Figure 8). Overall, samples with MAP presence and *PTPN2:rs478582* (N = 7) had an average relative mRNA expression in *IFN-γ* of 0.43 ± 0.32, while samples without MAP presence and the SNP (N = 14) was 0.29 ± 0.17. In RA samples, the average relative mRNA expression in *IFN-γ* in samples with MAP presence and *PTPN2:rs478582* (N = 6) was 0.39 ± 0.33 compared to 0.21 ±0.18 (N = 8) in RA samples without MAP presence and without the SNP. In healthy controls, the average relative mRNA expression in *IFN-γ* in samples with MAP presence and *PTPN2:rs478582* (N = 1) was 0.67 compared to 0.39 ± 0.10 in healthy controls without MAP presence and without the SNP (N = 6).

Correlation analyses were also performed to determine if MAP presence with *PTPN22:rs2476601* heterozygous (GA) or minor (AA) allele changes gene expression of *IFN-γ.* Overall, samples with MAP presence and *PTPN22:rs2476601* (N = 5) had an average relative mRNA expression in *IFN-γ* of 0.15 ± 0.07, while samples without MAP presence and the SNP (N = 34) was 0.32 ± 0.22. In healthy controls, there were no samples with both MAP presence

and *PTPN22:rs2476601* together. In RA patients, the average relative mRNA expression in *IFN-γ* in samples with MAP presence and the *PTPN22:rs2476601* SNP (N = 5) was 0.15 ± 0.07 compared to 0.27 ± 0.24 in RA patients without MAP presence and without the SNP (N = 17).

Only 3 RA samples had *PTPN2:rs478582, PTPN22:rs2476601* and were positive for MAP. *IFN-γ* expression and T-cell response were not significantly different from early observation.

Effect of Medication to Susceptibility to MAPbacteremia

The effect of the medications taken by RA participants, as shown in Table 1, were evaluated for the susceptibility of MAP. Four different medication groups were studied for MAP susceptibility: hydroxychloroquine (TLR repressor), methotrexate (anti-folate), prednisone (steroid), and anti-TNF-α medications (Humira®, Enbrel®, Simponi®, and Cimzia®). Out of 14 RA patients on hydroxychloroquine, 6 (42.9%) were positive for *MAPbacteremia*, while 12 out of 37 (32.4%) of RA patients on methotrexate also were positive for *MAPbacteremia*. For RA patients on prednisone, 4 out of 16 (25.0%) of RA patients were positive for *MAPbacteremia*, while 6 out of 23 (26.1%) RA patients on anti-TNF-α medications (Humira®, Enbrel®, Simponi®, and Cimzia®) also were positive for *MAPbacteremia*.

Discussion

Extensive efforts are ongoing to investigate pathogenesis and effective treatment for inflammatory diseases. Current medications are expensive and the side effects are lengthy. For example, RA etiology remains unknown, but there are established protocols for diagnosis of and management of symptoms. However, the side effects of all RA medications are serious and

undesirable [3][8][16]. Therefore, it is imperative that the pathogenesis of RA is deciphered in order to develop protocols for accurate and early detection and treatment of the disease. RA patients suffer from elevation of pro-inflammatory cytokines such as IFN-γ and TNF-α and their impact on apoptosis and development of chronic inflammation [1][3][8]. Only environmental factors and genetic predisposition mutations have been linked to RA [2][3][22][23]. This study is focused on investigating the effect of SNPs on key negative regulators genes such as *PTPN2/22* expression and their impact on upregulation of pro-inflammatory cytokines, apoptosis and inflammation. We hypothesized that heterozygous and/or homozygous minor allele mutation(s) in health-keeping genes such as *PTPN2/22* in RA lead to elevated IFN-γ, TNF-α, apoptosis, and development of inflammation. To our knowledge, this is the first study designed to elucidate the molecular cause of inflammation in RA in association with environmental triggers such as MAP. The latter has been associated with similar inflammation in CD, T1D, multiple sclerosis, and others [6][25][26][27][31]. This study is first to report the detection of MAP DNA in more than of one third of RA patients (Figure 3B). The data is significant, intriguing, and should be a motive to expand future investigations to include a larger pool of samples. As well-advertised, the incidence of *M. tuberculosis* infection in RA, is among the most accepted side effect of the treatment [1][3][16]. Therefore, detection of MAP infection in RA patients should be investigated further as to whether it is a complication of the treatment or a possible culprit of the disease. Although MAP *IS900* DNA is good in detecting MAP presence in patient samples, it does not provide information about the MAP bacteria viability. This in turn does not show accurate status of either active or previous infection in the patient sample. Thus, culturing of the blood from RA patients is necessary to determine if an active MAP infection is present in the patients.

Genetic predisposing is key for susceptibility to disease, severity of inflammation and possible ability to respond to treatment. Due to the large number of published SNPs in *PTPN2/22*, we selected 9 SNPs in this study based on shared occurrence in other diseases with similar approved treatment protocol [1][2][3][6]. Specifically, we focused on SNPs in *PTPN2/22*, which increase susceptibility to RA and CD. The latter is well-investigated in our laboratory in association with MAP [6][30][31]. This study identified *PTPN2:rs478582* to be significant in RA (p-values $\leq$ 0.05, OR = 2.28) compared to healthy controls (Figure 4A). Similarly, *PTPN22:rs2476601* was significant in RA (p-values ≤ 0.05, OR = 5.90) compared to healthy controls (Figure 4B). The data specifically linked *PTPN2:rs478582* minor (CC) allele to be more significantly associated with RA (p-values ≤ 0.05). In short, our data suggest to clinicians that minor (CC) allele in *PTPN2* increases the risk of acquiring RA by a fold of 2.1. Moreover, *PTPN22:rs2476601* heterozygous (GA) allele in *PTPN22* was more significant in RA (p-values ≤ 0.05), indicating that patients with this SNP are at risk of acquiring RA by a fold of 4.3. Further examination of RA genotyping showed that patient samples with both *PTPN2:rs478582* and *PTPN22:rs2476601* alleles (regardless of heterozygous or minor alleles) showed to be more significant (p-values $\leq$ 0.05) compared to healthy controls, showing a 6.5-fold increased risk of developing RA. Some of the limitations of looking into SNPs in a diverse population, such as from this study, is that it is difficult to determine the alterations of allelic distribution between different population groups. Thus, it is important that further population studies that focus on examining *PTPN2/22* SNPs from other subpopulation groups, such as race, country of origin, and age/gender in RA patients should be done.

The effect of SNPs on *PTPN2/22* gene expression and function have been debated heavily in the literature [6][33][34][35]. This study demonstrated that RA samples with either *PTPN2:rs478582/PTPN22:rs2476601* heterozygous or minor alleles could potentially alter the *PTPN2/22* gene or the protein activity of *PTPN2/22* in T-cells, thus could possibly void the negative regulatory function of *PTPN2/22*. The effects of *PTPN2:rs478582/PTPN22:rs2476601* in *PTPN2/22* were also examined further to determine the effect on T-cell and production of IFN-γ.

Since *PTPN2/22* is found in all T-cell types, we decided not to segregate the T-cell subpopulations and instead look into total T-cell activity. However, further studies need to be done on the effects of SNPs in *PTPN2/22* in different subpopulations of T-cells. Stimulation of T-cells from RA samples associated with *PTPN2:rs478582* and induced with PHA led marked increase in T-cell proliferation. T-cells from RA patients associated *PTPN22:rs2476601* had even more significant increase in T-cell proliferation (p-values ≤ 0.05). Moreover, it was shockingly surprising to see T-cell reactivity response treatment with MAP PPD-like. Specifically, T-cells, isolated from the blood of RA patients associated with *PTPN2:rs478582/PTPN22:rs2476601* combined SNPs, proliferated by several folds more than those cells from health controls. Thus, T-cells from RA samples associated with SNPs in *PTPN2/22* seem to demonstrate three characteristics: first, they are hyperactive, second they seem to lack a negative feedback control, and third they reacted to MAP PPD-like significantly indicating prior exposure to MAP antigens. Hyperactive T-cells with lack of negative feedback control may explain the marked increase in pro-inflammatory cytokines such as TNF-α and IFN-γ in RA. The examination of T-cells in this study has been an exploratory study, thus it is

necessary to examine bigger populations of RA and health control subjects in the future. Along with this, further investigation on the outcome of the other immune cells, such as B-cells, NK cells, and macrophages, need to be examined in RA patients with SNPs in *PTPN2/22* to conclude how the hyper-proliferative T-cells react to other immune cells.

The hyperactivity to MAP PPD-like in the RA T-cells may be correlated to presence of MAP in RA samples and possibly activation of *M. tuberculosis* in some RA patients with biologic drugs treatments. The study provided more evidence that SNPs in *PTPN2/22* may have increased susceptibility to MAP infection as shown in Figure 7. Specifically, *PTPN2:rs478582* correlated with MAP infection in 32.7% (OR = 7.05) RA patients. Similarly, *PTPN22:rs2476601* correlated with MAP infection in 35.0% (OR = 5.00) RA patients (p-values ≤ 0.05). The data also demonstrated that presence of MAP did not alter *PTPN2/22* expression.

To elucidate whether medications may have any effect on the outcome of this study, we examined the effect of current medications taken by the RA participants on the risk of susceptibility to MAP infection. As shown in Table 1, out of all the DMARDs (hydroxychloroquine, methotrexate, prednisone, Humira®, Enbrel®, etc.) that the RA patients were on, hydroxychloroquine was found to increase MAP susceptibility the most in RA patients by a 1.3-fold change compared to RA patients not on hydroxychloroquine and had MAPbacteremia. These findings suggest that more investigation is needed by testing larger number of patients with RA. We also discovered that *IFN-γ* expression was lower in RA patients who are on DMARDs treatment compared to RA patients who are on different treatments (Table 1). Specifically, blood samples from RA patients treated with Humira® expressed lower *IFN-γ*

(0.15 ± 0.10, N = 11) compared to blood samples from other RA patients (0.29 ± 0.27; *p*-values ≤ 0.05) or even healthy controls (0.44 ± 0.24; *p*-values ≤ 0.05). Moreover, RA samples associated with *PTPN2:rs478582/PTPN22:rs2476601* heterozygous or minor alleles had higher IFN-γ expression than RA group without (Figure 6). These finding demonstrates that SNPs in *PTPN2/22* may led to elevated *IFN-γ* levels and inflammation in RA patients. However, since we focused only on *PTPN2/22* on the control of *IFN-γ* expression in this study, we did not examine the other cytokines that control *IFN-γ* production. Further investigation is needed to examine the effects of both pro-inflammatory cytokines, such as TNF-α, and anti-inflammatory cytokines, such as IL-6, in subjects with SNPs in *PTPN2/22*.

Overall, the data supports our hypothesis that SNPs in *PTPN2/22* leads to loss functions of these genes resulting in hyper-proliferative T-cells and increase susceptibility to *Mycobacteria* including MAP. Along with genetic testing for SNPs and proper treatment, personalized treatment for RA is plausible. More studies are encouraged to explore the incidence and impact of these SNPs on health keeping genes and susceptibility to infection in RA.

Figures

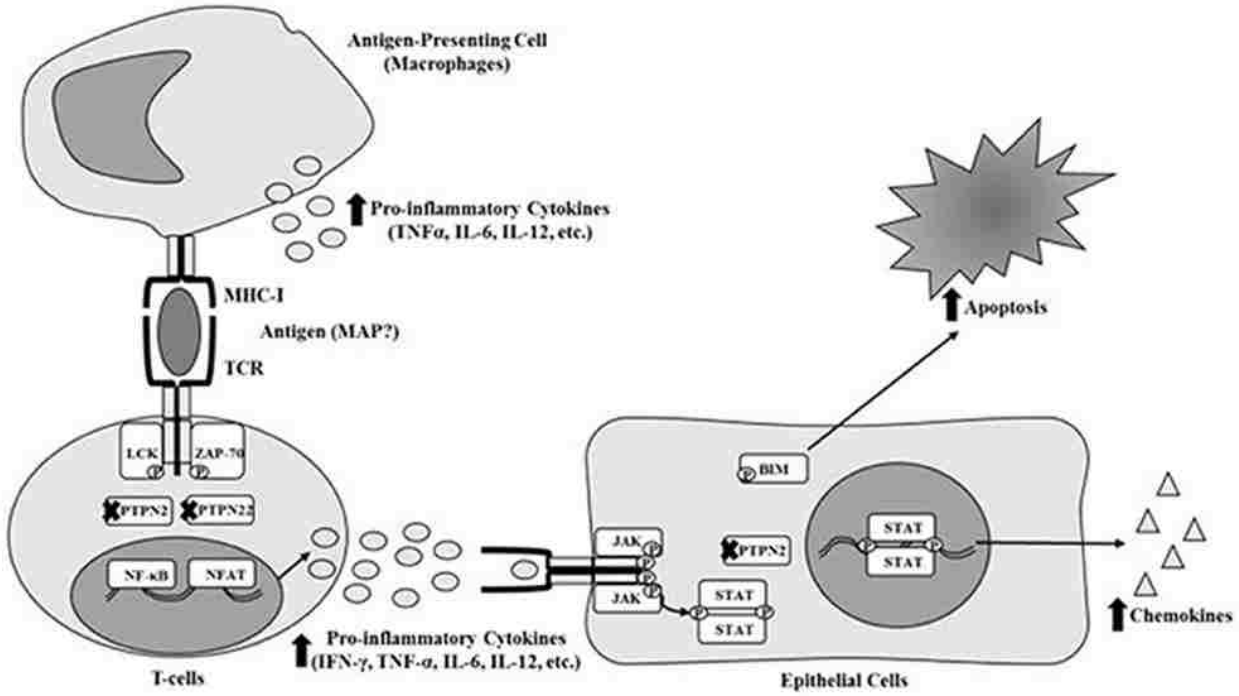

Figure 2. Effect of SNPs in *PTPN2/22* on T-cell Response

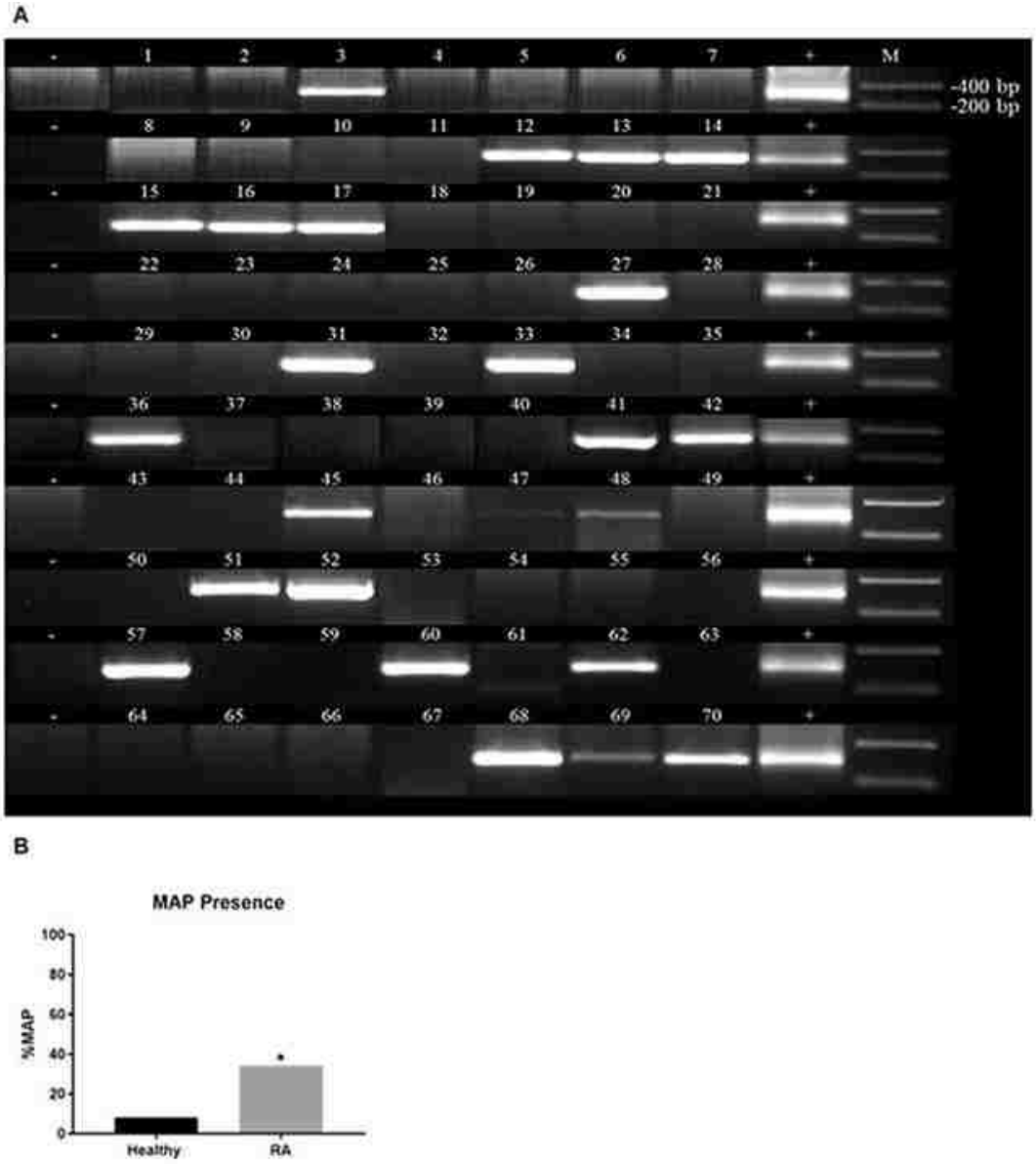

Figure 3. Detection of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in blood samples from RA

Nested PCR was performed on genomic DNA from blood samples from RA subjects (**A**) and healthy controls (**B**). DNA from MAP strain UCF4 was used as a positive control (+); a negative control (without DNA template) was also included (−). M corresponds with molecular weight marker. *:*P*-values ≤ 0.05.

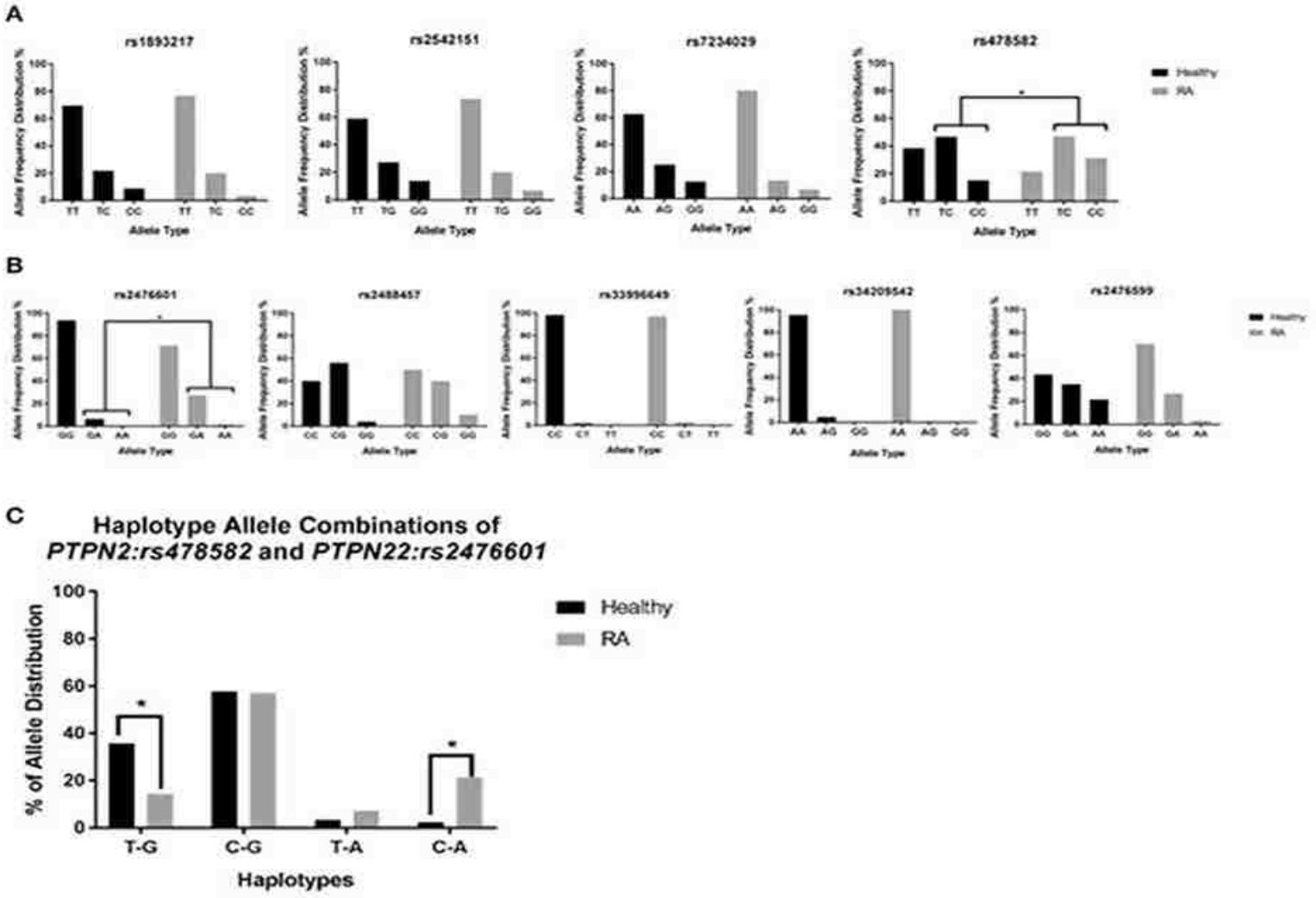

Figure 4. Genotyping of 9 SNPs in *PTPN2/22* in RA

(**A**) Represents the allele frequency in *PTPN2: rs1893217*, *rs2542151*, *rs7234029*, *rs478582*. (**B**) Represents the allele frequency in *PTPN22: rs2476601*, *rs2488457*, *rs33996649*, *rs34209542*, *rs2476599*. (**C**) Represents haplotype combinations between *PTPN2:rs478582* and *PTPN22:rs2476601* including T-G (major/major), C-G (heterozygous or minor/major), T-A (major/heterozygous or minor), and C-A (minor/minor). *:*P*-values ≤ 0.05.

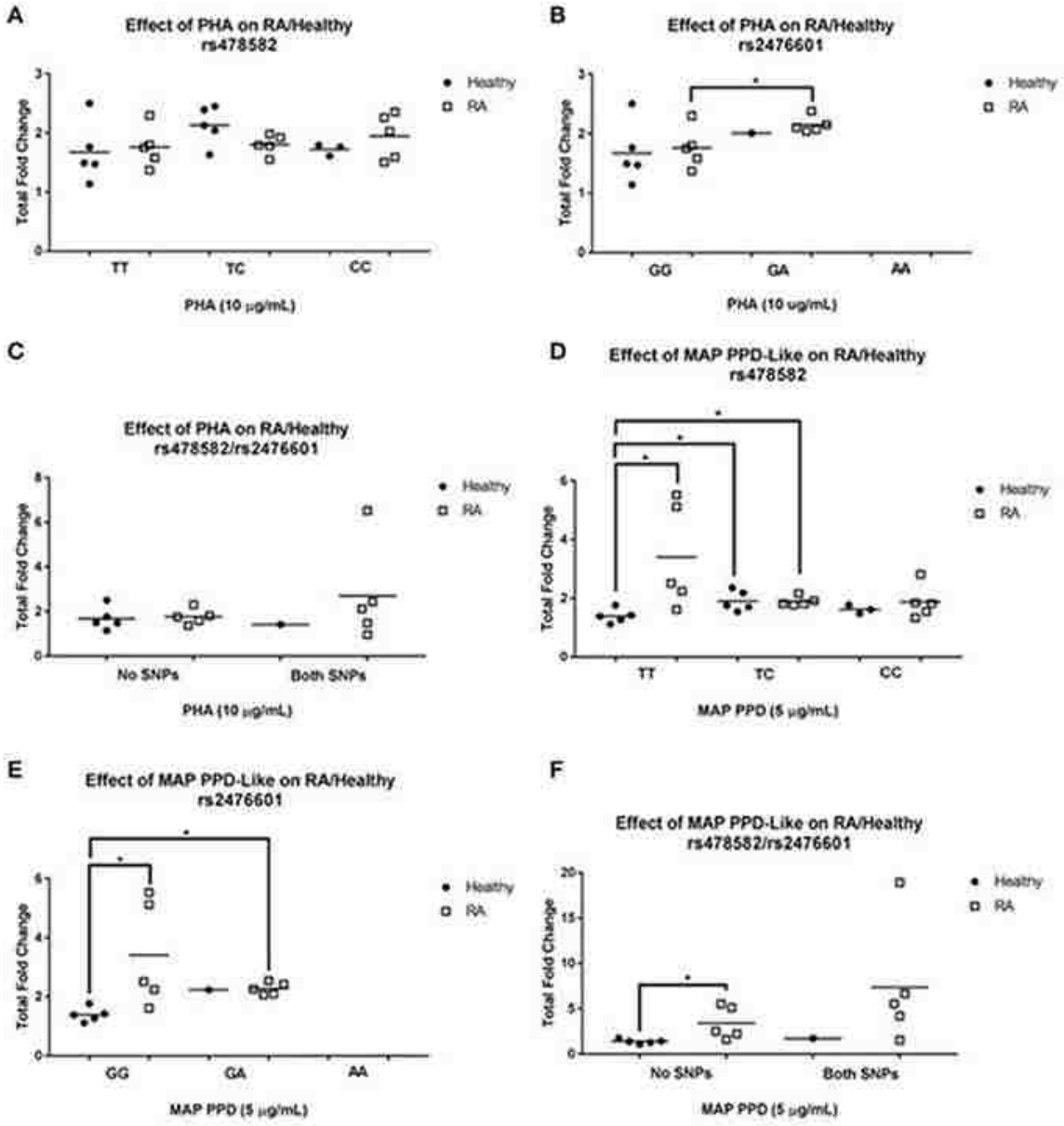

Figure 5. T-cell Response in RA Associated with *PTPN2:rs478582* and *PTPN22:rs2476601*

(**A,B**) Against Phytohematoagglutonin (PHA). (**D,E**) Against MAP Purified Protein Derivative-Like (PPD-Like). *PTPN2:rs478582*- heterozygous allele (TC), minor allele (CC), and wild type (TT). *PTPN22:rs2476601*-heterozygous allele (GA), minor allele (AA), and wild type (GG). The

effect of combined SNPs in *PTPN2/22* in T-cells induced with either PHA or MAP PPD-like is illustrated in (**C,F**). *: *P*-values ≤ 0.05.

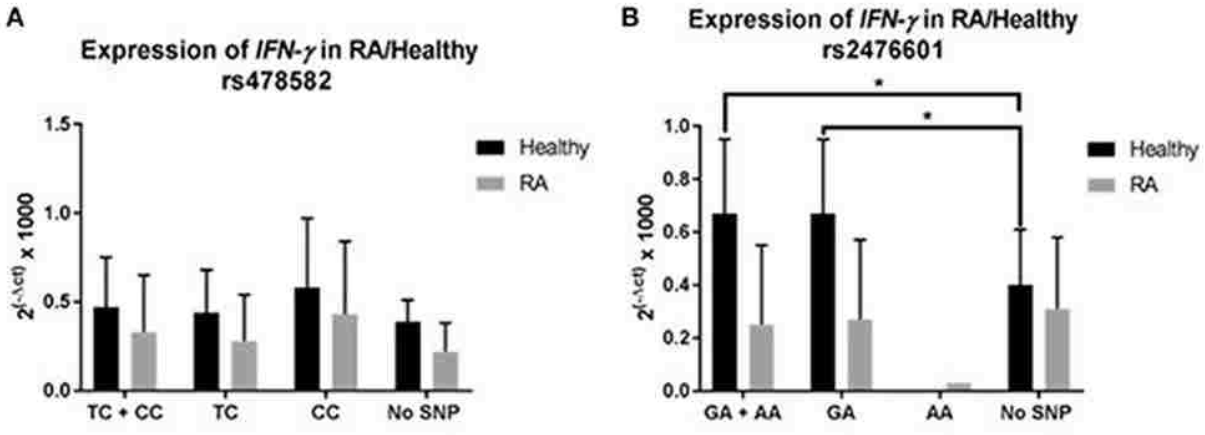

Figure 6. **Effect of *PTPN2:rs478582/PTPN22:rs2476601* on *IFN-γ* Expression in RA**

(**A**) *IFN-γ* expression in RA and healthy control subjects with *PTPN2:rs478582*. (**B**) *IFN-γ* expression in RA and healthy control subjects with *PTPN22:rs2476601*. *: *P*-values ≤ 0.05.

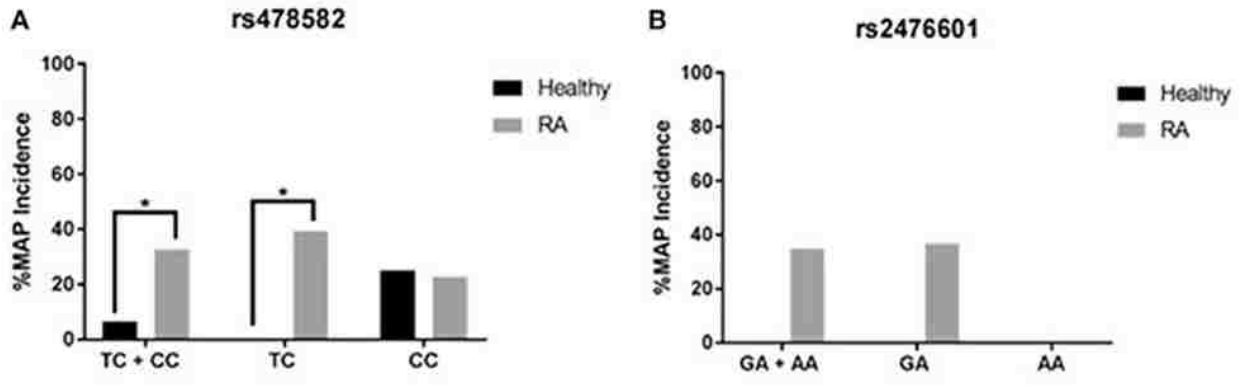

Figure 7. Effect of *PTPN2:rs478582* and *PTPN22:rs2476601* on Susceptibility to MAP Infection

(**A**) MAP in the blood from RA and healthy samples associated with *PTPN2:rs478582* [heterozygous allele (TC), minor allele (CC) and combined alleles (TC + CC)]. (**B**) MAP in the blood from RA and healthy samples-associated with *PTPN22:rs2476601* [heterozygous allele (GA), minor allele (AA), and combined alleles (GA + AA)]. *: *P*-values ≤ 0.05.

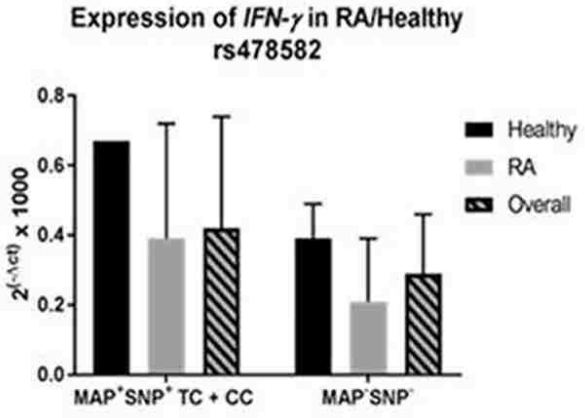

Figure 8. Combined Effect of MAP and *PTPN2:rs478582* on *IFN-γ* Expression in RA.

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CHAPTER THREE: ROLE OF *PTPN2/22* POLYMORPHISMS IN PATHOPHYSIOLOGY OF CROHN'S DISEASE

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Introduction

With a majority of autoimmune diseases sharing the SNPs with each other in immune regulatory genes, such as *PTPN2/22*, it is possible that the pathogenesis of these disorders could also share some of the same common environmental triggers with each other as well (Figure 9) [1][2][3][4][5]. Recent studies have shown that MAP infections have been associated with a variety of different inflammatory disorders including CD [6][7][8][9][10]. Mycobacterial infections causes problems in these autoimmune disease patients when the patient is genetically predisposed, causing the immune system to become dysregulated [6][7][8][9][10]. This dysregulation will lead to high amounts of pro-inflammatory cytokines, production of autoantibodies, and high amounts of apoptosis occurring in a variety of cell types, thus leading to chronic inflammation [6][7][8][9][10].

In addition to sharing the same genetic predispositions and environmental triggers, many autoimmune diseases share the same medical treatments as well. For instance, anti-TNF-α therapeutics such as adalimumab and infliximab are used for RA and CD [1][2]. However, anti-TNF-α medications along with NSAIDs, glucocorticoids, and other disease-modifying drugs cause several side effects [1][2][3][4][5]. These side effects include osteoporosis, hypertension, GI intolerance, autoantibodies against medications, and increased risk of developing

opportunistic infections, especially mycobacterial infections [1][2][3][4][5]. With the undesirable side effects of these medications, it is important that the pathophysiology of autoimmune diseases, such as CD, are thoroughly examined in order to develop more accurate detection of disease and to develop more personal treatment with little side effects.

In this study, we focus on the pathogenesis of CD, where we explore the effect of both the genetic predisposition of SNPs in *PTPN2/22* and the environmental trigger of MAP infection. We hypothesize that SNPs in *PTPN2/22* lead to loss of negative regulation in T-cells and, with a MAP infection, increases production of pro-inflammatory cytokines such as IFN-γ. This leads to an increase inflammation and apoptosis in the intestinal tissues of CD patients.

Materials and Methods

Clinical Samples

A total of 133 consented CD subjects and healthy controls donated two to three 4.0 mL K2-EDTA coded blood tubes for us in this study. The study was approved by the University of Central Florida Institutional Review Board #IRB00001138. Each subject completed and signed a written consent form before samples were collected. Healthy control subjects completed a survey that question if said subjects had any medical abnormality (CD, T1D, RA or "other diseases"). No healthy control subjects had any type of medical conditions to the best of their knowledge. The severities of the CD subjects' symptoms were scored from moderate to severe symptoms. The average age of CD subjects was 39.6 ± 14.3 with a gender ratio of 48.6% male and 51.4% female. The average age of healthy controls was 30.7 ± 13.4 with a gender ratio of 41.9% male and 58.1% female subjects. Table 6 lists age, gender and other demographic information for all

CD subjects in this study. From the blood tubes, the following procedures were done to the samples: *PTPN2/22* genotyping, gene expression profiling, MAP *IS900* nPCR detection, and T-cell proliferation assays.

Table 6. Demographics and Results of MAP Presence and Frequency of *PTPN2:rs478582*/*PTPN22:rs2476601* in CD Subjects

SAMPLE CODE	GENDER	AGE	DIAGNOSIS	MAP +/-	*PTPN2:rs478582*	*PTPN22:rs2476601*
RCS1	M	50	CD	-	TC	GA
RCS2	F	25	CD	-	TC	GA
RCS3	F	68	CD	+	TC	GG
RCS4	M	26	CD	+	CC	GG
RCS5	F	56	CD	+	CC	GG
RCS6	NA	NA	CD	+	TC	GG
RCS7	M	60	CD	+	TC	GG
RCS8	M	43	CD	+	TC	GG
RCS9	F	54	CD	-	CC	GG
RCS10	F	31	CD	NA	TC	GG
RCS11	M	21	CD	+	NA	GG
RCS12	M	25	CD	+	CC	GG
RCS13	F	40	CD	+	TC	GG
RCS14	M	36	CD	+	TC	GG
RCS15	NA	NA	CD	-	CC	GA
RCS16	F	25	CD	+	TC	GG
RCS17	F	27	CD	+	TC	GG
RCS18	M	20	CD	-	TT	GG
RCS19	M	25	CD	+	CC	GA
RCS20	F	41	CD	-	TC	GG
RCS21	M	20	CD	-	TT	GG
RCS22	M	40	CD	-	TC	GG
RCS23	M	30	CD	-	TC	GG
RCS24	F	60	CD	+	TC	GG
RCS25	F	39	CD	+	TT	GG

SAMPLE CODE	GENDER	AGE	DIAGNOSIS	MAP +/-	*PTPN2:rs478582*	*PTPN22:rs2476601*
RCS26	F	30	CD	+	CC	GA
RCS27	F	43	CD	+	CC	GG
RCS28	M	30	CD	+	TC	GA
RCS29	M	28	CD	+	TC	GG
RCS30	M	66	CD	+	TT	GG
RCS31	M	53	CD	-	TT	GG
RCS32	M	28	CD	-	TC	GA
RCS33	F	38	CD	+	CC	GG
RCS34	M	44	CD	-	CC	GA
RCS35	M	53	CD	-	TC	GG
RCS36	M	24	CD	+	TC	GG
RCS37	F	51	CD	+	TC	GG
RCS38	F	46	CD	+	TC	GG
RCS39	M	24	CD	-	CC	GG
RCS40	F	63	CD	+	TC	GG
RCS41	F	25	CD	-	TC	GG
RCS42	F	66	CD	-	TC	GG
RCS43	F	27	CD	+	TC	GG
RCS44	F	25	CD	+	TC	GG
RCS45	F	38	CD	+	TC	GG
RCS46	F	26	CD	-	CC	AA
RCS47	M	54	CD	+	TT	GA
RCS48	F	31	CD	+	TC	GG
RCS49	M	56	CD	-	CC	GG
RCS50	F	53	CD	-	TC	GG
RCS51	F	51	CD	-	TT	GA
RCS52	F	23	CD	+	TC	GG
RCS53	M	26	CD	+	TC	GG
RCS54	M	38	CD	-	TT	GG
RCS55	F	31	CD	+	TC	GG
RCS56	M	61	CD	+	TC	GG

SAMPLE CODE	GENDER	AGE	DIAGNOSIS	MAP +/-	*PTPN2:rs478582*	*PTPN22:rs2476601*
RCS57	F	24	CD	+	TC	GG
RCS58	M	57	CD	-	CC	GG
RCS59	F	30	CD	+	TT	GG
RCS60	M	51	CD	-	CC	GG
RCS61	F	55	CD	-	CC	GG
RCS62	F	61	CD	-	TT	GG
RCS63	F	31	CD	+	TC	GG
RCS64	F	56	CD	NA	TC	GG
RCS65	M	25	CD	+	NA	NA
RCS66	F	53	CD	+	NA	NA
RCS67	M	30	CD	-	TC	GG
RCS68	F	49	CD	-	CC	GG
RCS69	M	28	CD	+	TT	GG
RCS70	M	26	CD	+	TT	GG
RCS71	M	26	CD	+	CC	GG
RCS72	M	58	CD	+	CC	GG

CD: Crohn's Disease
*: TT = Homozygous Major Allele/No SNP
TC = Heterozygous Allele
CC = Homozygous Minor Allele
**: GG = Homozygous Major Allele/ No SNP
GA = Heterozygous Allele
AA = Homozygous Minor Allele

PTPN2/22 Genotyping

TaqMan™ SNP Genotyping Assays (Applied Biosystems™) were used to genotype nine SNPs in *PTPN2/22* from the isolated DNA from subjects' blood samples. Samples and reagents were sent to the University of Florida Pharmacotherapy and Translational Research Department (Gainesville, FL) to perform genotyping assays. Out of the nine SNPs, four SNPs were specific to *PTPN2* that includes *rs1893217, rs2542151, rs7234029, rs478582* along with five SNPs that were specific to *PTPN22* that includes *rs2476601*, *rs2488457*, *rs33996649*, *rs34209542*, *rs2476599*. Briefly, DNA was extracted from whole blood samples using QIAamp® DNA Blood Mini Kit (Qiagen™) following manufacturer's protocol. TaqMan™ genotyping assays for *PTPN2/22* SNPs were performed on DNA samples following manufacturer protocol (Applied Biosystems™). Briefly, DNA samples and the TaqMan™ SNP Genotyping Assays mixtures (primers with Vic and Fam fluorophore attachment) were transferred into a 384-well plate along with 2 × TaqMan™ Master Mix and 20 × Assay Working Stock in each well. Plates were treated to an RT-PCR protocol consisting of 95 °C for 10 min for 1 cycle, 92 °C for 15 s and 58 °C for 1 min for 50 cycles. The plates were then read for VIC (551 nm) and FAM (517 nm) fluorescence, where VIC or FAM alone determined allele 1 or allele 2 in the samples, while VIC and FAM together determined heterozygous for each allele in the samples.

PTPN2/22 and IFN-γ Gene Expression

Gene expression of *PTPN2/22* and *IFN-γ* was performed by converting RNA from subjects' whole blood samples to cDNA and performing RT-PCR. RNA from the subjects' blood samples

were isolated from peripheral leukocytes via TRIzol® Reagent (Invitrogen) per manufacturer's instruction. Briefly, 1.0 mL of whole blood from subjects' samples were transferred into a microcentrifuge tubes and centrifuged for 3,000 rpm for 15 min until the leukocytes formed a buffy coat layer, which was then transferred to new 2.0 mL RNase free microcentrifuge tubes. Tubes containing the leukocytes from subjects' samples were then suspended in 1.0 mL of TRIzol®, where the tubes were incubated and gently rocked for 15 min at room temperature. Next, 0.2 mL of chloroform was then mixed in each tube and then incubated at room temperature for 3 min. Tubes were then centrifuged at 11,400 rpm for 15 min at 4 °C, where afterwards the upper aqueous phase containing RNA was transferred to new 2.0 mL RNase free microcentrifuge tubes. Next, 0.5 mL of 100% isopropanol was added to the tubes containing subjects' RNA samples, where they were incubated at room temperature for 10 min. Tubes were then centrifuged at 11,400 rpm for 10 min at 4 °C, where afterwards the RNA pellets were washed in 1 mL of 75% ethanol. Washed RNA pellets were then centrifuged for 8700 rpm for 5 min at 4 °C and then air-dried until fully dried. Dried RNA pellets were then suspended in 20 µL of RNase free H_2O and boiled to 60 °C for 10 min.

Conversion of RNA to cDNA was done following the iScript™ Reverse Transcription (Bio-Rad®) manufacturer's instruction. RNA concentration from each subjects' samples were first quantified via NanoDrop ND-1000 Spectrophotometer (ThermoFisher Scientific®) and then diluted to 600 ng of total RNA. Next, diluted RNA samples were then added to PCR reaction tubes that contained 0.2 mL PCR reaction, 4 µL of iScript™ Reverse Transcription (Bio-Rad®), and up to 20 µL RNase free H2O. The PCR reaction tubes then underwent a PCR protocol

consisting of 5 min at 25 °C, 20 min at 46 °C and 1 min at 95 °C, where the final concentration of cDNA for each sample was 30 ng/μL.

For the RT-PCR reaction, 1 μL of cDNA (30 ng) was added to a 96-well microamp plates along with 10 μL of Fast SYBR Green Mastermix (ThermoFisher Scientific®), 1 μL of PrimePCR SYBR Green Assay mix (Bio-Rad®) specific to target gene, and 8 μL of sterile H2O. For the positive control for the RT-PCR reactions, the *18s* RNA gene was the target to determine if the reaction work and to obtain baseline CT readings. The oligonucleotide primers for the 18s RNA gene that were used for the RT-PCR reaction was the following: forward primer: 5'-GTA ACC CGT TGA ACC CCA TT-3' and reverse primer: 5'-CCA TCC AAT CGG TAG TAG CG-3'. RT-PCR reactions were performed using the 7500 Fast Real-Time PCR System (Applied Biosystems®), where relative gene expression levels were calculated using ΔCT (sample gene CT reading-*18s* RNA gene CT baseline reading) and using the equation ($2^{(-\Delta CT)} \times 1{,}000$).

Detection of MAP IS900 DNA

MAP *IS900* DNA was detected via nPCR from cultured peripheral leukocytes that were isolated from the subjects' blood samples as described previously [11]. Briefly, subjects' blood sample tubes were centrifuged for 3,000 rpm for 10 min at room temperature, where the buffy coat layer containing peripheral leukocytes was present and transferred to new sterile 2.0 mL microcentrifuge tubes. The peripheral leukocytes were then washed twice by adding double the volume of red cell lysis buffer (ammonium chloride solution, G-Biosciences®) to each tube and incubating/gently rocking for 10 min and then centrifuged at 5,000 rpm for 5 min at room temperature. The supernatant from each subjects' samples were then removed and the isolated

peripheral leukocyte pellets were re-suspended in TE buffer. The isolated pellets were then cultured in BD Bactec™ MGIT™ Para-TB medium (Becton, Dickinson and Company©) tubes supplemented with 800 uL of Bactec™ MGIT™ Para-TB Supplement (Becton, Dickinson and Company©) for six months at 37 °C in a BD Bactec™MGIT™ 320 Analyzer (Becton, Dickinson and Company©).

After six months of culturing, subjects' cultured samples underwent DNA extraction by using a modified DNAzol® (ThermoFisher Scientific®) extraction protocol as follows. A 2.0 mL sampling of culture from each subjects' tubes were obtained and pipetted into new sterile 2.0 mL microcentrifuge tubes. The tubes were then centrifuged at 13,000 rpm for 2.5 min, where afterwards the supernatant was discarded from the tubes and the culture pellets were saved. The subjects' culture pellet tubes were then mixed with 1.0 mL DNAzol® reagent and then mixed with 400 μL of 100% isopropanol. The tubes were then incubated for 15 min at room temperature followed by centrifugation at 8,000 rpm for 6 min, where afterwards the supernatant was discarded, leaving a DNA pellet. DNA pellets from the subjects' samples were then washed once with 500 μL DNAzol® reagent and centrifuged at 8,000 rpm for 5 min. Supernatant was then discarded from the tubes and the DNA pellets were then washed again with 1.0 mL of 75% ethanol, where they were centrifuged at 8,000 rpm for 5 min. DNA pellets were then dried after supernatant was removed via speedvac for 5 min. The dried DNA pellets were then dissolved in 50 μL of TE buffer.

MAP *IS900* DNA was then detected in each subjects' samples by the use of our nPCR protocol and nucleotide primers as described previously [11]. Subjects were considered to have MAP

presence when a 298 bp band on a 2% agarose gel is shown after nPCR reaction. The positive MAP DNA control that was used originated from our laboratory cultured clinical strain UCF4, which was isolated from a CD patient. The negative controls for each PCR step that was used contained all PCR reagents except for the DNA template used in the reactions.

T-cell Isolation and Proliferation Assay

T-cells were fully isolated from subjects' whole blood samples by the use of RosetteSep™ Human T-cell Enrichment Cocktail (StemCell™ Technology) as per manufacture's instruction. For the T-cell isolation and proliferation assays, the entire T-cell populations were examined in this study and were not segregated by subpopulations. Briefly, 50 µL/mL of RosetteSep™ Human T-cell Enrichment Cocktail was added to each subjects' whole blood samples and was incubated at 20 min at room temperature. Samples were then diluted with equal volumes of PBS with 2% fetal bovine serum (FBS, Sigma-Aldrich®) and mixed gently. The mixtures from each subjects' samples were then layered on top of a Lymphoprep™ (Axis-Shield®) density medium in a separated tube and centrifuged for 20 min at 2,500 rpm at room temperature. Separated T-cells from each subjects' samples were then found on top of the density medium layer and were collected into new sterile 2.0 mL microcentrifuge tubes and washed twice with PBS with 2% FBS.

Subjects' isolated T-cells were then plated on a 96-well plate, where T-cell proliferation assays were done using bromodeoxyuridine (BrdU) labeling proliferation ELISA kit (Roche Molecular Biochemicals®) as described previously [12]. To stimulate the subjects' isolated T-cells, phytohematoagglutunin (PHA) was used as a positive control mitogen. The test mitogen used in

the T-cell proliferation assays was purified protein derivative-like (PPD-like) from UCF4 MAP bacterial cultures that were prepared by purification of supernatant from sonicated protein extract. Briefly, 1×10^5 isolated T-cells from each subjects' samples were transferred in triplicates to 96-well plates and incubated in the following conditions: RPMI-1640 (Sigma-Aldrich®) only, PHA (10 μg/mL, Sigma-Aldrich®) or MAP PPD-like (5 μg/mL) along with respected subjects' plasma. The plates were then incubated for 72 h at 37 °C and 5% CO_2 and then labeled with 20 μL/well of BrdU and incubated again for 24 h at 37 °C and 5% CO_2. The T-cell proliferation assay was done through the Roche BrdU proliferation ELISA kit as described previously [12]. Relative T-cell proliferation levels of samples were compared to the control group (isolated T-cells in RPMI only) by examining the fold change in the absorbance reading of each well at 450 nm.

Statistical Analysis

Samples were analyzed for significance using unpaired, two-tailed *t*-tests; unpaired, two-tailed *z*-scores; and odds ratio. GraphPad Prism 7 was used for statistical analysis and creation of graphs. *P*-values < 0.05 were considered significant.

Results

PTPN2/22 SNP Allele Frequency in CD

Allele frequency of the nine SNPs examined in *PTPN2/22* found in both CD subjects and healthy controls are shown in Figure 10. All genotyped samples were found in Hardy-Weinberg equilibrium. Out of the four SNPs found in *PTPN2* (*rs1893217*, *rs2542151*, *rs7234029*, and *rs478582*), *rs478582* was significant in the CD, where heterozygous (TC) or minor (CC) alleles

when examined together were detected in 57/69 (82.6%) in CD compared to 36/59 (61.0%) healthy controls (OR = 3.03, 95% CI: 1.35-6.84, *P*-values < 0.05, Figure 10A). Specifically, the heterozygous (TC) alleles were detected in 38/69 (55.1%) CD compared to the 28/59 (47.5%) of healthy controls, while homozygous (CC) alleles were detected in 19/69 (27.5%) CD compared to 8/59 (13.6%) healthy controls. SNPs *rs1893217*, *rs2542151*, and *rs7234029* were found to be not significant in CD compared to the healthy controls. Out of the five SNPs specific to *PTPN22* (*rs2476601*, *rs2488457*, *rs33996649*, *rs34209542*, and *rs2476599*), none of SNPs were considered significant in CD compared to the healthy controls (Figure 10B). However, since *PTPN22:rs2476601* is found significantly in various inflammatory autoimmune diseases, we continued to investigate the SNP in more detail along with *PTPN2:rs478582* [6][13][14][15][16][17]. For *PTPN22:rs2476601*, CD with either heterozygous (GA) or minor (AA) alleles were detected in 11/70 (15.7%) subjects, while 4/62 (6.45%) was detected in healthy controls (OR = 2.7, 95% CI: 0.81-8.98, *P*-values > 0.05). Specifically, the heterozygous (GA) alleles were detected in 10/70 (14.3%) CD compared to the 4/62 (6.45%) of healthy controls, while homozygous (AA) alleles were rare in all samples.

For confirmation that CD subjects were significant in having SNP alleles for *PTPN2:rs478582* and *PTPN22:rs2476601*, determination of haplotype combinations were done (Figure 10C). Examination of the following haplotype combinations between *PTPN2:rs478582* and *PTPN22:rs2476601* were examined: T-G, C-G, T-A, and C-A. The T-G haplotype (major/major) was found more significantly in the healthy controls (21/59 = 35.6%) than in CD (10/69 = 14.5%, *P*-values < 0.05). The C-G haplotype (heterozygous or minor/major) and the C-A (heterozygous or minor/heterozygous or minor) were found more in CD (48/69 = 69.6%; 9/69 =

13.0%, respectively) than in healthy controls (34/59 = 57.6%; 2/59 = 3.39%, respectively). The C-A haplotype was found more significantly in CD than the healthy controls (*P*-values < 0.05).

Relationship of PTPN2:rs478582 and PTPN22:rs2476601 on Expression on PTPN2/22 and IFN-γ in CD

The average relative gene expression ($2^{(-\Delta CT)} \times 1{,}000$) of *PTPN2*, regardless of SNPs, in CD was significantly lower (5.27 ± 2.68, n = 38) than in healthy controls (10.5 ± 6.95, n = 30, *P*-values < 0.05, Figure 11A). Similarly, the average relative gene expression of *PTPN22*, regardless of SNPs, was also significantly lower in CD (1.76 ± 1.12, n = 38) than in healthy controls (3.24 ± 1.84, n = 30, *P*-values < 0.05, Figure 11B). The evaluation of the effect of *PTPN2:rs478582* and *PTPN22:rs2476601* on expression of *PTPN2/22* and *IFN-γ* was determined.

For subjects with either heterozygous (TC) or minor (CC) alleles in *PTPN2:rs478582*, regardless of disease, expression of *PTPN2* did not change when compared to the normal (TT) subjects. However, when examining the CD and healthy control subjects in each allele group, CD overall had a lower average relative gene expression of *PTPN2*. The average relative gene expression in CD with heterozygous (TC) or minor (CC) alleles in *PTPN2:rs478582* was significantly lower (5.34 ± 2.77, n = 31) compared to 10.2 ± 7.15 (n = 21) in healthy controls with similar SNPs (*P*-values < 0.05). Specifically, when examining subjects with heterozygous (TC) alleles in *PTPN2:rs478582*, CD average relative gene expression was 5.22 ± 2.57 (n = 22), which was significantly lower than the healthy controls with heterozygous (TC) alleles (10.5 ± 7.15, n = 17, *P*-values < 0.05). When examining subjects with homozygous (CC) alleles in *PTPN2:rs478582*, CD average relative gene expression was 5.64 ± 3.37 (n = 9), which was lower than the healthy controls with homozygous (CC) alleles (8.89 ± 8.03, n = 4).

For subjects with either heterozygous (GA) or minor (AA) alleles in *PTPN22:rs2476601*, regardless of disease, expression of *PTPN22* did not change when compared to the normal (GG) subjects. However, when examining the CD and healthy control subjects in each allele group, CD overall had a lower average relative gene expression of *PTPN22*. The average relative gene expression in CD with heterozygous (GA) or minor (AA) alleles in *PTPN22:rs2476601* was significantly lower (1.58 ± 0.93, n = 6) compared to 3.40 ± 1.19 (n = 4) in healthy controls with similar SNPs (*P*-values < 0.05). Specifically, when examining subjects with heterozygous (GA) alleles in *PTPN22:rs2476601*, CD average relative gene expression was 1.48 ± 1.00 (n = 5), which was significantly lower than the healthy controls with heterozygous (GA) alleles (3.40 ± 1.19, n = 4, $P < 0.05$). Minor (AA) alleles in *PTPN22:rs2476601* was rare in all subjects.

Correlation analyses were performed to determine if expression of relative gene expression of *IFN-γ* changed in subjects with *PTPN2:rs478582* or *PTPN22:rs2476601* (Figure 11C and 11D, respectively). The average relative gene expression of *IFN-γ* in CD subjects with the *PTPN2:rs478582* heterozygous (TC) or minor (CC) allele was 0.41 ± 0.31 (n = 38), which was significantly higher compared to the CD subjects with normal (TT) alleles (0.21 ± 0.22, n = 12, $P < 0.05$). Specifically, CD subjects with the heterozygous (TC) allele had significantly higher (0.41 ± 0.31, n = 24, $P < 0.05$) *IFN-γ* relative gene expression than CD subjects with normal (TT) alleles, while CD subjects with the minor (CC) alleles had higher gene expression as well (0.40 ± 0.31, n = 14). There was no significant change in *IFN-γ* relative gene expression in the CD subjects with the *PTPN22:rs2476601* heterozygous (GA) or minor (AA) alleles. However, in healthy controls, subjects with the heterozygous (GA) or minor (AA) alleles had a significantly

higher gene expression (0.67 ± 0.28, n = 4, $P < 0.05$) than healthy controls with normal (GG) alleles (0.40 ± 0.21, n = 20).

Effect of PTPN2:rs478582 and PTPN22:rs2476601 on Susceptibility of MAP Infection in CD

Overall detection of MAP *IS900* DNA was found in CD and healthy control subjects and were correlated with *PTPN2:rs478582* and *PTPN22:rs2476601* (Table 7). Out of 70 CD subjects, 43 (61.4%) were positive for *MAPbacteremia* compared to only 4/48 (9.33%) of healthy controls ($P < 0.05$, OR = 17.5, 95% CI: 5.65-54.3).

Table 7. MAP *IS900* nPCR Presence and Correlation with *PTPN2:rs478582*/*PTPN22:rs2476601* in Clinical Subjects

	MAP Presence		
	Healthy	**CD**	**OR (95% CI)**
Overall	4/48= 9.33%	**43/70= 61.4% ***	**17.5 (5.65–54.3) ***
rs478582			
TT	2/17= 11.8%	**6/12= 50% ***	**7.5 (1.17-48.2) ***
TC	0/22= 0.00%	**25/37= 67.6% ***	**91.8 (5.14-1604.3) ***
CC	2/8= 25%	9/19= 47.4%	2.7 (0.43-16.9)
TC + CC	2/30 = 6.67%	**34/56 = 60.7% ***	**21.6 (4.68-100.1) ***
rs2476601			
GG	4/59 = 6.78%	**33/59 = 55.9% ***	**17.6 (5.59-54.4) ***
GA	0/4 = 0.00%	3/10 = 30.0%	4.2 (0.17-101.5)
AA	0/1 = 0.00%	0/1 = 0.00%	1.00 (0.02-92.4)
GA + AA	0/5 = 0.00%	3/11 = 27.3%	4.53 (0.19-105.8)
Haplotypes			
T-G	2/15 = 13.3%	**5/10 = 50.0% ***	**6.5 (0.94-45.1) ***
C-G	2/29 = 6.90%	**31/46 = 67.4% ***	**30.0 (6.3-142.6) ***
T-A	0/2 = 0.00%	1/2 = 50.0%	5.00 (0.11-220.6)
C-A	0/2 =0.00%	3/9 = 33.3%	2.69 (0.1-73.2)

[*] = *P*-value < 0.05

Correlation analyses with *PTPN2:rs478582* and *PTPN22:rs2476601* along with MAP infection was done on CD and healthy controls to see if these SNPs increase MAP susceptibility (Table 7). For CD subjects with heterozygous (TC) or minor (CC) alleles in *PTPN2:rs478582*, 34/56 (60.7%) had *MAPbacteremia* presence compared to only 2/30 (6.67%) in healthy controls with similar SNPs ($P < 0.05$, OR = 21.6, 95% CI: 4.68-100.1). Specifically, CD subjects with heterozygous (TC) alleles in *PTPN2:rs478582* was 25/37 (67.6%) compared to 0/22 (0.00%) in

healthy controls with heterozygous (TC) alleles ($P < 0.05$, OR = 91.8, 95% CI: 5.14-1640.3). The CD subjects with heterozygous (TC) or minor (CC) alleles group (34/56 = 60.7%) and CD subjects with heterozygous (TC) allele group (25/37 = 67.6%) in *PTPN2:rs478582* had higher *MAPbacteremia* compared to CD subjects with normal (TT) alleles (6/12 = 50%).

For CD subjects with heterozygous (GA) alleles in *PTPN22:rs2476601*, 3/10 (30.0%) had *MAPbacteremia* compared to 0/4 (0.00%) in healthy controls with heterozygous (GA) alleles (OR = 4.2, 95% CI: 0.17-101.5). Presence of *MAPbacteremia* was rare in all subjects with the minor (AA) allele.

Correlation of haplotype combinations of *PTPN2:rs478582* and *PTPN22:rs2476601* alleles on susceptibility to *MAPbacteremia* was analyzed, where CD subjects with the C-G haplotype (heterozygous or minor/major) had 31/46 (67.4%) with *MAPbacteremia* presence compared to 2/29 (6.90%) of healthy controls with the C-G haplotype (*P*-values < 0.05, OR = 30.0, 95%CI: 6.3-142.6). The T-A haplotype (major/heterozygous or minor) and the C-A haplotype (heterozygous or minor/heterozygous or minor) was rare in all samples. However, CD subjects with the T-A haplotype had 1/2 (50.0%) with *MAPbacteremia* presence compared to the 0/2 (0.00%) in healthy controls with the T-A haplotype, while CD subjects with the C-A haplotype had 3/9 (33.3%) with *MAPbacteremia* presence compared to the 0/2 (0.00%) in healthy controls with the C-A haplotype.

Relationship of Combined MAP Presence with PTPN2:rs478582 and PTPN22:rs2476601 on Expression of PTPN2/22 and IFN-γ in CD

When examining CD and healthy control subjects with or without *MAPbacteremia* presence alone, there was no change in *PTPN2/22* and *IFN-γ* relative gene expression when examining correlation data. However, *PTPN2/22* was significantly lower in CD subjects than in the health control subjects regardless of *MAPbacteremia* presence or not. CD subjects who had *MAPbacteremia* presence had an average relative gene expression of 5.25 ± 2.58 (n = 21) in *PTPN2* compared to the healthy controls with *MAPbacteremia* presence (11.9 ± 10.5, n =3, $P < 0.05$). CD subjects who had an absence of *MAPbacteremia* presence had an average relative gene expression of 5.28 ± 2.87 (n = 17) in *PTPN2* compared to the healthy controls without *MAPbacteremia* presence (10.3 ± 6.71, n = 27, $P < 0.05$). For *PTPN22* average relative gene expression, CD subjects with *MAPbacteremia* presence had 1.73 ± 0.97 (n = 21) compared to healthy controls with *MAPbacteremia* presence (2.83 ± 1.94, n = 3). CD subjects without *MAPbacteremia* presence had an average relative gene expression of 1.81 ± 1.31 (n = 17) in *PTPN22* compared to the healthy controls without *MAPbacteremia* presence (3.29 ± 1.86, n = 27, *P*-values < 0.05).

The effect of combined *MAPbacteremia* presence and either *PTPN2:rs478582* or *PTPN22:rs2476601* did not significantly change *PTPN2/22* expression in all CD and healthy control samples. However, when examining the combined effects of *MAPbacteremia* presence and either *PTPN2:rs478582* or *PTPN22:rs2476601*, the average relative gene expression of *IFN-γ* does increase in subjects compared to subjects without *MAPbacteremia* presence and no SNPs. For CD subjects with both *MAPbacteremia* and heterozygous (TC) or minor (CC) alleles in

PTPN2:rs478582, the average relative gene expression of *IFN-γ* was higher (0.40 ± 0.29 n = 22) compared to the CD subjects without *MAPbacteremia* and *PTPN2:rs478582* (0.23 ± 0.31, n = 5, Figure 12). For CD subjects with both *MAPbacteremia* and heterozygous (GA) or minor (CC) alleles in *PTPN22:rs2476601*, the average relative gene expression of *IFN-γ* was higher (0.42 ± 0.32, n = 4) compared to the CD subjects without *MAPbacteremia* and *PTPN22:rs2476601* (0.37 ± 0.31, n = 18).

T-cell Proliferation Response in CD

T-cell functionality when SNPs and *MAPbacteremia* was presented in subjects was determined in five CD and five healthy control subjects. All five CD subjects that had their T-cell response tested had SNPs in either *PTPN2:rs478582* and/or *PTPN22:rs2476601*, while the five healthy control subjects had no observed SNPs present. Overall, when the subjects' T-cells were treated with PHA, the average overall fold change in the CD subjects was 2.22 ± 1.36 (n = 5) fold increase compared to the healthy controls (1.67 ± 0.51 fold increase, n = 5). Similarly, when the same T-cells were treated with MAP PPD-like, the average overall fold change in CD subjects was 2.01 ± 0.79 (n = 5) compared to the healthy controls (1.39 ± 0.24 fold increase, n = 5).

Out of the five CD subjects, 3/5 were tested for having *MAPbacteremia* presence. When examining T-cells treated with PHA from CD subjects tested positive for *MAPbacteremia* presence, the average overall fold change was 2.7 ± 1.65 (n = 3) compared to the CD subjects' T-cells that were absence of *MAPbacteremia* presence and treated with PHA (1.51 ± 0.51 fold increase, n = 2). Similarly, when the same T-cells were treated with MAP PPD-like, the average

overall fold change in CD subjects with *MAPbacteremia* was 2.5 ± 0.59 (n = 3) compared to the CD subjects' T-cells without *MAPbacteremia* presence (1.27 ± 0.12 fold increase, n = 2).

Discussion

The pathogenesis of CD, as with other autoimmune diseases, involves both genetic pre-disposition leading to higher immune responses and an environmental trigger that exacerbates the immune response. However, with current diagnosis and treatment, it has been difficult to treat CD symptoms due to loss of treatment response and many side effects [1][2][3][4][5]. Thus, understanding the key elements of CD pathogenesis (genetic SNPs and environmental triggers), it is possible to find new treatment targets for the disease and new diagnosis techniques as well. CD pathogenesis is very dependent on the overproduction of pro-inflammatory cytokines such as TNF-α and IFN-γ, which promote chronic inflammation, increased granuloma formation, and increased apoptosis of intestinal tissues [3][4][18][19]. Since the majority of CD medications are blocking pro-inflammatory cytokines such as TNF-α and IFN-γ, other types of targets has been ignored [1][2][3][4][5] [18][19]. This study is focused on finding new targets for both diagnosis and treatment of CD, where we looked into the SNPs of negative regulatory genes *PTPN2/22* and their impact on: increased production of pro-inflammatory cytokines, apoptosis, mycobacterial susceptibility, and inflammation. To our knowledge, this is the first study to look into SNPs in both *PTPN2/22* together along with correlation with gene expression and MAP susceptibility in CD.

The effect of SNPs in *PTPN2/22* in CD pathogenesis has been highly debated in the literature, thus we selected nine SNPs that not only was found associated with CD, but with other diseases

as well [6][14][15][16][17][20][21][22]. Out of the nine SNPs examined in this study, *PTPN2:rs478582* was found to be significant in CD (*P*-values < 0.05, OR = 3.03) compared to the healthy controls (Figure 10A). Although *PTPN22:rs2476601* was found to not be significant to CD (*P* > 0.05, OR = 2.7) compared to the healthy controls, we continued to study the effects of the SNP along with *PTPN2:rs478582* due to *PTPN22:rs2476601* being associated with autoimmune diseases in general (Figure 10B) [6][13][14][15][16][17][20][21][22]. Since a diverse population (no restriction on race, place of origin, age, or gender) was used in this study, alterations of allele distribution in the SNPs could possibly happen due to SNPs overall fluctuating between different population groups[6][13][14][15][16][17][20][21][22]. Further isolated population studies on *PTPN2/22* SNPs in CD subjects need to be investigated more. Knowledge of which SNP is more associated with CD could possibly be used as a diagnosis tool for clinicians when examining patients with CD like symptoms.

Gene expression of *PTPN2/22* correlated with the SNPs *PTPN2:rs478582* and *PTPN22:rs2476601* was also done to determine if the SNPs did change *PTPN2/22* levels. Although overall *PTPN2/22* expression was significantly decreased in CD subjects (*P* < 0.05, Figure 11A and 11B), the SNPs *PTPN2:rs478582* and *PTPN22:rs2476601* did not change gene expression between normal, heterozygous, or minor alleles. However, *IFN-γ* gene expression was found significantly higher in both CD and healthy controls (*P* < 0.05) along with an overall increased T-cell activity in subjects that had heterozygous/minor alleles in either *PTPN2:rs478582* and/or *PTPN22:rs2476601* (Figure 11C and 11D). These correlation analyzes shows that the SNPs *PTPN2:rs478582* and *PTPN22:rs2476601* may not necessarily change the regulation of the *PTPN2/22* gene, but could possible disrupt the protein activity of *PTPN2/22*.

For the *PTPN2:rs478582* SNP, a base change (T > C) in intron 3 occurs, where it is theorized that splicing problems could occur during the RNA splicing [23][24][25][26]. This could lead to loss of activity in the protein once fully translated [23][24][25][26]. The *PTPN22:rs2476601* SNP is a base change (G > A) that occurs in exon 14, which physically changes the amino acid arginine (R) to a tryptophan (W) on the 620 amino acid residue on the catalytic portion of the PTPN22 protein [17][24][25][26]. It has been highly debated what the R620W does to the PTPN22 protein, but it is suspected to cause the protein to be less active [17][24][25][26]. Overall, the SNPs *PTPN2:rs478582* and *PTPN22:rs2476601* seem to cause a loss of function in *PTPN2/22*, thus leading to less negative regulated T-cells. This will lead to a high production of pro-inflammatory cytokines, which will lead to increased inflammation/apoptosis in intestinal tissues in CD subjects. Other SNPs in *PTPN2/22* will need to be studied further to see if those SNPs will alter gene expression of *PTPN2/22* instead of *PTPN2:rs478582* and *PTPN22:rs2476601* just altering protein activity. Although we only examined the effect of *PTPN2/22* on the expression of *IFN-γ*, other factors do control *IFN-γ* expression and production. These include cytokines, such as TNF-α and IL-12, which stimulate T-cell production of IFN-γ and cytokines, such as IL-6 and IL-10, which decrease T-cell production of IFN-γ [27]. However, since CD and other inflammatory autoimmune disorders are T-cell mediated, we focused only on PTPN2/22 regulation on *IFN-γ* expression. This is due to PTPN2/22 ultimately acting as negative regulators of T-cell activity and thus controlling IFN-γ production from T-cells. Further investigation of the effect of these other regulatory IFN-γ production cytokines in subjects with SNPs in *PTPN2/22* is needed.

Although the role MAP has been studied in CD pathogenesis extensively, correlation studies with SNPs in *PTPN2/22* and MAP susceptibility have not been done before until this study [4][6][7][8][9][10][11][12][18]. Overall, the correlation analyzes of SNPs in *PTPN2/22* and *MAPbacteremia* presence showed that the SNPs might have increased susceptibility in CD subjects (Table 7). Specifically, 60.7% (OR = 21.6, $P < 0.05$) of CD subjects with *PTPN2:rs478582* SNP (heterozygous or minor group) had *MAPbacteremia* presence, while 27.3% (OR = 4.53) subjects with the *PTPN22:rs2476601* SNP (heterozygous or minor group) had *MAPbacteremia*. Limitations however in the detection of MAP *IS900* DNA from the blood of subjects' samples do not provided the information that the MAP bacteria is alive or dead, thus does not show active infection or previous infection. Further culturing of the blood from the subjects is necessary to determine live MAP infection in the subjects examined. The findings found in this study suggest that SNPs in *PTPN2/22* increases susceptibility to *MAPbacteremia*, which is possible due to the lack of negative regulation in the T-cells. Since T-cells control macrophage activity and mycobacterial species such as MAP can survive in infected macrophages, it is important that the T-cells are regulated correctly in order to prevent MAP infection [28][29][30][31][32]. If problems involving the *PTPN2/22* gene regulation or function the PTPN2/22 protein occurs, T-cells will be overactive and in turn will make macrophages overactive as well (Figure 13) [28][29][30][31][32]. This increased activity of macrophages will not only lead to increased pro-inflammatory cytokines like TNF-α, but could allow MAP and other intracellular pathogens to survive and grow faster due to the increased activation of newer macrophages [28][29][30][31][32].This is why SNPs in *PTPN2/22* and the hyperactivity of T-cells should increase susceptibility to intracellular pathogens such as MAP.

To further test if T-cells from the CD subjects with the *PTPN2:rs478582* and the *PTPN22:rs2476601* were overactive, we induced isolated T-cells from CD subjects with either PHA or MAP PPD-like. Although we did not isolate out total T-cell populations from mucosal intestinal tissues and instead from peripheral blood draws, we believe that T-cell proliferation will be the same regardless of the source of origin. This is possible due to *PTPN2/22* being found in every T-cell population, regardless of the site of isolation, thus SNPs in *PTPN2/22* should affect all T-cells in the body in the same way. Overall, CD subjects with the SNPs proliferated more than healthy controls without the SNPs. In addition, CD subjects who had *MAPbacteremia* presence and SNPs in *PTPN2/22* proliferated more than CD subjects who did not have *MAPbacteremia* presence. These analyzes showed that for T-cells to become overactive, both SNPs in *PTPN2/22* and the presence of *MAPbacteremia* is required to induce the pathogenesis process of CD. This is further evidence that for the pathogenesis of any autoimmune disease, both genetic predisposition and an environmental trigger are needed to cause disease. Further investigation in gene expression of pro-inflammatory cytokines produced (IFN-γ for example) by T-cells after being induced with antigens need to be examined. Along with this, further investigation of subpopulations of T-cell activity is needed to determine which T-cell population is more active in subjects with SNPs in *PTPN2/22*.

Overall, SNPs in *PTPN2/22* lead to overactive T-cell activity and increased susceptibility to intracellular pathogens such as MAP. With genetic testing for SNPs and detection/treatment for mycobacterial infections such as MAP, it is possible for personalized treatment of CD to be an option. Further studies in SNPs in *PTPN2/22* and other immunity specific genes need to be researched and correlated with bacterial infections to improve CD diagnosis and treatment.

Figures

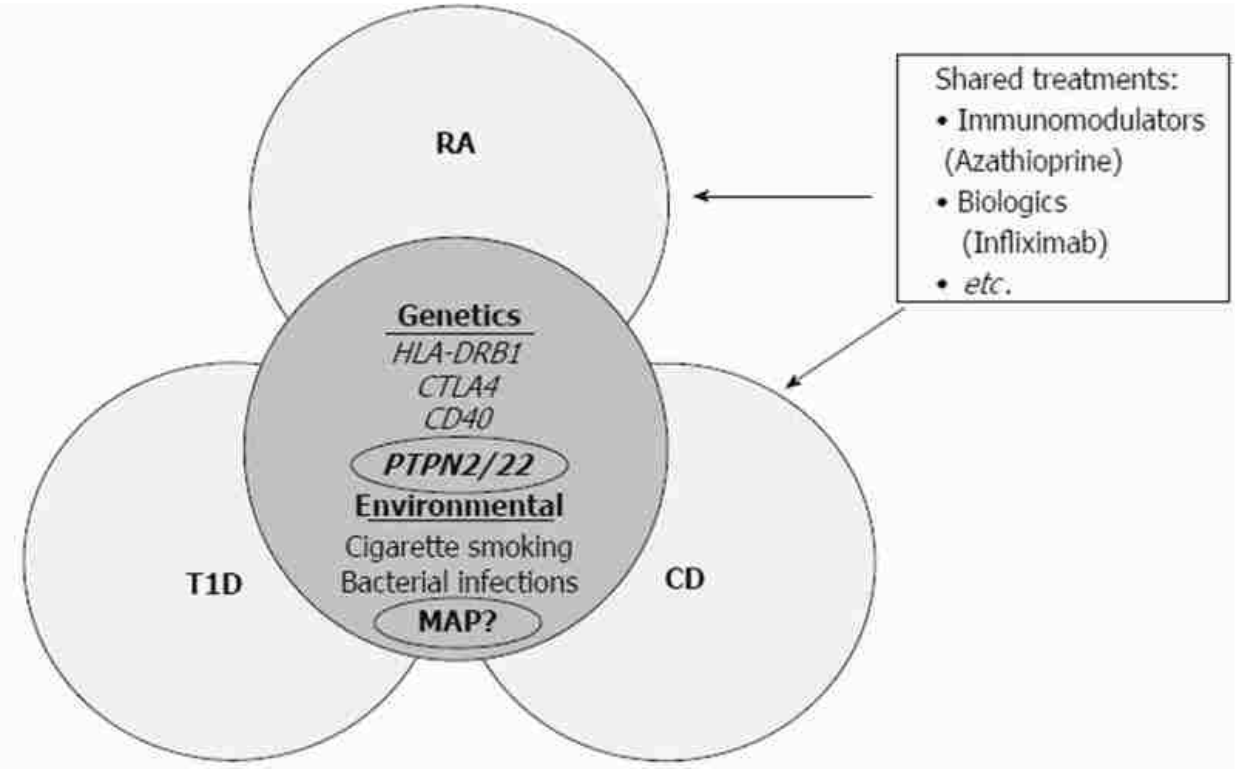

Figure 9. Shared Genetic Predispositions and Environmental Triggers between Common Autoimmune Diseases

For autoimmune diseases, many share the same treatments and some of the same genetic single nucleotide polymorphisms in specific immunity genes. Thus, it is possible that these disorders share the same environmental triggers as well, such as *Mycobacterium avium* subspecies *paratuberculosis* (MAP) bacterial infection.

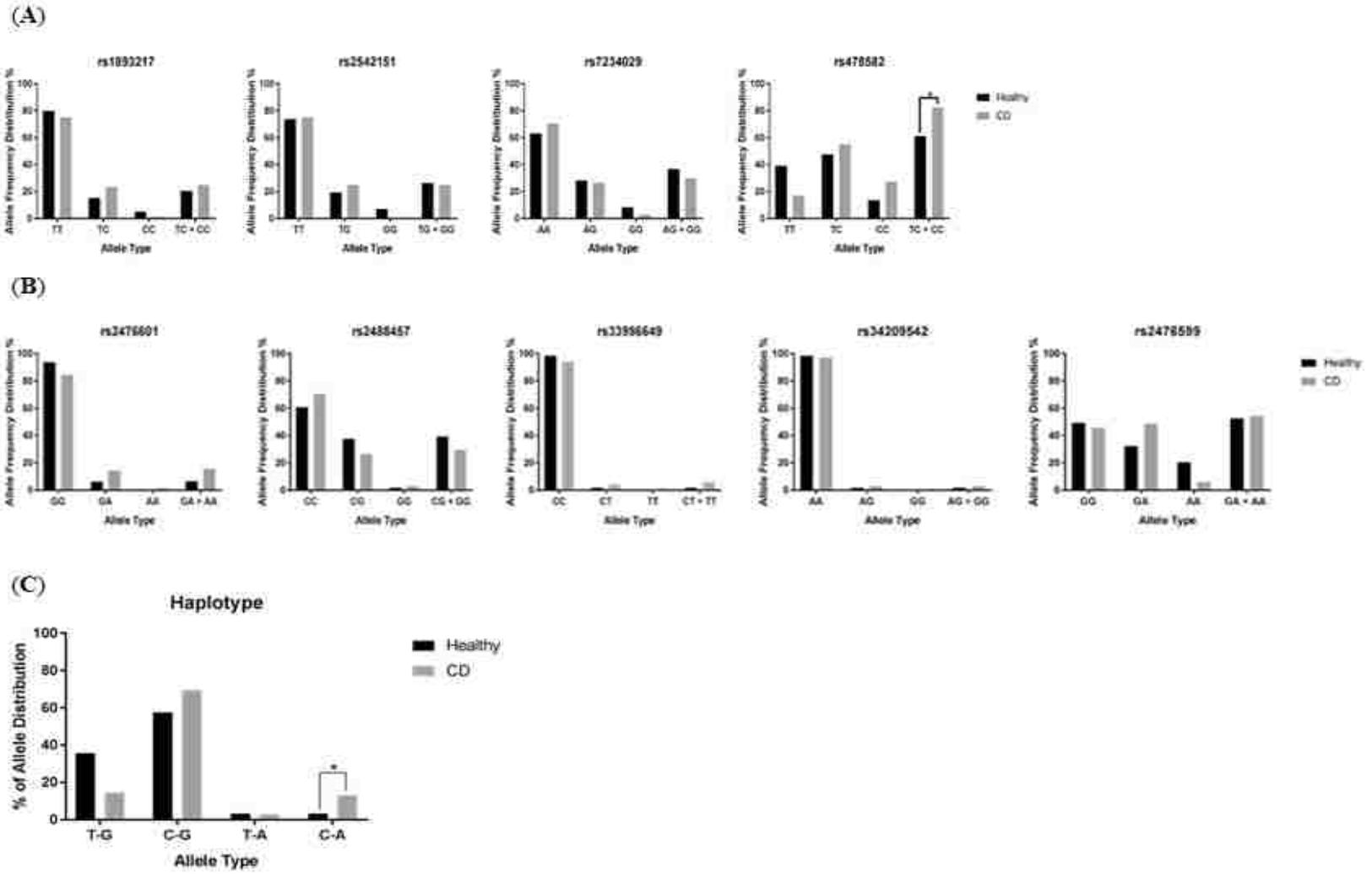

Figure 10. Allele Frequency in Nine Single Nucleotide Polymorphisms in Crohn's Disease and Healthy Control Subjects

A: Represents allele frequency of *PTPN2* SNPs*: rs1893217*, *rs2542151*, *rs7234029*, *rs478582*; **B**: Represents allele frequency of *PTPN22* SNPs: *rs2476601*, *rs2488457*, *rs33996649*, *rs34209542*, *rs2476599*; **C**: Represents haplotype combinations *PTPN2:rs478582* and *PTPN22:rs2476601*. *: $P < 0.05$, healthy vs CD. T-G: Major/major; C-G: SNP/major; T-A: Major/SNP; C-A: SNP/SNP.

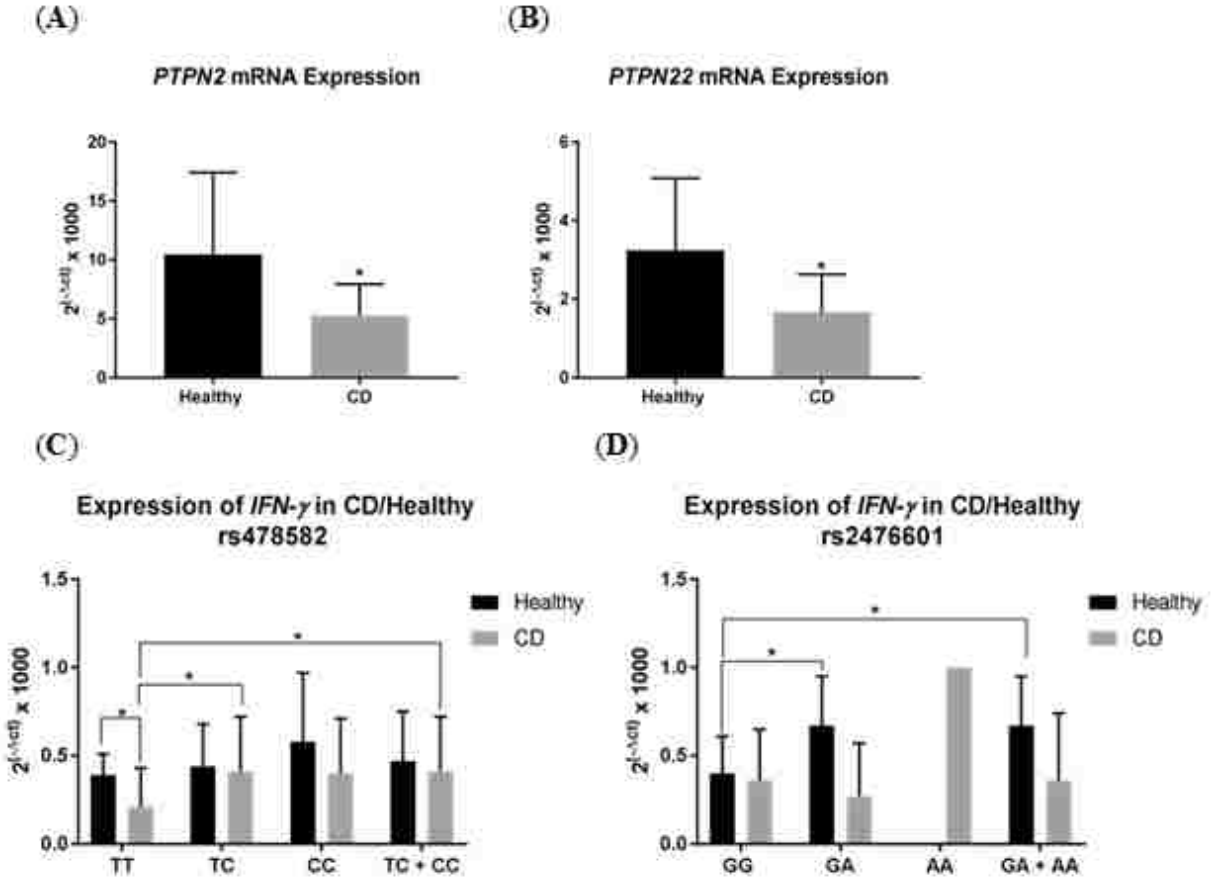

Figure 11. Relative mRNA Expression ($2^{(-\Delta CT)} \times 1000$) of *PTPN2, PTPN22* and *IFN-γ*

Relative mRNA expression of *PTPN2* (**A**) and *PTPN22* (**B**) in CD and healthy control subjects. Relative mRNA expression of *IFN-γ* was correlated with CD and healthy control subjects with either *PTPN2:rs478582* (**C**) or *PTPN22:2476601* (**D**). *: $P < 0.05$.

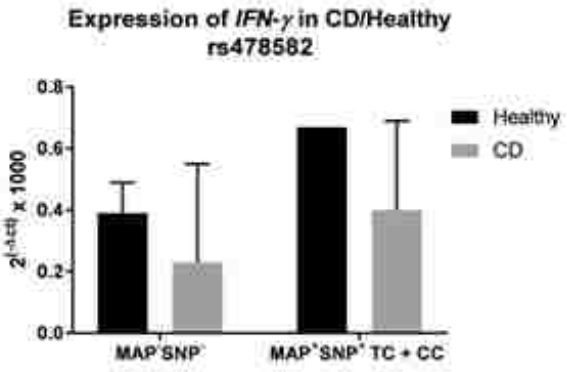

Figure 12. The Effect of Both *Mycobacterium avium* subspecies *paratuberculosis* and *PTPN2:rs478582* on *IFN-γ* Gene Expression in Crohn's Disease and Healthy Control Subjects

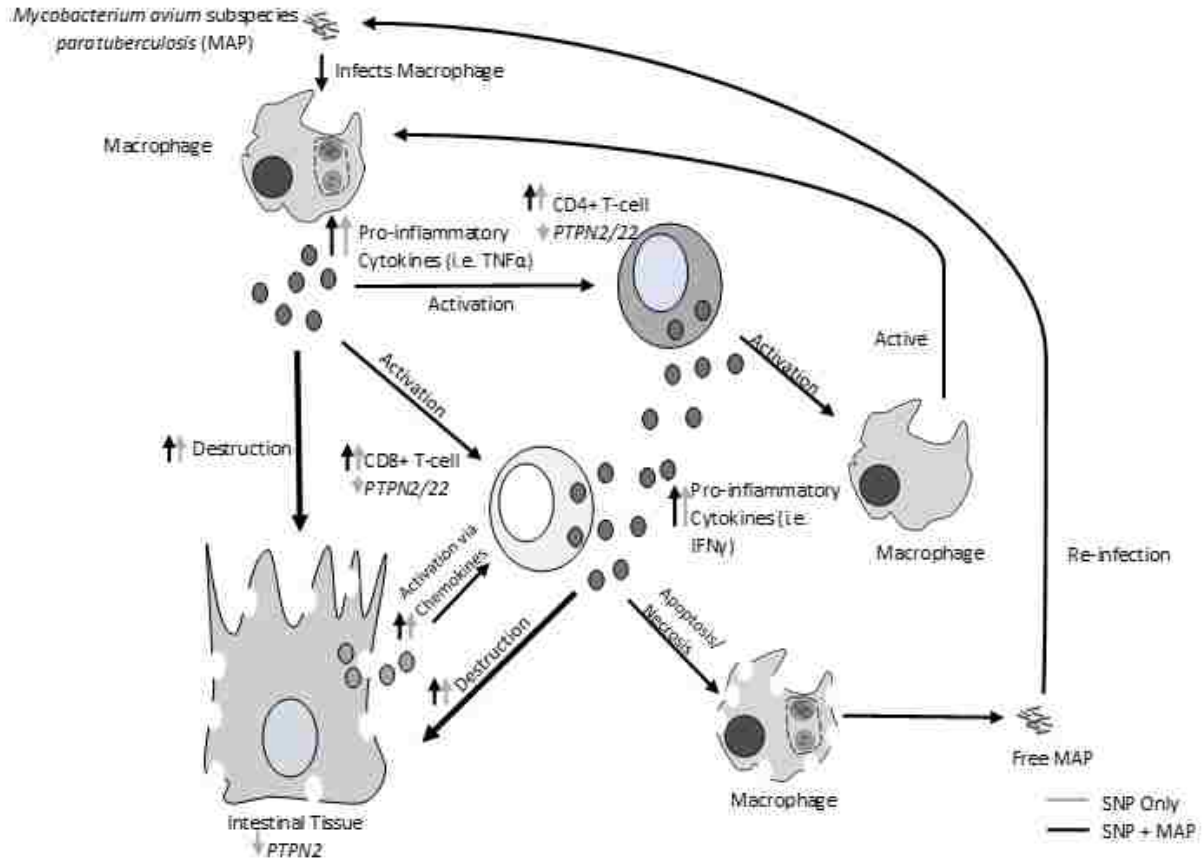

Figure 13. Complex Interaction of Crohn's Disease Pathophysiology

The effect of single nucleotide polymorphisms (SNPs) in *protein tyrosine phosphatase non-receptor type 2 and 22* (*PTPN2/22*) and *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in a dysregulated immune response in Crohn's disease (CD).

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CHAPTER FOUR: MULTIPLEX PCR AND FLUORESCENT IN SITU HYBRIDIZATION (FISH) COUPLED PROTOCOL FOR DETECTION OF PATHOGENS INVOLVED IN INFLAMMATORY BOWEL DISEASE PATHOGENESIS

Introduction

IBD, which consists of UC and CD, share a variety of different genetic factors, environmental triggers, and treatment plans [1-5]. Multiple recurring reports from us and others provided evidence that environmental triggers, including enteric pathogens, may cause IBD pathogenesis. Specifically, MAP, AIEC strain LF82, and *K. pneumoniae* have been implicated as causative agents in CD [5-12].

For instance, MAP was first isolated as the causative agent for Johne's disease, a CD-like enteritis in cattle [8,9,13,14]. MAP is an intracellular acid-fast pathogen that infects macrophages and dendritic cells and inhibits phagosome-lysosome fusion [15,16]. Interestingly, MAP was detected in the blood, milk and intestinal biopsies from patients with CD and most recently RA [5,6,8,9]. AIEC strain LF82 is a gram-negative bacillus pathogen that has also been isolated from intestinal tissue from patients with CD [10,12,17,18]. The LF82 strain of AIEC has been studied intensely due to its ability to infiltrate intestinal tissue and increase pro-inflammatory cytokines level in CD patients [10,12,17,18]. Like MAP, AIEC strain LF82 resists phagosome-lysosome fusion and acidification [10,12,17,18]. *K. pneumoniae* is a gram-negative, facultative anaerobic bacillus pathogen that causes pneumonia in immunocompromised patients [11,19]. Recent studies showed that *K. pneumoniae* is also associated with IBD pathogenesis [11,19]. *K. pneumoniae* colonizes the intestine of IBD patients with an imbalance in gut flora leading to elevated humoral immune response [11,19].

Although there are numerous studies investigating these pathogens individually in association with IBD, none of them has envisioned or examined the co-occurrence multiple pathogens in clinical samples obtained from CD or UC patients nor did they have a reliable, time saving diagnostic tool to attempt such endeavor. This limitation in the current literature prompted our group to develop a protocol to evaluate clinical samples for the presence of co-infection. We developed a multi-color FISH protocol, using pathogen-specific nucleotide probes and confocal scanning laser microscopy (CSLM). Additionally, we also developed a multiplex PCR technique based on a rapid modified DNAzol® extraction protocol and pathogens-based oligonucleotide primers. The multiplex PCR assay is coupled with the FISH protocol in order to verify results obtained from CSLM images. More importantly, multiplex PCR is evaluated for possible use in rapid testing of clinical samples for the presence of multiple pathogens in same tissue section.

Materials and Methods

Bacterial Cultures

A total of ten MAP strains, five other *Mycobacterium* species, and four non-*Mycobacterium* species were used in this study (Table 8). *Mycobacterium* species including MAP were cultured in BD Bactec™ MGIT™ Para-TB medium (Becton, Dickinson and Company©) tubes supplemented with 800 uL of Bactec™ MGIT™ Para-TB Supplement (Becton, Dickinson and Company©) at 37°C until optimal growth was achieved. *E.coli*, *Staphylococcus aureus* and *K. pneumoniae* were cultured in Luria broth (LB broth, Fisher Scientific®) at 37°C. *Listeria monocytogenes* was cultured in brain heart infusion broth (BHI broth, Fisher Scientific®) at 37°C.

Table 8. Bacteria Cultures Used in Study

Bacteria Species	Source
MAP Strain 1	Milk
MAP Strain 3	CD Tissue
MAP Strain 8B	CD Blood
MAP Para 18	ATCC 19698
MAP UCF3	CD Tissue
MAP UCF4	CD Tissue
MAP UCF5	CD Tissue
MAP UCF7	CD Tissue
MAP Linda	ATCC 43015
MAP MS137	CD Tissue
Mycobacterium smegmatis (*M. smegmatis*)	ATCC 27199
Mycobacterium avium subspecies *avium* (*M. avium*)	ATCC 25291
M. avium JF7	HIV Blood
Mycobacterium xenopi (*M. xenopi*)	ATCC 19971
Mycobacterium fortuitum subspecies *fortuitum* (*M. fortuitum*)	ATCC 23031
Escherichia coli (*E.coli*)	ATCC 8739
Staphylococcus aureus (*S.aureus*)	ATCC 25932
Klebsiella pneumoniae (*K. pneumoniae*)	ATCC 13883
Listeria monocytogenes (*L. monocytogenes*)	ATCC 19112

Bacteria Species	Source
MAP: *Mycobacterium avium* subspecies *paratuberculosis*	

Intestinal Tissue

Intestinal tissue from one UC (RS1) and from five CD (RS2, RS3, RS4, RS5, RS6) patients were used in this study. Tissue samples were stored at -80°C in Dr. Saleh A. Naser's laboratory and were obtained following the University of Central Florida Institutional Review Board #IRB00001138 approval.

DNA Extraction

Preparation of Cell Pellets/Intestinal Tissue Lysate for DNA Extraction

A volume of 1 mL of bacterial culture in 1.5 mL microcentrifuge tube was centrifuged at 13,000 RPMs for 2.5 min at room temperature. Supernatant was discarded and bacterial cell pellet was re-suspended in 500 uL of tris-EDTA buffer (TE buffer). For intestinal tissue, approximately 1 g tissue block was placed in tissue grinder (Precision™) with 1 mL of saline solution and was homogenized for 15 min. The tissue homogenate was then added to a lysing matrix B tubes (MP Biomedicals©) and was subjected to sonication using FastPrep FP120 Cell Disrupter at 6.0 m/sec for 30 sec in a (Thermo Savant™). The lysate was then centrifuged at 13,000 RPMs for 20 min. The supernatant was then removed from each tube and stored at -20°C until further use.

DNA extractions of both bacterial cell pellets and intestinal tissue lysate were performed following our modified DNAzol® (ThermoFisher Scientific®) DNA extraction protocol and our traditional phenol/chloroform/isoamyl DNA extraction method as described previously [5,6,8].

Modified DNAzol® DNA Extraction Protocol

Each tube containing bacterial culture pellet or intestinal tissue lysate in 500 uL of TE was subjected to DNA extraction by a protocol that utilizes DNAzol® as previously described [5,6]. Briefly, a total of 1.0 mL of DNAzol® was added to bacterial culture pellets or intestinal tissue lysates suspended in 500 uL TE. After mixing, a 400 uL of 100% isopropanol was added to each tube and then incubated for 15 min at room temperature. Following centrifugation at 8,000 RPMs for 6 min, the supernatant was discarded and DNA pellets were then washed with 500 uL of DNAzol® at 8,000 RPMs for 5 min. DNA pellets were then washed again in 1.0 mL of 75% ethanol at 8,000 RPMs for 5 min and then dried *via* a speedvac for 5 min. Dried DNA pellets were then dissolved in 50 uL of TE buffer and stored at -20°C until further use.

Phenol/Chloroform/Isoamyl DNA Extraction Protocol

Each tube containing bacterial culture pellet or tissue lysate in 500 uL of TE was subjected to DNA extraction by a protocol that utilizes phenol/chloroform/isoamyl alcohol as previously described [8]. Briefly, tubes were incubated in a heat block for 30 min at 100°C and then placed on ice for 15 min. Tubes were then centrifuged at 12,000 RPMs at 4°C for 10 min. Supernatants were transferred into 2.0 mL Phase Lock Gel™ tubes (Fisher Scientific®) and then mixed with 200 uL of Phenol/Chloroform/Isoamyl-Alcohol (Fisher Scientific®). Tubes were centrifuged at 12,000 RPMs at 4°C for 5 min, where supernatants transferred into new 1.5 mL microcentrifuge

tubes containing 100% chilled ethanol and stored at -20°C overnight. Next day, tubes are thawed and centrifuged at 12,000 RPMs at 4°C for 10 min and the supernatants discarded. DNA pellets were washed with 80% chilled ethanol, dried in a speedvac for 15 min and re-suspended in 50 uL TE buffer for storage at -20°C until further use.

Validation of DNAzol® Extraction Method by MAP IS900 nPCR

MAP-specific nPCR based on *IS900* derived oligonucleotide primers (Table 9) was used to evaluate the efficiency of the modified DNAzol® protocol compared to the phenol/chloroform/isoamyl alcohol protocol. In the first round, the PCR reaction consisted of 25 uL master mix (2x solution containing *Taq* DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers, Promega©), 5 uL betaine (Sigma-Aldrich©), 1 ul of P90 and 1 ul of P91 oligonucleotide primers, 8 uL of millipore H_2O, and 10 ul of DNA. The PCR cycling conditions were: 95°C for 5 min; 35 cycles of 95°C for 60 sec, 58°C for 90 sec, and 72°C for 90 sec; and a final extension of 72°C for 10 min. For the second round of PCR, the same reagents were used from the first round with 5 uL of P90/P91 product and AV1/AV2 oligonucleotide primers. The PCR cycling conditions were: 95°C for 5 min; 35 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 60 sec; and a final extension of 72°C for 10 min. Amplified DNA was then analyzed on a 2% agarose gel, and a 298 bp band was considered positive for MAP. DNA from MAP clinical strain UCF4 was used a positive control. The negative control consisted of all reagents except DNA.

Table 9. Nucleotide Primers Used in *IS900* nPCR, Multiplex PCR and FISH Probes

Bacterial Gene Target	nPCR Primers	Multiplex PCR Primers	FISH Probe
Mycobacterium avium subspecies *paratuberculosis* (MAP) *IS900*	P90: 5'-GTTCGGGGCCGTCGCTTAGG-3' (BLAST E-value: 2e-04) P91: 5'-GAGGTCGATCGCCCACGTGA-3' (BLAST E-value: 2e-04) AV1: 5'-ATGTGGTTGCTGTGTTGGATGG-3' (BLAST E-value: 1e-05) AV2: 5'-CCGCCGCAATCAACTCCAG-3' (BLAST E-value: 5e-04)	AV1: 5'-ATGTGGTTGCTGTGTTGGATGG-3' (BLAST E-value: 1e-05) AV2: 5'-CCGCCGCAATCAACTCCAG-3' (BLAST E-value: 5e-04)	5'-AF488 ATGTGGTTGCTGTGTTGGATGG-3' (BLAST E-value: 1e-05)

Bacterial Gene Target	**nPCR Primers**	**Multiplex PCR Primers**	**FISH Probe**
Mycobacterium avium complex (MAC) *IS1311*	NA	Forward: 5'-AAACGACCAAGGATCACTACCGAG-3' (BLAST E-value: 1e-06) Reverse: 5'-GTCGAGGAACACATACGGGAAGT-3' (BLAST E-value: 4e-06)	NA
Non-pathogenic *E.coli* *18s*	NA	Forward: 5'-CCGCATAACGTCGCAAGACC-3' (BLAST E-value: 6e-04) Reverse: 5'-CGTAGGAGTCTGGACCGTGTC-3' (BLAST E-value: 2e-04)	5'-AF647 GGTCTTGCGACGTTATGCGG-3' (BLAST E-value: 6e-04)
AIEC strain LF82 *GipA*	NA	Forward: 5'-GCTGTGTGCGCTTCGTCTAC-3' (BLAST E-value: 4e-08) Reverse: 5'-GATGGTAATTCTCGACTCCAGCGA-3' (BLAST E-value: 2e-07)	5'-AF565 GTAGACGAAGCGCACACAGC-3' (BLAST E-value: 4e-08)

Bacterial Gene Target	nPCR Primers	Multiplex PCR Primers	FISH Probe
K.pneumoniae *23s*	NA	Forward: 5'-TGGCAGTCAGAGGCGATGAAG-3' (BLAST E-value: 1e-04) Reverse: 5'-CTTTCCCTCACGGTACTGGTTCA-3' (BLAST E-value: 0.002)	5'-AF546 CTTCATCGCCTCTGACTGCCA-3' (BLAST E-value: 0.001)
BLAST E-value: Basic Local Alignment Search Tool Expected Value			

Development of Multiplex PCR

All oligonucleotide primers were designed and then purchased from Eurofins Genomics© (Table 9). Briefly, 10 uL of DNA containing 17 ng/uL of bacterial DNA or 50 ng/uL tissue DNA were added into a 200 uL-microcentrifuge tube containing 25 uL of PCR Master Mix (2x solution containing *Taq* DNA polymerase, dNTPs, MgCl2 and reaction buffers, Promega©), 5 uL of betaine (Sigma-Aldrich©), and 1 uL of each oligonucleotide primer (10 uM forward and 10 uM reverse primer for each bacterial species (MAP, MAC, non-pathogenic *E. coli*, AIEC strain LF82, and *K. pneumoniae*). The PCR cycling conditions were: 95°C for 5 min; 35 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 60 sec; and a final extension of 72°C for 10 min. The products of the multiplex PCR were analyzed on a 3% agarose gel, where the following base pairs bands are considered positive for the bacterial species tested: 171 bp (non-pathogenic *E.coli*), 298 bp (MAP), 357 bp (AIEC strain LF82), 493 bp (*K. pneumoniae*), and 543 bp (MAC). For the bacterial DNA positive controls, all tested bacterial species were first separated into individual tubes and underwent DNA extraction/multiplex PCR. Once successful multiplex PCR was done on the bacterial DNA positive controls individually, all tested bacterial species were then added into one tube and then underwent DNA extraction/multiplex PCR. The negative control for the multiplex PCR step that was used had all of the necessary PCR reagents except for the DNA.

Development of Fluorescent in situ Hybridization (FISH) for Imaging for Gut Bacteria

Preparation of Bacterial Slides for FISH

A volume of 1 mL of bacterial culture in 1.5 mL microcentrifuge tube was centrifuged at 13,000 RPMs for 2.5 min at room temperature. The supernatant was removed and the culture pellet was washed with 500 uL of TE and then centrifuged again at 13,000 RPMs for 2.5 min at room temperature. After centrifugation, the supernatant was removed and the culture pellet was re-suspended in 100 uL of TE. Of which, 20 uL suspension was placed on each slide, air dried and then heat fixed. Slides were then incubated in 4% paraformaldehyde (PFA, Fisher Scientific®) overnight at 4°C on a shaker at 50 rpm.

Preparation of Intestinal Tissue Slides for FISH

Intestinal tissue sections were obtained using previously established protocols [20,21]. In brief, PFA fixed tissue specimens (1 g each) were placed in perforated cassettes and immersed in ascending concentrations of ethanol (70%, 90%, and 100%) on a Leica processing system (TP 1020) for dehydration followed by clearing in (Xylene 50:Ethanol 50), and 100% Xylene solutions. Next, intestinal tissue was embedded in melted paraffin (60^{o}C) and allowed to solidify to 4^{o}C. Solidified blocks were then cut using tissue microtome (HM 325 Microm; Medical Equipment Source) into consistent 5 um thickness serial tissue sections and placed on Colorfrost Plus microscope slides (Fisher Scientific®). Before histology evaluation, sections were put in 60^{o}C incubator for 30 minutes, and immersed into in xylene solution (Sigma-Aldrich©) for 10 min to remove extra wax and three separate washes with 100% ethanol, 95% ethanol, and 70% ethanol for 10 min each to rehydrate the tissue section. The tissue slides were air-dried after

removal of paraffin, and incubated overnight at 4°C with 4% PFA (Fisher Scientific®) on a shaker.

Bacterial FISH on Bacterial Slides and IBD Patient's Biopsy Slides

After fixation with 4% PFA, the slides were then washed three separate times in 1x phosphate buffer saline (PBS) for 10 min each on a shaker. Next, a solution comprising of 100 uL of 1% sodium dodecyl sulfate (SDS) with 2 uL of 20 mg/mL of Proteinase K (Thermo Scientific™) was added directly to the slides in hybridization chambers (Corning®). The slides in the hybridization chambers were then incubated at 55°C for 30 min. Inactivation of the Proteinase K was done by adding 200 uL of 0.2% glycine (Sigma-Aldrich©) to each slide and incubated for 3 min on a shaker. After inactivation, the slides were then washed three separate times in 1x PBS for 5 min each on a shaker. The slides were washed again in the following solutions for 1 min each on a shaker: 50% ethanol, 80% ethanol, 100% ethanol and then xylene. Next, the slides were washed once again with the following solutions for 1 min each on a shaker: 100% ethanol, 80% ethanol, and 50% ethanol. After the ethanol/xylene washes, the slides are then incubated in 1x PBS for 60 min on a shaker. The slides were then incubated in a pre-hybridization solution consisting of 2x saline-sodium citrate (SSC, Sigma-Aldrich©), 20% dextran sulfate (Fisher Scientific®), 50% formamide (Sigma-Aldrich©), 50 mM NaH_2PO_4 (Sigma-Aldrich©), and 1 mM EDTA (Fisher Scientific®) for 10 min at 50°C. After incubation in the pre-hybridization solution, the slides are then placed in hybridization chambers and 20 uL of hybridization solution (pre-hybridization solution without the 20% dextran sulfate) was added directly to the samples. The hybridization solution also included 3 uL of the 1 ug/uL oligonucleotide fluorescent probe per slide for the bacterial species being detected. Oligonucleotide probes used in this study are

listed in Table 9 and were purchased from Eurofins Genomics©. All FISH oligonucleotide probes were prepared by diluting probes to 1 ug/uL in TE buffer. After the hybridization solution was added to the slides in the hybridization chambers, the slides were then incubated for 60°C for 60 min and then 37°C overnight. After incubation overnight, the slides were then washed with the following solutions for 15 min each on a shaker: 2x SSC, 1x SSC, 0.3x SSC in 40°C water bath, and 0.3x SSC in room temperature in the dark. The slides were then washed three separate times with H_2O for 15 min each on a shaker and then air-dried in the dark. A solution of DAPI/mounting medium (Vectashield®) was added to the slides and were sealed with a slide cover. The slides were analyzed using confocal scanning laser microscopy (CSLM). The images created were analyzed on ImageJ (National Institute of Health).

Results

Specificity and Sensitivity between DNAzol® and Phenol/Chloroform/Isoamyl-Alcohol DNA Extraction Protocols

Comparison between DNAzol® and phenol/chloroform/isoamyl-alcohol DNA extraction techniques was done by examining the specificity and sensitivity of *IS900* nested PCR (nPCR) on various bacterial cultures. This was done in order to evaluate the new, modified DNAzol® DNA extraction protocol. Specificity of the techniques wase done by comparing both DNA extraction protocols on a variety of different bacterial species and MAP strains after *IS900* nPCR. Based on the analysis of the 2% agarose gel and the 298 bp target sequence shown in Figure 1A and 1B, the *IS900* nPCR is confirmed to be specific to the MAP strains and not to the other bacterial species. When examining the specificity of the *IS900* nPCR between the two DNA extraction protocols, the modified DNAzol® (Figure 14A, Gel II) showed more intense

DNA bands than the phenol/chloroform/isoamyl-alcohol DNA protocol did after the nPCR was done (Figure 14B, Gel I).

Sensitivity of both the *IS900* nPCR and the DNA extraction techniques was done by comparing between the protocols on serial dilutions of MAP UCF4 culture and MAP UCF4 isolated DNA (Figure 14C and 14D). Based on the analysis of the 2% agarose gel and the 298 bp target sequence shown in Figure 14C, the *IS900* nPCR shows that the technique can detect a minimum of 2-3 colony forming units (CFU) of MAP culture. In addition, the *IS900* nPCR can detect MAP DNA at a minimum of up to 3.17 fg/uL-317 ag/uL (Figure 14D). When comping the *IS900* nPCR sensitivity between the two DNA extraction protocols, the modified DNAzol® protocol (Figure 14C, Gel II) detected lower amounts of MAP UCF4 CFU culture, while the phenol/chloroform/isoamyl-alcohol DNA extraction protocol (Figure 14D, Gel 1) detected lower amounts of pure MAP UCF4 DNA.

Multiplex PCR on Bacterial Species and IBD Patient Biopsy Samples

We engineered an all in one multiplex PCR protocol that facilitates the detection of multiple bacterial species in one single biopsy sample. For the positive controls, a tube with all of the tested bacteria species (*E.coli*, *K. pneumonia*, and MAP) were mixed and DNA was extracted for multiplex PCR. All positive controls had successful multiplex PCR reactions with either single set of bacterial gene primers alone or with all bacterial gene primers together in one tube (Figure 15). Due to laboratory biosafety level restrictions, a positive control bacterial culture for AIEC strain LF82 could not be obtained. In lieu of this, primers for AIEC strain LF82 that did not bind

onto the other bacterial cultures, such as non-pathogenic *E.coli*, should thus theoretically only amplify AIEC strain LF82 DNA. *Mycobacterium avium* complex (MAC) primers were used to confirm presence of *Mycobacterial* species in either positive controls or IBD patient samples.

After the establishment of the positive controls, two IBD biopsy tissues (RS1: UC patient and RS2: CD patient) were used for multiplex PCR (Table 10). As seen in Table 10 and Figure 16, both non-pathogenic *E.coli* (171 bp) and *K. pneumoniae* (493 bp) were detected in both RS1 and RS2 samples. MAP (298 bp) was only detected in RS2 and not RS1. AIEC strain LF82 (357 bp) was not detected in either of the samples.

Table 10. Multiplex PCR and FISH Results for IBD Patient Samples

Bacterial Species Detected	**Patient Sample**	
	RS1	**RS2**
Multiplex PCR		
Non-pathogenic E.coli	+	+
AIEC strain LF82	-	-
MAP	-	+
K. pneumoniae	+	+
MAC	-	-
FISH		
Non-pathogenic E.coli	+	+
AIEC strain LF82	-	-
MAP	-	+
K. pneumoniae	+	+

AIEC: Adherent-invasive *Escherichia coli*

MAP: *Mycobacterium avium* subspecies *paratuberculosis*

MAC: *Mycobacterium avium* complex

FISH: Fluorescent *in situ* hybridization

FISH Procedure on Bacterial Species and IBD Patient Biopsy Samples

A FISH protocol was created in order to visually identify multiple bacterial species in a single biopsy sample. Different probes were used for detecting four different bacterial species: non-pathogenic *E.coli* (AF647 fluorophore, blue fluorescence), AIEC strain LF82 (AF568 fluorophore, yellow fluorescence), MAP (AF488 fluorophore, green fluorescence), and *K. pneumoniae* (AF546 fluorophore, magenta fluorescence) (Figure 17). For positive controls, bacterial cultures were heat-fixed on microscope slides and were treated with their respected FISH probes. Since no positive control could be used for the AIEC strain LF82 bacterial cultures, non-pathogenic *E.coli* was used to determine negative binding of the probes. Gram stains (Figure 17, a-d) and acid-fast stains (Figure 17, e-h) were done to confirm bacteria were heat-fixed on microscope slide. Overall, all FISH probes were successful in binding onto their respective bacterial species and did not have any cross-reactivity with each other (Figure 17 i-l).

For the IBD patient biopsy samples (RS1 and RS2), individual FISH probes were used along with DAPI (red fluorescence) to stain both the targeted bacteria and the tissue (Figure 18). As with the multiplex PCR, FISH for non-pathogenic *E.coli* and *K. pneumoniae* showed positive signaling in RS1 and RS2 tissue biopsies (Table 10, Figure 18A, a and e; Figure 5D, d and h), while only MAP was found in the RS2 sample (Table 10, Figure 18C, c and g). The AIEC strain LF82 bacterial species was not detected in either RS1 or RS2 tissue biopsies (Table 10, Figure 18B, b and f).

Detection of Both Non-Pathogenic E.coli and MAP Coinfection in CD Patient Biopsy Samples Using FISH

After optimization of the FISH protocol, we used two set of probes together to attempt to identify the presence of multiple bacterial species in individual CD patient biopsy samples (RS3, RS4, RS5, RS6). As seen in Figure 19, the tissues were stained with DAPI (blue fluorescence, Figure 19, a, e, i, m) and treated with both non-pathogenic *E.coli* (AF657 fluorophore, red fluorescence, Figure 19, b, f, j, n) and MAP (AF488 fluorophore, green fluorescence, Figure 19, c, g, k, o) FISH probes on four different CD patient biopsy slides. For RS3 and RS4, both non-pathogenic *E.coli* and MAP bacteria were successfully found together in the patient biopsy slides (Figure 19A and Figure 19B). Both images (Figure 19A, d and Figure 19B, h) showed positive signals from both bacterial probes coincide with each other along with DAPI signaling in a single section of the biopsy samples. For RS5 and RS6, only one bacterial signal was detected in each of the biopsies. RS5 (Figure 19C, l) had only positive signaling for MAP, while RS6 (Figure 19D, p) had only positive signaling for non-pathogenic *E.coli*.

Discussion

Recent studies have strongly supported the role of microbial infection in IBD development [7-12]. It is still unclear which pathogen is found more readily in either UC or CD patients, where recent studies are more focused on detecting one pathogen at a time [8,10,11,18,19]. In this study, the investigation of the presence of multiple pathogens including MAP, *E. coli* strains, and *K. pneumoniae,* was done to fully understand IBD pathogenesis.

The development of a multiplex PCR protocol coupled with a FISH detection protocol was done, which together can detect multiple bacterial pathogens in a single sample. Along with this, a

newly modified DNA extraction protocol was used in order to process the samples faster (~1 hour) than previously used techniques (2-3 days) [5,6,8]. Overall, combining all three protocols has produced a faster, more efficient way into detecting multiple bacterial species in one test sample.

The effectiveness of the two DNA extractions techniques that were used in this study was established, where the modified DNAzol® technique showed similar specificity and sensitivity to the "traditional" phenol/chloroform/isoamyl-alcohol DNA extraction technique (Figure 14). The modified DNAzol® DNA extraction technique however has the advantage over the "traditional" DNA extraction due to the simplistic and the less time consuming protocol. This can lead to more samples being processed and could potentially be used in a clinical setting due to the cost-effectiveness of the modified protocol.

The multiplex PCR was able to detect multiple bacterial DNA individually and when they were all mixed in one sample tube (Figure 15 and Figure 16). Overall, the data shows that all the bacterial culture positive controls did have positive multiplex PCR reactions with both respected individual bacterial DNA primers in separate tubes or with all bacterial DNA primers in one tube (Figure 15). This demonstrated that the multiplex PCR protocol that was created was successful and thus was used on IBD patient samples (Table 10 and Figure 16). The use of the FISH probes derived from the multiplex PCR primers confirmed the results of the both the positive controls and the IBD patient biopsy samples' results (Figure 17 and Figure 18).

The IBD patients multiplex PCR and FISH analysis showed that both RS1 (UC patient) and RS2 (CD patient) had both non-pathogenic *E. coli* and *K. pneumoniae*, but, RS1 was not positive for MAP, unlike RS2 (Table 10, Figure 16, Figure 18C). For the MAP multiplex PCR and FISH results for RS1 and RS2, it has been previously shown that a majority of CD patients do have a higher association with MAP infections than UC patients, which was confirmed again in this study [5,6,8,9,13,22]. Astonishing, both multiplex PCR and FISH did not detected the AIEC strain LF82 in the two IBD patients samples, where previous studies have shown that this bacteria has been associated with IBD pathogenesis (Table 10, Figure 16 and Figure 18B) [7,10,12,18]. This could be due to the small sample size in this study, but also could be due to the possibility that the *gipA* gene being amplified in the AIEC strain LF82 could be a low copy gene target [23,24]. The virulent gene *gipA* was chosen as the target gene for amplification in AIEC strain LF82 is due to its association with AIEC infection in Peyer's patches of CD patients [23,24].

When comparing the multiplex PCR DNA bands and the FISH signaling of non-pathogenic *E.coli* with the *K.pneumoniae* in both patient samples, it is evident that the influx of commensal *E.coli* does play a role in *K.pneumoniae* presence (Figure 16 and Figure 18A and 18D). The data suggests that the more *K.pneumoniae* is present in a patient sample, the less non-pathogenic *E.coli* is present. This could suggest and confirm that *K.pneumoniae* infections can occur if there is a dysfunction of the microbiome in IBD patients [11,19,25,26]. With the increasing influx of *K.pneumoniae* in the CD patient more so in the UC patient, it could be a potential pathogen to investigate for CD pathogenesis studies.

The FISH assay with the four other CD patients (RS3-RS6) that were examined in this study showed that it is possible to visualize multiple bacterial species in a single biopsy sample (Figure 19). When FISH probes for both non-pathogenic *E.coli* and MAP were used together on the biopsy samples, it was evident that signaling from both bacterial species was shown. Further testing with different combinations of FISH probes for different bacterial species together on individual biopsy samples need to be done to finally elucidate which bacterial pathogen is more predominant in IBD pathogenesis.

This study was done as a pilot study in order to verify and examine these newly developed protocols, and as such, more IBD patient samples are required for future examination. Overall, this study shows that the protocols created to detect multiple pathogens was successful and that the data suggest that bacterial presence is potentially different among UC and CD pathogenesis.

Figures

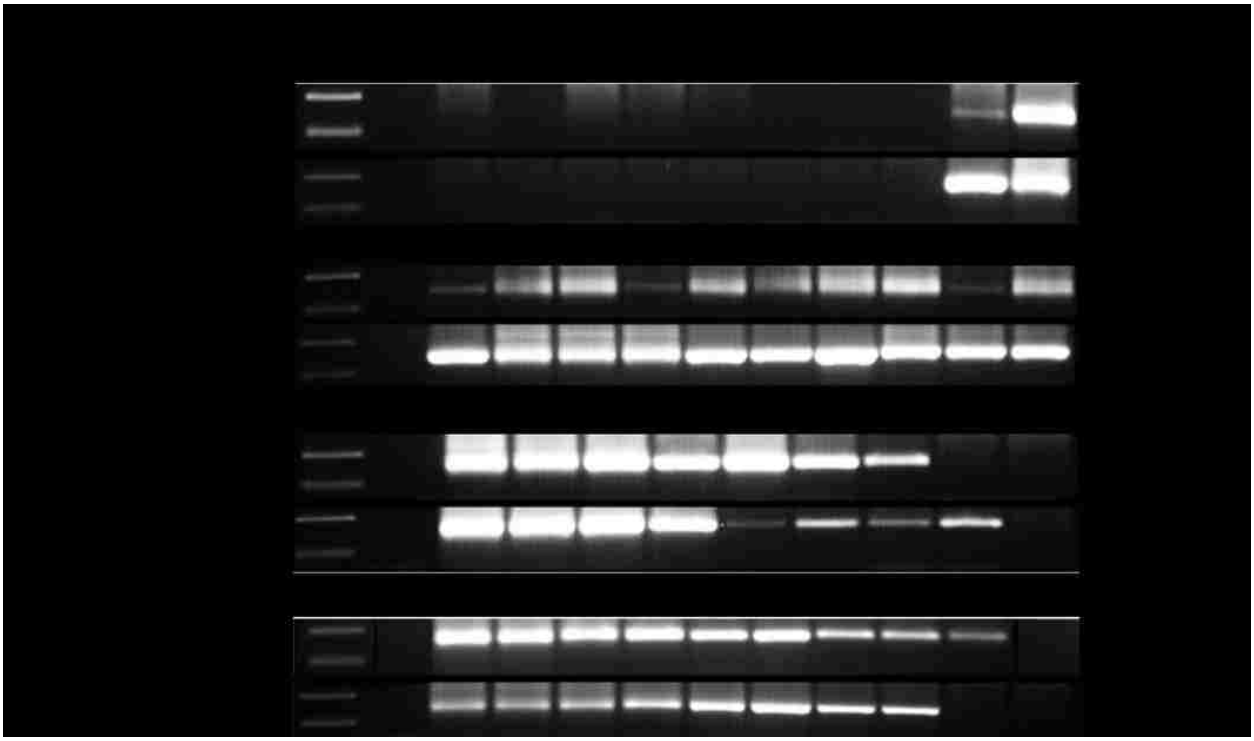

Figure 14. Comparison of Specificity and Sensitivity of *IS900* nPCR between DNAzol® and Phenol/Chloroform/Isoamyl-Alcohol DNA Extraction Protocols

Gel images comparing between the specificity and sensitivity of the *Mycobacterium avium* subspecies *paratuberculosis* (MAP) *IS900* nested polymerase chain reaction (nPCR) after DNA extraction from either phenol/chloroform/isoamyl-alcohol DNA extraction (**Gel I.**) or modified DNAzol® DNA extraction (**Gel II.**). Figure **1A** shows specificity of the nPCR: M: DNA marker; N: Negative control; 1: *E.coli*; 2: *S.aureus*; 3: *L. monocytogenes*; 4: *K. pneumoniae*; 5: *M. smegmatis*; 6: *M. avium*; 7: *M. xenopi*; 8: *M. fortuitum*; 9: *M. avium* JF7; +: MAP UCF4. Figure **1B** shows further specificity of the nPCR: M: DNA marker; N: Negative control; 1: MAP Strain 1; 2: MAP Strain 3; 3: MAP Strain 8B; 4: MAP Para 18; 5: MAP UCF3; 6: MAP UCF5; 7: MAP UCF7; 8: MAP Linda; 9: MAP MS137; +: MAP UCF4. Figure **1C** shows sensitivity of the nPCR on serial dilutions of MAP UCF4 CFU: M: DNA marker; N: Negative control; 1: 5.42 x

10^6 CFU; 2: 5.42 x 10^5 CFU; 3: 5.42 x 10^4 CFU; 4: 5.42 x 10^3 CFU; 5: 5.42 x 10^2 CFU; 6: ~50 CFU; 7: ~5 CFU; 8 & 9: ~2-3 CFU. Figure **1D** shows sensitivity of the nPCR on serial dilutions of MAP UCF4 DNA: M: DNA marker; N: Negative control; 1: 31.7 ng/uL; 3.17 ng/uL; 3: 317 pg/uL; 4: 31.7 pg/uL; 5: 3.17 pg/uL; 6: 317 fg/uL; 7: 31.7 fg/uL; 8: 3.17 fg/uL; 9: 317 ag/uL; 10: 31.7 ag/uL.

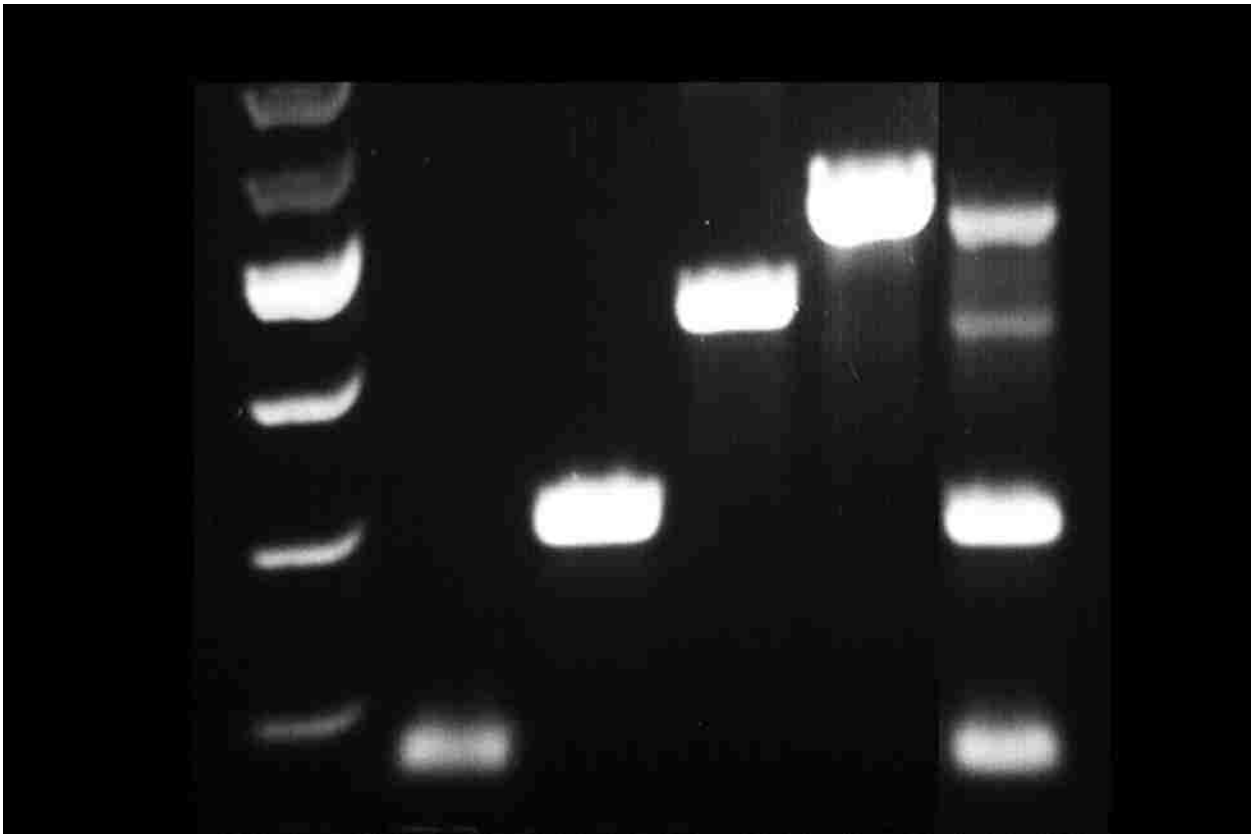

Figure 15. Multiplex PCR for Bacterial Positive Controls

Multiplex polymerase chain reaction (PCR) was done on a tube of bacterial cultures mixed together (*Escherichia coli* (*E.coli*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Mycobacterium avium* subspecies *paratuberculosis* (MAP)). M: DNA Marker; 1: *E.coli* primers only (171 bp); 2: MAP primers only (298 bp); 3: *K. pneumoniae* primers only (493 bp); 4: *Mycobacterium avium* complex (MAC) primers only (534 bp); 5: all primers together (171 bp, 298 bp, 493 bp, 534 bp).

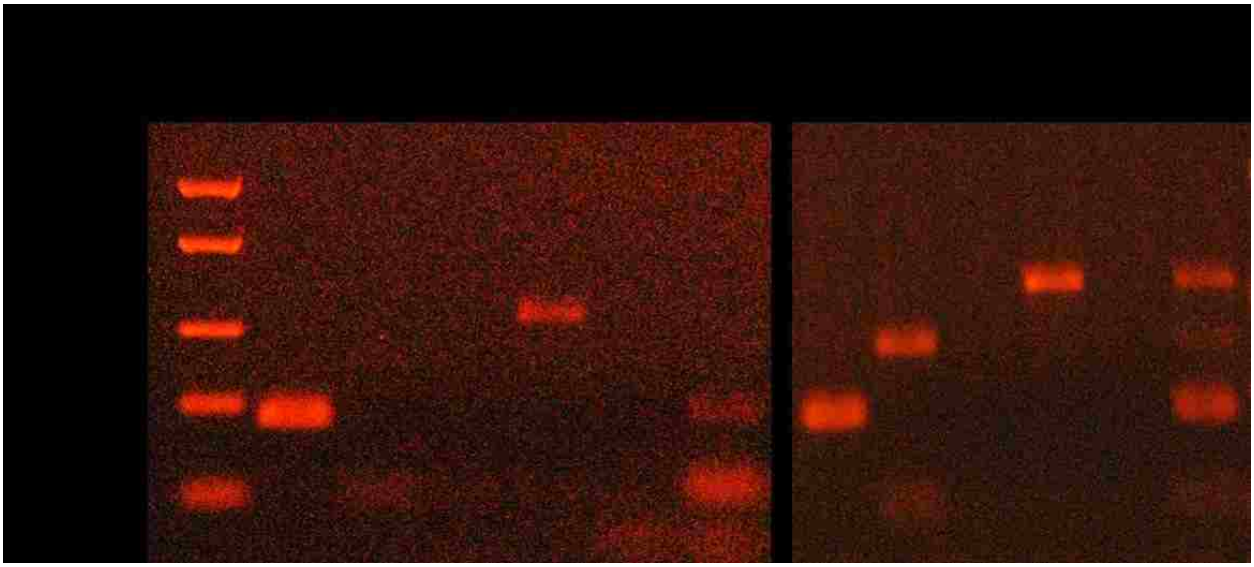

Figure 16. Multiplex PCR for IBD Patient Biopsy Samples

Multiplex polymerase chain reaction (PCR) was done on two inflammatory bowel disease (IBD) patients (RS1 and RS2). RS1: ulcerative colitis (UC) patient; RS2: Crohn's disease (CD) patient; M: DNA Marker; 1: *Escherichia coli* (*E.coli*) primers only (171 bp); 2: *Mycobacterium avium* subspecies *paratuberculosis* (MAP) primers only (298 bp); 3: adherent-invasive *Escherichia coli* (AIEC) strain LF82 primers only (357 bp); 4: *Klebsiella pneumoniae* (*K. pneumoniae*) primers only (493 bp); 5: *Mycobacterium avium* complex (MAC) primers only (534 bp); 6: all primers together (171 bp, 298 bp, 357 bp, 493 bp, 534 bp).

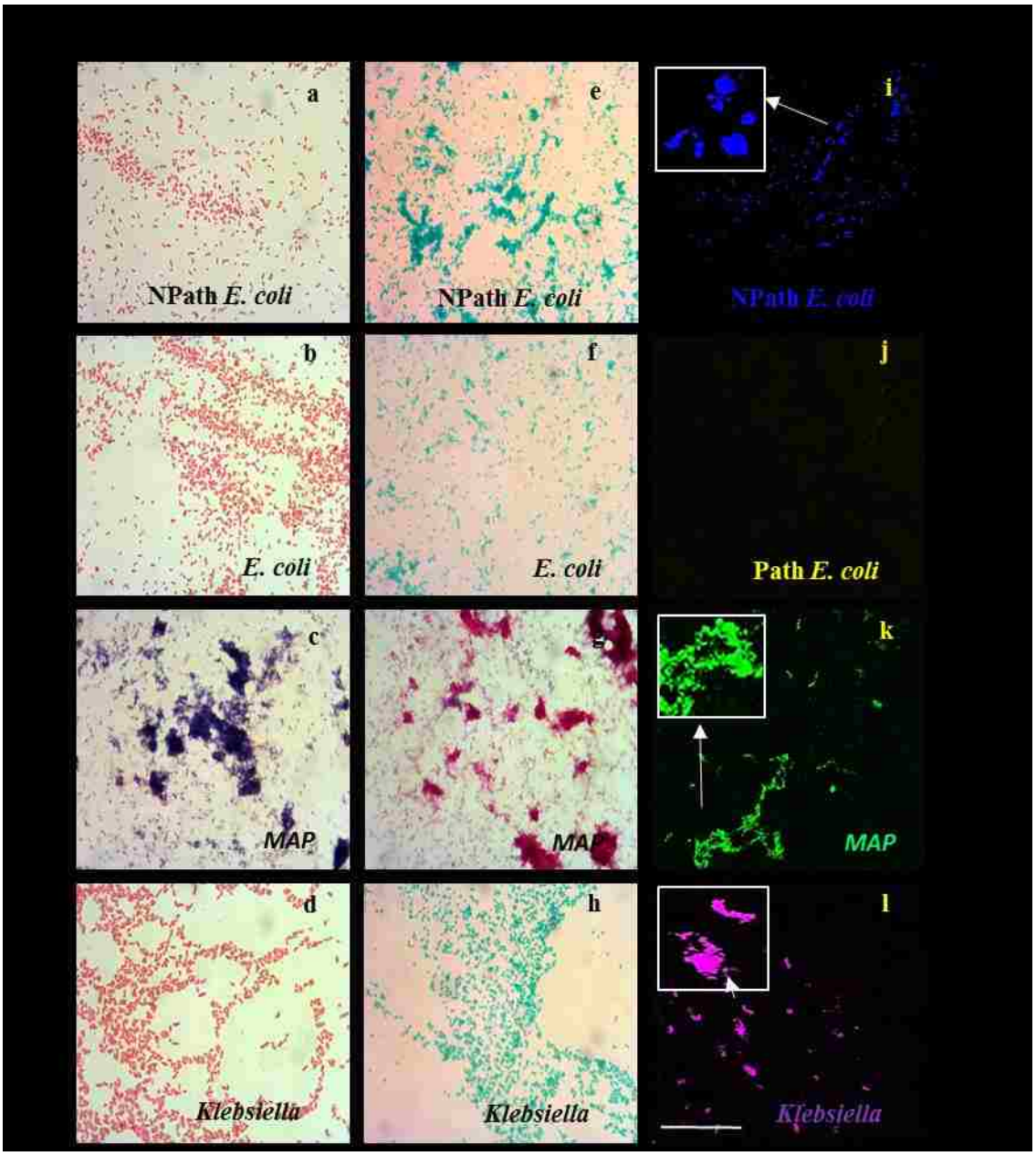

Figure 17. Gram Stain, Acid-Fast Stain and FISH for Bacterial Cultures

Gram stain (**a-d**), Acid-Fast stain (**e-h**), and fluorescent *in situ* hybridization (FISH) (**i-l**) images of the following bacterial cultures: **A**: *Escherichia coli* (*E.coli*) with non-pathogenic *E.coli* FISH probe; **B**: *E.coli* with adherent-invasive *Escherichia coli* (AIEC) strain LF82 FISH probe; **C**:

Mycobacterium avium subspecies *paratuberculosis* (MAP) with MAP FISH probe; **D**: *Klebsiella pneumoniae* (*K. pneumoniae*) with *K. pneumoniae* FISH probe.

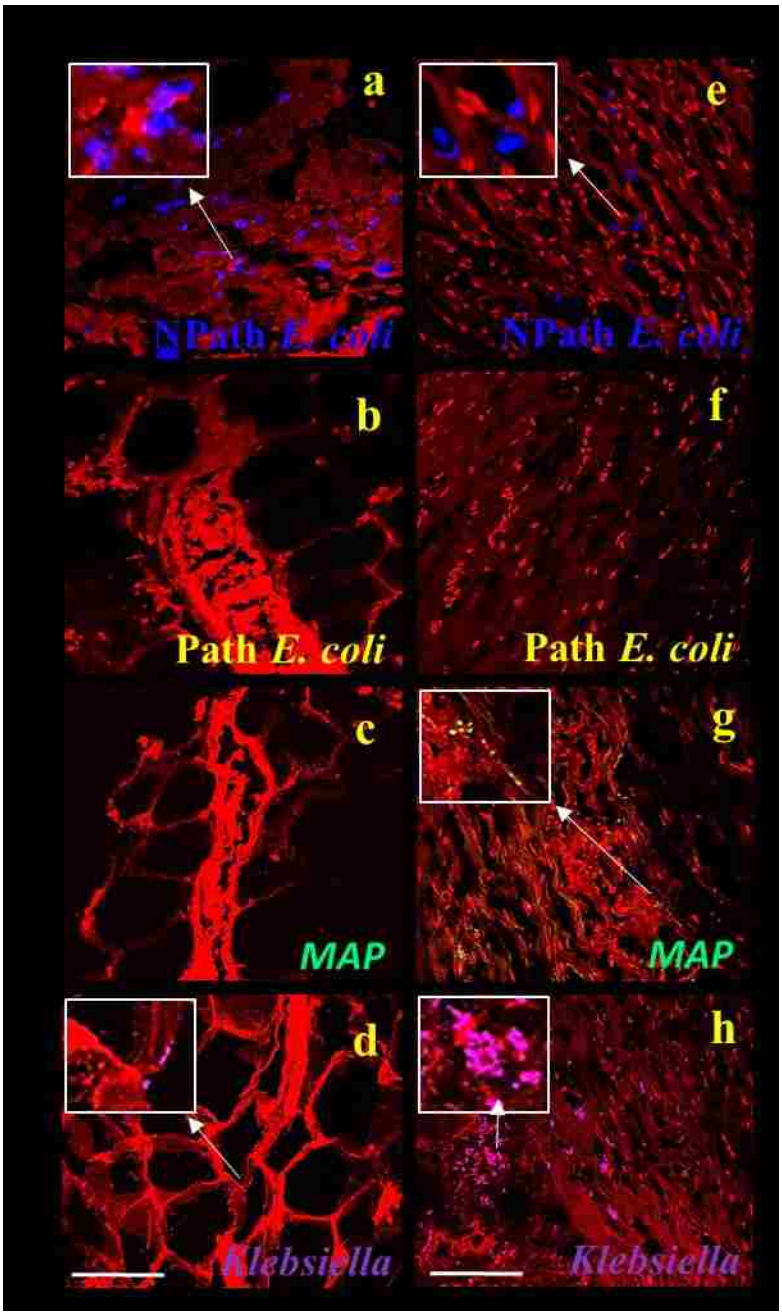

Figure 18. Detection of Bacterial Species via FISH in IBD Patient Biopsy Samples

Fluorescent *in situ* hybridization (FISH) images for two IBD patient biopsy samples (RS1: ulcerative colitis (UC) patient; RS2: Crohn's disease (CD) patient) stained with DAPI (red fluorescence) and treated with FISH bacterial probes. **A**: Non-pathogenic *Escherichia coli* (*E.coli*) FISH probe (**a and e**); **B**: adherent-invasive *Escherichia coli* (AIEC) strain LF82 FISH

probe (**b and f**); **C**: *Mycobacterium avium* subspecies *paratuberculosis* (MAP) FISH probe (**c and g**); **D**: *Klebsiella pneumoniae* (*K. pneumoniae*) FISH probe (**d and h**).

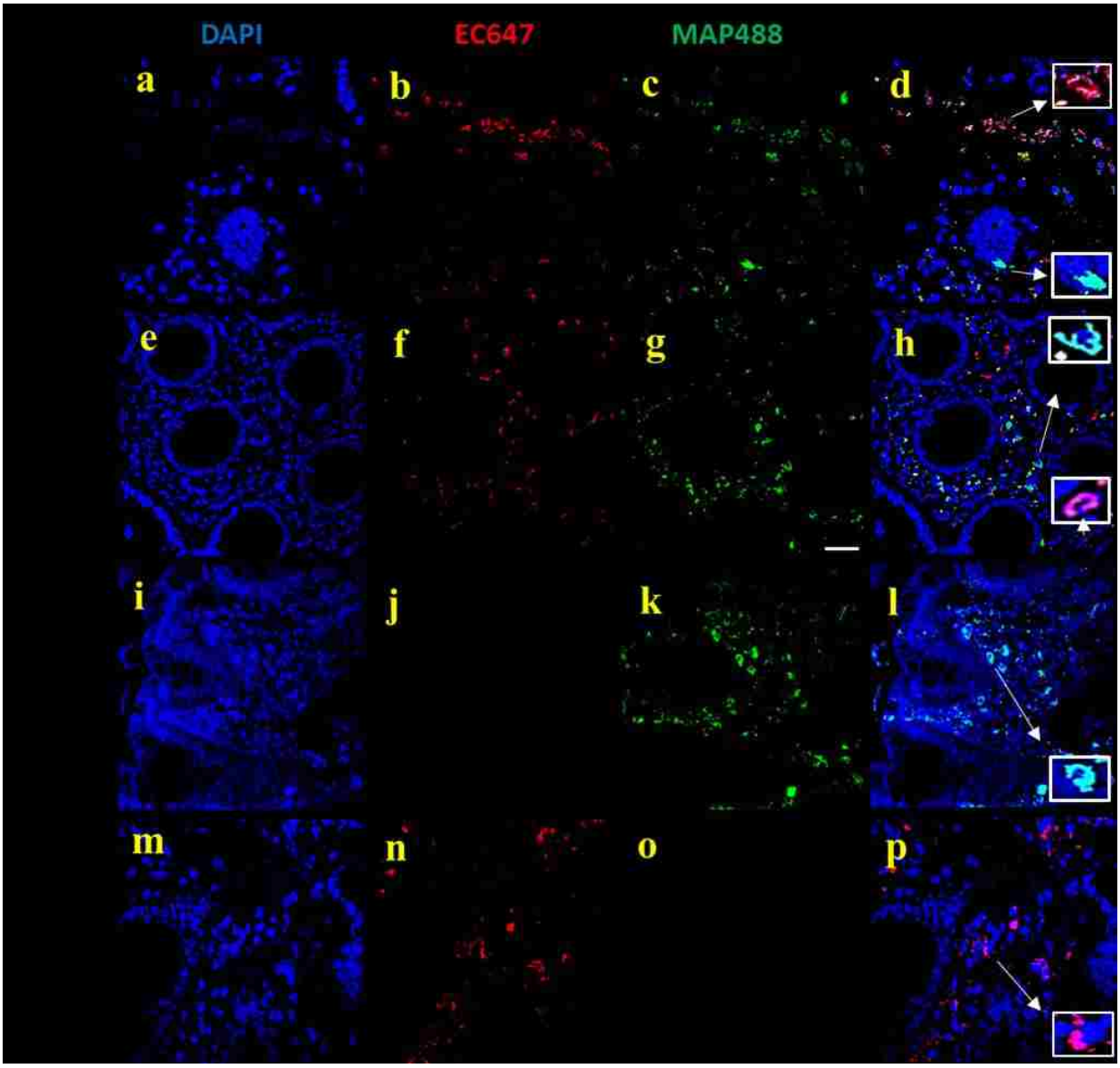

Figure 19. Detection of Multiple Bacterial Species via FISH in CD Patient Biopsy Samples

Fluorescent *in situ* hybridization (FISH) images for four CD patient biopsy samples: **A**: RS3, **B**: RS4, **C**: RS5, and **D**: RS6. DAPI (**a, e, i, m**), non-pathogenic *Escherichia coli* (*E.coli*) FISH probe (**b, f, j, n**), and *Mycobacterium avium* subspecies *paratuberculosis* (MAP) FISH probe (**c, g, k, o**) were used together on single CD patient biopsy samples. Merged images were created (**d,**

h, l, p) that overlapped the DAPI with the *E.coli* and MAP FISH probes signaling into a single image for each CD patient biopsy sample.

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CHAPTER FIVE: CONCLUSION/FUTURE DIRECTIONS

Knowledge of the pathophysiology of autoimmune diseases, such as RA and CD, is vital in the development of new diagnosis techniques and new treatment options for these diseases. In this study, the examination of SNPs found in the negative immunoregulatory genes *PTPN2/22* and their effects on the immune system when introduced to a mycobacterial infection with MAP was done in order to investigate future targets for diagnosis/treatment for autoimmune diseases. With the significant SNPs (*PTPN2:rs478582* and *PTPN22:rs2476601*) that were found more prevalent in the autoimmune disease patients than the healthy controls, these SNPs could potentially be used as biomarkers for diagnosis of these diseases. This could also be said about the significance of MAP infection found in the autoimmune disease patients than the healthy controls.

SNPs in *PTPN2/22* and MAP infection could also be used as treatment targets due to both factors together showing immunoregulatory problems in autoimmune disease patients. Eradication of MAP with antibiotics, such as RHB-104, could potentially be used in patients that are found to be MAP positive *via* the *IS900* nPCR [1][2]. With the removal of the MAP infection with these antibiotics, there will be no environmental trigger that could potentially start the dysregulation of the immune response in genetically predisposed individuals, thus providing relieve of symptoms and possible remission in these patients. Also, with the knowledge that PTPN2/22 proteins could potentially be non-functional or have lower activation in patients with SNPs in the *PTPN2/22* genes, the ability to alieve the effects of these SNPs is crucial for treatment.

For both treatment for SNPs in *PTPN2/22* and MAP infection in autoimmune disease patients, a new type of treatment needs to found. Potentially, the use of exogenous polyamines or polyamine derivatives can be used to not only help with removing MAP infection in the body, but could also be used to alleviate the effects of SNPs in *PTPN2/22* [3][4][5][6][7][8]. Polyamines, which consist of mainly putrescine, spermidine, and spermine (Figure 20) are found in a majority of living tissues, microorganisms, and certain foods [6]. Naturally, polyamines are produced in a variety of cell types, where they play a role in cell growth and survival [6][7][8]. These polyamines have also been found to increase susceptibility to antibiotics in a variety of microorganisms when used at a high amount [3][4][5]. More specifically, polyamines have shown to enhanced susceptibility to antibiotics, such as rifampicin, to *Mycobacteria* species [5]. For polyamine studies on *PTPN2/22*, it is shown that these compounds enhance the effect of the phosphatase activity of PTPN2/22, where there is an increase in anti-inflammatory activity in tested cell lines [7][8].

With the promise of an anti-microbial effect on mycobacterial infections and the anti-inflammatory effect on the immune system, polyamine treatment could be a possibility for RA and CD patients with both SNPs in *PTPN2/22* and MAP infection (Figure 21). Future studies on the effect of polyamines on T-cell activity and tissue apoptosis from patient samples with SNPs in *PTPN2/22* need to be done in order to examine if polyamines can alleviate the effect of these SNPs. Along with this, examination of the increase of susceptibility to antibiotics like RHB-104 to MAP when paired with polyamines need to be examined as well.

Figures

Figure 20. Role of Polyamines in Humans

Polyamines are found in a majority of living tissues, where they have a wide variety of effects on the body. These effects range from immune modulation, autophagy inducing, and cardio protection.

Figure 21. Potential Use of Polyamines in Autoimmune Disease Patients

Future directions for the use of polyamines on patient samples with SNPs in *PTPN2/22* is to induce T-cells and tissue cells from these patients with putrescine, spermidine, and spermine to see potential decrease in PTPN2/22 activity. This should lead to decrease activity of the T-cells and decrease apoptosis occurring in the patient samples. Also, MAP cultures will be treated with both RHB-104 and the polyamines together to see if there is an increase of antibiotic susceptibility to MAP than with RHB-104 alone.

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APPENDIX: CONSENTS FOR PUBLICATIONS

We, the authors, give our permission to include data and materials described in Sharp et. al. 2015 (below) in the dissertation contents of Mr. Robert C. Sharp for Doctor of Philosophy in Biomedical Sciences at the University of Central Florida.

Article Title: Genetic variations of *PTPN2* and *PTPN22:* role in the pathogenesis of type 1 diabetes and Crohn's disease

Authors: Robert C. Sharp, Muna Abdulrahim, Ebraheem S. Naser and Saleh A. Naser

Journal: Frontiers in Cellular and Infection Microbiology

DOI: https://doi.org/10.3389/fcimb.2015.00095

Published: 24 December 2015

We, the authors, give our permission to include data and materials described in Sharp et. al. 2018 (below) in the dissertation contents of Mr. Robert C. Sharp for Doctor of Philosophy in Biomedical Sciences at the University of Central Florida.

Article Title: Polymorphisms in *protein tyrosine phosphatase non-receptor type 2* and *22* (*PTPN2/22*) are linked to hyper-proliferative T-cells and susceptibility to *Mycobacteria* in rheumatoid arthritis

Authors: Robert C. Sharp, Shazia A. Beg and Saleh A. Naser

Journal: Frontiers in Cellular and Infection Microbiology

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