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Downregulation in IFNGR1 Increases Suspectiblity to Mycobacterium Avium Subspecies Paratuberculosis Infection in Crohn's Disease

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DOWNREGULATION IN *INFGR1* INCREASES SUSCEPTIBILITY TO *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* IN CROHN'S DISEASE

by

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ABSTRACT

BACKGROUND: Crohn's disease (CD) is an inflammatory bowel disease (IBD) and has been associated with *Mycobacterium avium* subspecies *paratuberculosis (*MAP). MAP has been detected in stool, tissue and blood samples from patients with CD. Gamma interferon (γ-IFN) is an inflammatory cytokine that plays a crucial role in killing intracellular pathogens like MAP, and its receptor (IFNGR1) mutations cause immunodeficiency and severe disseminated mycobacterial infections. The role of MAP in association with *IFNGR1* mutation in CD patients have not been investigated.

METHODS: In this study, we investigated blood samples of 79 human subjects for MAP infection in association with *IFNGR1* gene dysfunction. Samples were divided into 22 CD, 6 Ulcerative colitis (UC), 32 normal healthy and 19 non-inflammatory bowel disease (NIBD). Five variants of *IFNGR1* single nucleotide polymorphisms (SNP) were investigated using Taqman Genotyping assay, then *IFNGR1* expression measured by RT-PCR and serum IFNGR1 and γ-IFN levels were measured using ELISA. MAP infection was detected using nested PCR

RESULTS: Among 28 IBD patients, 4/6 (66.67%) of UC and 18/22 (81.82%) of CD are tested positive for at least one SNP homozygous minor form compared to 21.88% and 47.37%% in 32 healthy and 19 NIBD ($P < 0.05$). *IFNGR1* gene expression was downregulated 1.4-fold in IBD patients ($P = 0.07$) and 1.7-fold downregulated in MAP positive IBD patients compared to MAP negative IBD patients ($P = 0.06$). Serum IFNGR1 protein levels were downregulated 1.53-fold in IBD patients compared to normal, and 1.4-fold downregulated in MAP positive IBD patients

compared to MAP negative IBD patients. MAP infection is more common in rs2234711 SNP positive patients (5/7 =71.42%) ($P<0.05$). Serum γ-IFN levels were not elevated in both groups

CONCLUSION: *IFNGR1* SNP's, MAP infection and *IFNGR1* downregulation were found in higher incidence in IBD, suggesting role of *IFNGR1* in susceptibility of MAP infection in IBD patients.

I dedicate this work to my parents, and my beloved sister Thi Thi Khine. Without their endless support, it wouldn’t have been possible for me to complete this work.

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LIST OF ABBREVIATIONS

CD: Crohn's disease

cDNA: complementary Deoxy-ribonucleic acid

DNA: Deoxy-ribonucleic acid

DM: Diabetes mellitus

ELISA: Enzyme linked immunoabsorbent assay

γ-IFN: Interferon gamma

IBD: Inflammatory bowel disease

IFNGR1: Interferon gamma receptor subunit 1

IL: Interleukin

MAP: Mycobacterium avium subspecies Para tuberculosis

MGIT: Mycobacterial growth indicator tube

NIBD: non-inflammatory bowel disease

PCR: Polymerase chain reaction

RT-PCR: reverse transcription polymerase chain reaction

RA: rheumatoid arthritis

RNA: Ribonucleic acid

SNP: single nucleotide polymorphism

UC: Ulcerative colitis

CHAPTER ONE: INTRODUCTION

Crohn's Disease

Crohn's disease (CD) is a chronic relapsing inflammatory disease of gastrointestinal tract occurring most commonly in Europe and Northern America[1]. Currently in USA, as per Centre of Disease Prevention and Control (CDC), 1 to 1.3 million of people are suffering from inflammatory bowel disease. 2.5 million of people in Europe are also estimated to have IBD and the prevalence is rising in newly industrialized countries such as Asia, South America and Middle East. CD is usually diagnosed early in adulthood and it keeps remising and relapsing throughout life, hence the prevalence keeps rising. CD adds significant burden to global healthcare because of high cost of therapeutic drugs and prolonged disease duration, its effects on quality of life and career impediments.

CD, together with ulcerative colitis, forms two major categories of Inflammatory Bowel Diseases (IBD). However, CD is different from ulcerative colitis in that CD affects the entire gastrointestinal tract from mouth to perianal area, and it affects the entire mucosal thickness. Affected patients suffer from chronic relapsing episodes of diarrhea, abdominal pain, rectal bleeding, fever and weight loss [2].

There is no cure for CD so far and treatment for it focuses on anti-inflammatory drugs, immunosuppressive drugs, and biological agents that inhibit the functions of inflammatory cytokines. Antibiotics have been proved to be beneficial in treatment of CD[3].

Pathogenesis of Crohn's Disease

The etiology of CD has been much debated. Defective innate and adaptive immune response, genetic mutations, environmental factors, alterations in normal intestinal flora proportions,

several infectious agents including *Mycobacterium avium s*ubspecies *paratuberculosis* (MAP) have been suggested to play a role in causation of Crohn's disease [4]. Development of abnormal immune responses in Th1 and IL-17 producing Th17 responses are predominately observed in pathogenesis of CD[5]. Activation of macrophages and dendritic cells stimulates secretion of inflammatory cytokines gamma interferon (INF-γ), IL-1, IL-6 and IL-23 and results in mucosal inflammation and skip lesions.

Many genetic mutations have been proved to be associated with CD incidence as well. Variations of genes associated with immune functions such as ATG16L1, IRGM, NOD2 and IL23R have been shown to be associated with increased risk of CD[6-8]. Genetic variations of PTPN2 and PTPN 22 has significance in pathogenesis of Crohn's disease too[9]. Environmental factors such as smoking, oral contraceptives, diet, vaccinations and childhood hygiene have been suggested to play a role in CD[10]. In this study, we screened gene expression of 30,000 human genes using DNA microarray technique to look for genes that are differentially expressed in IBD patients compared to normal controls.

The role of microbes in etiology of CD have been widely investigated by researchers. Adherent-invasive *Escherichia coli*, *Pseudomonas* species, *Listeria monocytogenes, Enterococcus fecalis, Klebsiella pneumoniae, Mycoplasma* species and *Saccharomyces cerevisiae* have shown association with CD[11]. MAP have been the most intensely investigated microbe because of its causation of CD-like chronic granulomatous gastrointestinal inflammatory disease called Johne's disease in ruminants. Our Lab focuses on establishing the role of MAP in the etiology of Crohn's disease.

Mycobacterium avium paratuberculosis (MAP) And Crohn's Disease (CD)

Mycobaterium avium subspecies *paratuberculosis* (MAP) is an intracellular pathogenic bacterium and an acid-fast bacillus. It is the causative agent for Johne's disease in cattle and there has been extensive evidence that suggests it is causal agent of CD as well. The clinical features of Johne's disease closely resemble CD in human. Symptoms include fever, abdominal pain, diarrhea and perianal fistulas. Furthermore, pathology findings of Johne's disease such as granulomatous ileitis, bowel inflammation and ulcerations have been comparable to those of CD[12]. MAP has been detected in milk secreted from infected cattle and shown to survive pasteurization process depending on types of strain and concentration in the milk, hence predisposing high infection rate in human[13]. It has been isolated from blood, intestinal tissue and breast milk from CD patients[14, 15].

MAP infection could be detected in human by acid-fast staining and microscopy, detection of serum antibodies using ELISA, cultures using solid medium like Löwenstein- Jensen media or liquid medium with automated incubation and reading system like BACTEC MGIT 960 system. However, the most specific method of detection of MAP in human is proved to be amplification of IS900 gene sequence specific to MAP using nested PCR technique[16]. Several researchers have reported increased detection rates of MAP in CD and genetically susceptible individuals compared to normal healthy humans but wide-spread population studies still need to be done to determine the prevalence of MAP infection in human[16-18].

Our lab has isolated MAP from blood, intestinal biopsy and breastmilk from CD patients and we have published several papers establishing association of MAP with CD[14, 15, 19-22]. Currently, our lab collaborated with RedHill Biopharma Ltd to run a Phase III clinical trial, RHB104, an oral antibiotics combination therapy against MAP on CD patients[23]. In this

project, we investigated the role of gamma interferon receptor subunit 1, *IFNGR1*, mutation increasing susceptibility to MAP infection in CD patients.

CHAPTER TWO: ROLE OF IFNGR1 IN CAUSING SUSCEPTIBILITY TO MAP INFECTION IN CD

Summary

Crohn's disease has multiple factors in etiology, among which infection and genetic predisposition are of utmost importance. MAP has been shown to play a role in CD by several studies[17, 24, 25]. In this study, we researched 79 human subjects including 22 CD patients and we identified that four *IFNGR1* SNP's are associated with defective immune response against mycobacterial infections, to be more common in CD patients and their gene expressions downregulated. Furthermore, these SNP positive patients have higher MAP infection rate. Hence, this study demonstrated a new genetic predisposition that make individuals susceptible to MAP infection and CD development.

Introduction

CD patients have elevated levels of pro-inflammatory cytokines including interferon gamma (γ-IFN) and tumour necrosis factor alpha (TNF-α). Interestingly, γ-IFN is crucial to defend against intracellular pathogens. Like in tuberculosis, it initiates granuloma formation which supports a possible MAP association with CD. Fais. S. et al have reported an elevated γ-IFN response in peripheral lymphocytes from CD patients, suggesting prior sensitization with mycobacterial antigens[25]. IFNGR1, which is the major subunit of the two subunits of γ-IFN receptor, have shown mutations, single nucleotide polymorphisms (SNP), deletions and they all have been identified to cause immunodeficiency and susceptibility to various infections including *Mycobacterium tuberculosis*, atypical mycobacteria and intracellular organisms such as *Salmonella, Malaria*, and *Leishmania [26-29]*. No studies have investigated the role of *IFNGR1* in CD.

Differentially expressed genes in CD and UC

Previously in our lab, Dr. Romero Claudia performed DNA microarray technique to screen for differentially expressed genes in 2 CD and 1 UC patients compared to 8 healthy individuals. We found that 17% (5630 genes) were differentially expressed in both IBD disorders (Figure 1). Most of these genes belong to four biological functions classified as cellular processes (38%) physiological processes (26%), intracellular genes (18%), and genes involved in metabolism (18%). 238 genes that are differentially expressed belong to immune response processes. As shown in Table 1 and 2, seven genes expression were different in both UC and CD compared to normal subjects and eight genes were differentially expressed in either UC or CD. Among them, *IFNGR1* will be focused in our study because CD and UC are diseases arisen by abnormal immune response mechanisms

Mechanism of *IFNGR1* mutation

γ-IFN is secreted by Th1 lymphocytes, natural killer cells and antigen presenting cells. Its secretion is initiated by mycobacterial cell wall components such as purified protein derivative (PPD), and peptidoglycan[30]. It induces up-regulation of MHC class 1 cell surface receptors, hence increasing potential for cytotoxic T cell recognition and destruction of host cells infected with MAP. γ-IFN produced from Th1 cells promotes antigen presentation and IL-1 and IL-12 release from macrophages, hence resulting in further activation of Th1 cells, further γ-IFN production and aggregation of Th1 cells around macrophages and granuloma formation[31]. Ultimately it results in intracellular bacteria killing. γ-IFN receptor is a heterodimeric type II cytokine receptor and is composed of IFNGR1 and IFNGR2. Two subunits of IFNGR1 binds to ligand and two more subunits of IFNGR2 carry out signal transduction via JAK/STAT pathway. IFNGR1 is also known as Cluster of Differentiation 119 (CD119). It has two forms, soluble form

and classic membrane bound form. *IFNGR1* gene is mapped to human chromosome 6 at region 6q23–6q24[32]. Inactivating mutations in *IFNGR1* have shown defective defense against intracellular pathogens in mouse models[33]. In human counterparts, significant clinical manifestations have been reported too. Small deletions in coding regions of *IFNGR1* causes dominant susceptibility to non-virulent mycobacterial infections in children[34]. These children are shown to suffer from disseminated and fatal mycobacterial infections with Bacillus Calmette-Guérin (BCG) vaccination which is made from *Mycobacterium bovis.* Some children with *IFNGR1* autosomal recessive pattern mutations suffer from a condition called Familial Atypical Mycobacteriosis; an immunodeficiency that predispose them to infection of various atypical mycobacteria (M. fortuitum, M. chelonei, and two strains of M. avium), and prolonged salmonellosis [26-28, 35-37].

Single nucleotide polymorphisms (SNP) are variations in a single nucleotide at a specific position of a gene. Each variation is present to some extent in the population and phenotypic expression can vary from benign to genetic susceptibility to disease, higher risk of infections, different response to therapeutic regimes and severity of a disease. SNP rs2234711 is located at transcription factor AP4 binding site of *IFNGR1* gene while rs7749390 is at exon-intron splicing site. Association studies have shown that both SNP are associated with increased risk for *Mycobacterium tuberculosis* infection in Chinese Han population and contributes to increased susceptibility to post kala-azar dermal leishmaniasis in Sudanese population[28, 38]. Marker rs7749390 is shown to be associated with Eczema herpeticum, a *Herpes Simplex* virus infection to pre-existing atopic dermatitis[39].

However, the role of *IFNGR1* mutations and SNP on susceptibility to MAP infection or their incidence in inflammatory bowel disease patients and autoimmune disease patients has never

been investigated. In this study, we investigated the presence of 5 SNP's (rs1327474, rs1327475, rs2234711, rs7749390, rs3799488) in Crohn's disease patients and their status of MAP infection compared to normal healthy controls. Their effect on *IFNGR1* gene expression and serum level of γ-IFN were also investigated. From this study, we hope to identify novel therapeutic targets for Crohn's disease patients by uncovering more underlying factors in disease causation.

Materials and Methods

Detection of *IFNGR1* SNP type and frequency using Taqman

EDTA blood from IRB approved patients recruited from Univeristy of Central Florida Health, and Digestive and Liver Center of Florida were collected and a total of 79 patients including 22 CD, 6 UC, 14 healthy subjects and 16 NIBD are analysed for the presence of SNP. Taqman SNP Genotyping Assay (Thermo fisher Scientific) were used for 5 *IFNGR1* SNP's rs1327474, rs1327475, rs2234711, rs7749390 and rs3799488. Assay IDs, their location on *IFNGR1* gene and nucleotide sequencing are shown in Table 3. 1ml of whole blood samples were stored at -20C and sent to University of Florida for genotyping analysis. Taqman® Genotyping Assay utilizes 5' exonuclease activity of Taq DNA polymerase. It includes two oligonucleotide Taqman Probes each specific to one allele of the target gene that have fluorescence reporter dye on 5' end and non-fluorescent quencher (NFQ) with minor groove binder (MGB) at 3'end. One probe is labelled with VIC dye and detects allele 1 sequence, another is labelled with FAM dye and detects the allele 2 sequence. In its unbound form, fluorescent signal at 5' end is quenched by 3'end quencher due to proximity. When the probe binds to the target allele, 5' fluorescence is hybridized by 5'exonuclease activity of Taq Polymerase and emits fluorescence [40](Figure 2).

Taqman SNP genotyping was done by adding 1 to 20ng of purified gDNA sample extracted from whole blood, 40X Taqman Genotyping Assay diluted to 20X working stock, and 2X Taqman Universal PCR Mastermix. PCR reaction was run in thermal cycler with AmpliTaq Gold Enzyme Activation step of 10 mins holding at 950C, followed by 40 cycles of PCR (15 seconds denaturation at 920C and 1 minute annealing/extension at 600C). The fluorescence was picked up by the machine and the data is analyzed to detect presence of homozygous major allele, homozygous minor allele or heterozygous state.

Measurement of *IFNGR1* gene expression

Measurement of *IFNGR1* expression includes 3 steps (1) RNA extraction (2) cDNA synthesis and (3) Real Time quantitative PCR analysis.

RNA extraction was done using Trizol® Reagent (Invitrogen) using manufacturer's protocol. 1 ml of Trizol® Reagent was used to extract RNA from buffy coat from 1ml of blood. RNA was separated from DNA and proteins by adding choloroform. Supernatant containing RNA was centrifuged and RNA pallet was washed with 100% propanol and then again with 75% ethanol. Finally RNA pallet was suspended in RNase free water treated by Diethyl Pyrocarbonate (≥97% (NMR) Sigma-Aldrich) and heated at 55-60^{o}C for 15 minutes for denaturation. RNA quality is accessed by running 6% urea-polyacrylamide gel. RNA concentration is measured by NanoDrop ND 1000 Spectrophotometer (Thermo Fisher).

cDNA was synthesized using iScript^{TM} Reverse Transcription Supermix for RT-qPCR kit (BioRad) per manufacturer's protocol. 4uL of 5X RT supermix is added to 600ng of extracted total RNA and DEPC treated sterile water is added to make 20 ul reaction. Then the complete reaction mix is incubated in thermal cycler for 5 minutes at 25^{o}C for priming, 20

mins at 46^{o}C for reverse transcription, 1 min at 95^{o}C for reverse transcriptase activation. The cDNA synthesized is stored at -20^{o}C for further processing.

1ul of cDNA was added into 10ul of Fast SYBR® Green Supermix (Applied Biosystems) and 1ul of PrimePCR assay for *IFNGR1*. Then, water was added to make 20 ul reaction.

The primers used were from PrimePCR SYBR Green assay IFNGR1, Human, unique assay ID: qHsaCID0013339 (Bio-rad). PrimePCR Template for SYBR Green assay: IFNGR1, Human(Bio-rad) was used as positive control reaction to check melting temperature and melting curve.

Human 18S ribosome was used as control reaction, reactions are prepared as above using specific primers. Applied Biosystems 7500 real time PCR system was used for qPCR analysis with manufacturer recommended protocol of stage 1: 20 seconds holding at 95^{o}C, stage 2: 40 cycles of 3 seconds denaturation at 95^{0}C, 30 seconds of annealing/extension at 60^{0}C, and at annealing/extension stage, fluorescence was captured. Dissociation stage was run from 95^{0}C to 60^{0}C, with 15 seconds increments, and melting temperature for *IFNGR1* gene was 73^{0}C in accordance with positive template control reaction. The size of amplicon is 84 base pairs.

Ct value was captured and ΔCt value was calculated and compared with normal controls.

Measurement of IFNGR1 protein level in serum

We used Human IFNGR1 ELISA kit (RayBiotech) for detection of IFNGR1 soluble form in serum. Reagents and standards were prepared according to manufacturer's protocol and 100 ul of serum collected from blood samples were added to wells, then incubated for 2.5 hours at RT. Then they were washed with 300 ul of 1X wash solution for 4 times and blotted

against clean paper towels. 100 ul of 1X biotinylated antibody was added, incubated for 1 hour at RT and washed. Then, 100 ul of streptovidin solution was added, incubated for 45 minutes and washed. Finally, 100 ul of TMB one-step substrate reagent was added, incubated for 30 mins, then stop solution was added and read immediately at 450 nm. Standard curve was created using software and results were calculated from the standard curve obtained.

Measurement of γ-IFN protein level in serum

γ-IFN was measured in serum using γ-IFN ELISA kit (Thermo Fischer) following manufacturer's protocol.

Detection of MAP infection

Nested polymerase chain reaction (PCR) and culturing in BACTEC media for sensitivity and specificity of MAP detection was used. Insertion sequence IS900 is specific only to MAP strains infecting human. DNA extraction was done from extracted buffy coats from whole blood using DNAzol® BD Reagent (Invitrogen). Then, nested PCR technique with primers P90 and P91 (P90 5' GTT CGG GGC CGT CGC TTA GG 3' and P91 5' GAG GTC GAT CGC CCA CGT GA 3') for first PCR cycle and primers AV1 and AV2 (AV1 5' ATG TGG TTG CTG TGT TGG ATG G 3' and AV2 5' CCG CCG CAA TCA ACT CCA G 3') for second PCR cycle were used. This increases the specificity of PCR reaction for MAP. Finally, the PCR products were electrophoresed on 2.5% agarose gel and stained with ethidium bromide for visualization. 298 base pairs band in gel were considered MAP positive.

The positive controls we used in our experiments were MAP bacteria grown in Mycobacteria Growth Indicator Tubes (MGIT). MGIT contains Middlebrook 7H9 broth base for culturing

of *Mycobacteria* and a fluorescent compound is embedded in the silicon on the bottom of the tube. We use BBL™ MGIT™ tubes (Becton, Dickinson and Company). BACTEC™ MGIT™ Growth Supplement (BD) and Mycobactin J (BD) is added to help grow MAP. Mycobactin J is the iron-binding and transporting factor that is necessary for in vitro growth of MAP. The extracted buffy coat is dissolved in 200 ul of TE buffer and added into prepared MGIT tube and cultured in BACTEC™ MGIT™ 320 System at 37°C. The system checks for fluorescence every 60 minutes and 10^5 to 10^6 colonies forming units per milliliter (CFU/ml) is reported as positive. The culture tubes that shows no growth for a minimum of 42 days are regarded as negative. The BACTEC™ MGIT™ 320 technology is superior to traditional culture medias in that it is more sensitive and less time consuming.

Results

Investigation for prevalence of IFNGR1 SNP's

We detected presence of five IFNGR1 SNP's in 32 healthy subjects, 28 IBD patients including 6 UC patients, 22 CD patients. As controls, we additionally investigated SNP's presence in 19 non IBD (NIBD) patients. NIBD patients are patients they have some other diagnoses other than CD or UC. For example, some patients have RA, while others have type 1 or type 2 diabetes, and/or irritable bowel syndrome. Five SNP's investigated are rs1327474, rs1327475, rs2234711, rs7749390 and rs3799488.

As seen in Figure 3, rs1327474 is tested positive for homozygous in minor allele in 2 out of 14 normal subjects (14.3%), 0% in NIBD, 4 out of 21 CD (19%), and 1 out of 6 UC (16.7%). CD and UC are combined to be IBD group, so the prevalence of IBD groups for rs1327474 is 5/27 (18.5%). Rs1327475 is tested homozygous minor in none of the samples

tested. Hence prevalence of rs1327474 and rs1327474 is relatively same in all groups. Homozygous minor allele status for rs2234711 is found in 5 out of 32 healthy (15.6%), 3 out of 19 NICD (15.8%), 5 out of 22 CD (22.7%) and 1 out of 6 UC (16.7%), in IBD group, it is 21.4%. Rs7749390 is seen homozygous minor allele positive in 6/32 normal (18.8%), 6 NIBD (31.6%), 12 CD (54.5%) and 3 UC (50%), in combined IBD group, it becomes 53.6%. Similarly, homozygous minor allele for rs3799488 is found in 2 CD (9.1%) and 0 UC, in IBD it is 3.6% compared to 0% in both healthy and NIBD group. We can see that homozygous minor status is clearly more common in CD and UC patients than healthy and NIBD patients for rs2234711, rs7749390 and rs3799488 and it is significant in rs7749390 ($P < 0.05$).

However, heterozygous status for major and minor allele has a different distribution (Figure 4). Prevalence for heterozygous alleles is higher in healthy and NIBD population than CD and UC position in most SNP's. Heterozygous rs1327474 is seen in 7 out of 14 healthy (50%), 8 out of 16 NIBD (50%), and 10 out of 21 CD (47.62%) and 3 out of 6UC (50%), bringing IBD prevalence to 48.15% against 50% in both healthy and NIBD group. So, as with homozygous minor rs 1327474, there is no difference in prevalence of its heterozygous status. Rs1327475 heterozygous status is seen only in one healthy subject and this is the only minor allele seen in all samples tested. Both rs2234711 and rs7749390 are seen in 17 out of 32 healthy, 8 out of 19 NIBD, 3 out of 22 CD (13.6%) and 2 out of 6 UC (33.3%). Hence IBD incidence is 17.9 % compared to 53.1% healthy and 42.1% NIBD. Rs3799488 heterozygosity is seen in 6/32 (18.6%) healthy and 3/19 (15.8%) NIBD, whereas in IBD patients, there is 2/22 (9.1%) in CD and 1/6 UC (16.7%) respectively.

Overall speaking, out of 32 healthy, 7 people (21.9 %) and 9 out of 19 NIBD (47.4%) have at least one SNP homozygous minor allele present. However, in IBD group, 4/6 (66.7%) UC and 18/22 (81.8%) CD has at least one SNP homozygous minor allele present (Figure 5). So, incidence of homozygous minor alleles of 5 SNP's under the study is much more common in both CD and UC ($P<0.05$).

Measurement of *IFNGR1* gene expression

We measured gene expression of *IFNGR1* gene using RT-PCR in 30 healthy, 14 NIBD and 54 IBD samples. Among them, 24 healthy, 14 NIBD, 24 IBD were detected for presence of MAP and 30 healthy, 14 NIBD and 23 IBD were detected for the presence of SNP. The gene expression levels were reported as mean of $(2^{(-\Delta CT)} \times 1000) \pm 95\%$ CI. Mean of healthy group is 5.3 ± 2.4 compared to 4.5 ± 0.9 of NIBD and 3.8 ± 0.9 of IBD. IBD samples were 1.4-fold downregulated ($P=0.07$) whereas NIBD samples were 1.2-fold downregulated ($P = 0.32$) (Table 4). But we classified them into MAP positive and MAP negative in each group (Table 5), as well as SNP positive and SNP negative in each group (Table 6) and analyzed them separately. Both SNP and MAP causes downregulation in IBD group, 1.5-fold in presence of SNP ($P =0.1$) and 1.7-fold in presence of MAP ($P = 0.08$). The group that has co-existence of SNP and MAP together still causes 1.7-fold downregulation. ($P =0.08$).

Measurement of IFNGR1 protein level in serum

We carried out ELISA to measure the amount of IFNGR1 protein in serum in 6 healthy, 9 NIBD and 24 IBD patients, then classified them into SNP positive and negative groups and compared against each other. Mean of IFNGR1 proteins levels along with 95% CI is found

as follows. Healthy group has 810.8 ± 1024.5 pg/ml whereas IBD group has 530.3 ± 190.5 pg/ml and NIBD group is 502.7 ± 263.2 pg/ml. IBD group is 1.53-fold lower (P = 0.15) and NIBD group is 1.61-fold lower (P = 0.21) than healthy group (Table 7) Considering MAP infection status, it causes 1.4-fold downregulation in both IBD and healthy group (P = 0.5) (Table 8). The presence of SNP and MAP together causes 1.42-fold lower levels of IFNGR1 protein in healthy, 2.14-fold in NIBD and 1.51-fold in IBD (Table 9). However, presence of SNP alone doesn't appear to lower IFNGR1 levels. Healthy subjects have 1.3-fold higher levels, NIBD subjects have similar levels and IBD patients have 1.1 times higher levels (Table 10).

Measurement of γ-IFN protein level in serum

In 2005, we tested 5 controls for serum γ--IFN levels using ELISA and 7 IBD as preliminary studies. Average values are 6.7 ± 4.1 pg/ml in healthy compared to 77.7 ±123.1 pg/ml. IBD samples are 11.6-fold upregulated compared to control samples (P>0.1).

In 2017, we analyzed 29 controls versus 25 IBD. Except for one outlier in healthy group that has elevated γ--IFN level of 364.6 pg/ml, all other samples in both groups show either low or undetectable levels of γ--IFN. Mean value for control group is 0.7± 1.1 pg/ml while IBD group mean serum γ--IFN shows 0.3±0.7 pg/ml. Thus, there is no significant elevation of serum γ--IFN anymore in 2017 in IBD group, as seen in figure 6. We assume these changes are due to wide-spread use of biological agents which block functions and secretion of cytokines.

Detection of MAP infection

MAP infection is detected by either nested PCR from buffy coats extracted from freshly drawn blood, or nested PCR from MAP cultures done from buffy coats. 4 out of 32 healthy are MAP positive (12.5%) as well as 6/19 of NIBD (18.8%). In IBD group, 11 out of 28 CD are MAP positive (34.4%). We see from the data that MAP incidence is more common in IBD group compared to healthy ($P < 0.05$) (Figure 7).

Of the MAP positive patients, 1/4 healthy (25%) subjects in healthy and 2/3 (33.3%) NIBD has at least one homozygous minor allele SNP. In IBD group, 9 out of 11 MAP positive patients have at least one homozygous minor allele SNP (81.8%), making it clear that presence of SNP makes an individual more prone to MAP infection in IBD patients ($P<0.05$) (Figure 8).

Co-existence of SNP and MAP infection

In homozygous minor allele SNP's, none but 1 out of 4 CD (25%) in rs1327474 is associated with MAP. Rs1327475 has no homozygous minor alleles detected. For rs2234711, none of 1 healthy and 3 NIBD patients that has SNP positive has MAP infection. However, 1 UC with SNP has MAP detected (100%), 4 out of 6 CD is infected with MAP (66.67%). Therefore, we can see that MAP detection is more common in IBD patients (71.4%) compared to 0% in healthy and NIBD. Rs7749390 homozygous minor SNPs positive subjects were tested MAP positive in 1/5 (20%) of healthy, 2/6 (33.33%) of NIBD, and 1/3 (33.33%) in UC and 3/12 (25%) in CD. For rs3799488, only 2 CD has SNP positive for homozygous minor status out of all subjects and 1 (50%) is associated with MAP. Hence, we can see that MAP infection is more common in rs2234711 homozygous minor patients ($P < 0.05$), though MAP infection is not significantly different in rs1327474 and rs774939 (Table 11).

Odds ratio for having at least one SNP in IBD group is 13.1 times more than control group. ($P < 0.05$) (95% confidence interval = 3.8 to 44.9). Odds ratio for presence of MAP in the blood is also 4.5 times higher in IBD patients ($P < 0.05$) (95% CI = 1.24 to 16.5). Calculations using Spearman's rho test show that presence of MAP and SNP are positively correlated with rho value of 1. ($P < 0.05$). Presence of MAP and gene expression are weakly negatively correlated, rho = -0.125. ($P = 0.234$).

Discussion

Investigation for prevalence of IFNGR1 SNP's

As seen in Table 1, all 5 SNP's investigated in this study are somehow related with susceptibility to *Mycobacterium tuberculosis*, *Helicobacter pylori*, parasites such as *Leishmania* and *Schistosom*a, and viral infections such as *Herpes simplex* in different ethnicities and population. But our study is the first to investigate the role of *IFNGR1* SNPs in IBD patients, as well as role of *IFNGR1* SNPs in susceptibility to MAP infection. As seen in our data, the incidence of these homozygous minor SNPs is higher in IBD patients, hence suggesting the role of IFNGR1 mutations in the causation of CD. Four SNPs, rs1327474, rs2234711, rs7749390, rs3799488 have been associated with *Mycobacterium tuberculosis* susceptibility in literature and in our study, those SNPs are also found in higher incidence in CD patients. Rs7749390 is showing statistical significance ($P < 0.05$) and larger sample size is needed to show significance in other SNPs too. We also found statistical significance when all SNPs are analyzed together that IBD patients are 13.1 times more likely to have underlying IFNGR1 homozygous minor SNPs compared to other NIBD and healthy subjects. MAP infection is seen in higher incidence

with patients with homozygous minor SNP. Thus, it shows that IFNGR1 SNPs are contributing to susceptibility of MAP infection in CD patients. Our study serves as first indicator to point out effect of IFNGR1 mutation to MAP susceptibility in CD patients and more studies with bigger sample size should be done to establish its role in MAP infection. MAP has been long suggested as causal agent of CD and this study explains how genetic and infectious factors work together to bring about the etiology of CD and UC.

The incidence of heterozygous SNP's is lower in IBD group, suggesting the recessive pattern of the SNPs to show phenotypic effects. It could also suggest that heterozygous variants might have a protective action against MAP infection unlike homozygous minor variants which cause susceptibility.

Patients with presence of SNP are found to be more infected with MAP. This suggests that defective □-IFN signaling pathways with IFNGR1 defects could be responsible for immune system's failure to defend MAP infection, hence the bacteria opportunity to induce more inflammation and more damage inside the gastro-intestinal mucosa. Further studies need to be done how IFNGR1 mutation affect □-IFN signaling pathways to affect defense against intracellular infections such as MAP. Our study could be the initial demonstration of a possible novel therapeutic target of IFNGR1 for CD patients and it emphasizes the existing role of MAP infection in CD causation.

Measurement of *IFNGR1* gene expression

The RT-PCR result for comparing IFNGR1 gene expression in IBD against healthy and NIBD shows downregulation in IBD patients 1.4-fold ($P = 0.07$) compared to 1.2-fold of NIBD ($P = 0.32$). This suggests the mechanism of SNPs in causing MAP susceptibility could be through downregulation of gene expression, hence causing loss of functional

receptors. When we classify them into SNP positive, negative groups and MAP positive, negative group, we can clearly see that more downregulation is seen with SNP positive patients, and MAP positive patients even among IBD patients. This is evidence that MAP infection is accompanied by more severe downregulation in IBD patients, again supporting the hypothesis that *IFNGR1* gene dysfunction is playing a role in MAP susceptibility and hence contributing of MAP's role in the complex nature of initiation of CD inflammatory process.

Measurement of IFNGR1 protein level in serum

The trend of downregulation in *IFNGR1* gene expression is replicated by serum levels of IFNGR1 proteins. IFNGR1 protein has two forms, cell surface forms and soluble forms. Even though cell surface forms are the main function form, we measure soluble forms in serum as an indirect indicator of IFNGR1 protein translation levels. As we can see in Table 7,8 and 9, IBD patients and NIBD patients are downregulated compared to healthy, and MAP positive patients are downregulated more than MAP negative patients. For SNP positive patients, there is no downregulation unless they are paired with MAP infection. Even though sample size is too small (control = 15, IBD = 23 in total for IFNGR1 ELISA) to show statistical significance, more studies are suggested to establish the statistical significance and phenomenon of downregulation in IFNGR1 SNP positive and MAP positive IBD patients. This data also indicate that even though soluble receptors amount are not significantly reduced, their functionality might be impaired, or the effect is on cell surface receptors but not on soluble receptors

Measurement of γ-IFN protein level in serum

When we measured the serum γ-IFN protein levels back in 2005, serum levels were elevated in IBD patients by 11.59-fold ($P > 0.1$). When we repeated the experiment in 2017, surprisingly, there is no elevation of γ-IFN in IBD patients anymore. We hypothesize that widespread use of biologic agents in last decade plays an essential role in it. Apart from infliximab (Remicade) which was approved in 1998 by FDA, all the other biologic agents were approved after 2006, when our experiments were performed[41, 42]. Hence we can assume that biologic agents were not accessible and easily available back in 2005. Thus γ-IFN levels in IBD patients were not suppressed. In 2017, biologic agents become accessible and affordable, covered by health insurances, and medical treatment for IBD improved in potency and application, hence IBD patients would be under the influence of immunosuppressive, anti-inflammatory and biologic agents to control their signs and symptom of IBD and to maintain their clinical remission[42]. Thus, we detect no difference in γ-IFN levels in IBD patients in 2017 anymore. While this phenomenon highlights improvement of medical therapy in the treatment of IBD, it also indicates that we need to exercise extra-caution in detecting cytokines in patients' blood as well as interpreting the data.

Detection of MAP infection

MAP infection is more common in NIBD group but the prevalence is highest in CD and UC. It appears that patients with immune dysregulation like RA or Type 1 diabetes are more predisposed to MAP infection, but IBD patients are much more common. Thus, in addition to immune dysregulation, it is reasonable to consider that there are factors that predispose IBD

patients to MAP infection. Seeing as *IFNGR1* SNPs are more common in patients with MAP infection, IFNGR1 is one of the susceptibility factors making IBD patients get infected.

CHAPTER THREE: GENERAL DISCUSSION

Crohn's disease (CD) and ulcerative colitis (UC) are inflammatory bowel diseases (IBD) whose etiology has been suggested as multifactorial. CD etiology is influenced by immunodysregulation, genetic mutations and microbial infections. This study uncovers the role of a novel IFNGR1 mutation that causes susceptibility to various infections in CD and links *Mycobacterim avium paratuberculosis* infection to Crohn's disease through *IFNGR1* mutation and its associated immune dysfunction. *IFNGR1* single nucleotide poloymorphisms (SNP) prevalence is higher in IBD patients, and those SNP positive patients are more susceptible to MAP infection as evident by higher MAP infection rates in these patients. In addition, SNP positive and MAP positive patients are more downregulated in the gene transcription as well as translation into serum IFNGR1 protein. MAP infection is found in higher incidence in IBD patients and retrospective studies showed that those MAP infected patients have more SNP's detected. Serum γ-IFN levels used to be elevated in IBD patients in 2005, hence suggesting the mechanism of *IFNGR1* mutation in initiating CD, but their levels flatten out in 2017, which might be the effect of biologic agents which were in widespread use in the years after 2005. All in all, the study highlights the presence of *IFNGR1* mutation as well as MAP infection in IBD patients and gives an insight into the mechanism of *IFNGR1* SNP's contribution to CD inflammation. Further studies with larger sample size as well as cell and molecular studies need to be done on this matter and we hope that *IFNGR1* and MAP therapy will be the start of novel therapeutic targets in helping CD patients maintain remission and eradication of disease.

APPENDIX A: FIGURES

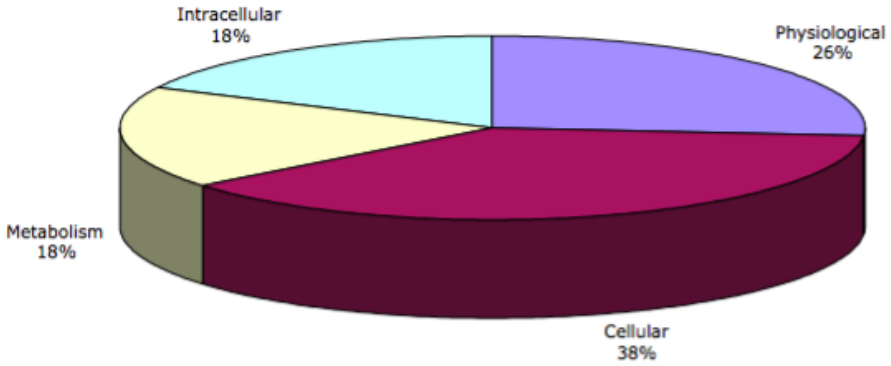

Figure 1: Biological themes predicted by DNA microarray technique for differentially expressed genes in IBD.

17% (5630 genes) were differentially expressed in both IBD disorders, which consists of 4 biological themes, cellular processes (38%), physiological processes (26%), intracellular genes (18%) and metabolic genes (18%). (Figure obtained from Doctor Romero Claudia PhD thesis)

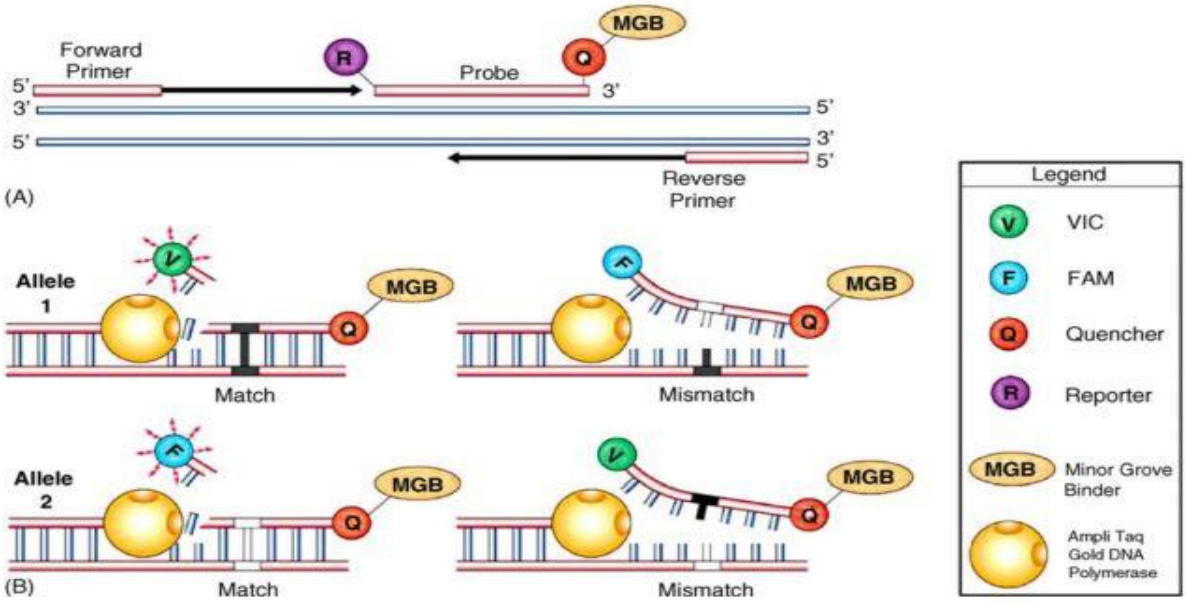

Figure 2: (A) Probe Binding and primer extension in a Taqman SNP Genotyping assay (B) Allelic discrimation is achieved by the selective annealing of matching probe and template sequences, which generates an allele-specific (fluorescent dye-specific) signal.

Picture from Vega, F.M.D.L., et al., Assessment of two flexible and compatible SNP genotyping platforms: TaqMan® SNP Genotyping Assays and the SNPlex™ Genotyping System. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2005. 573(1–2): p. 111-135

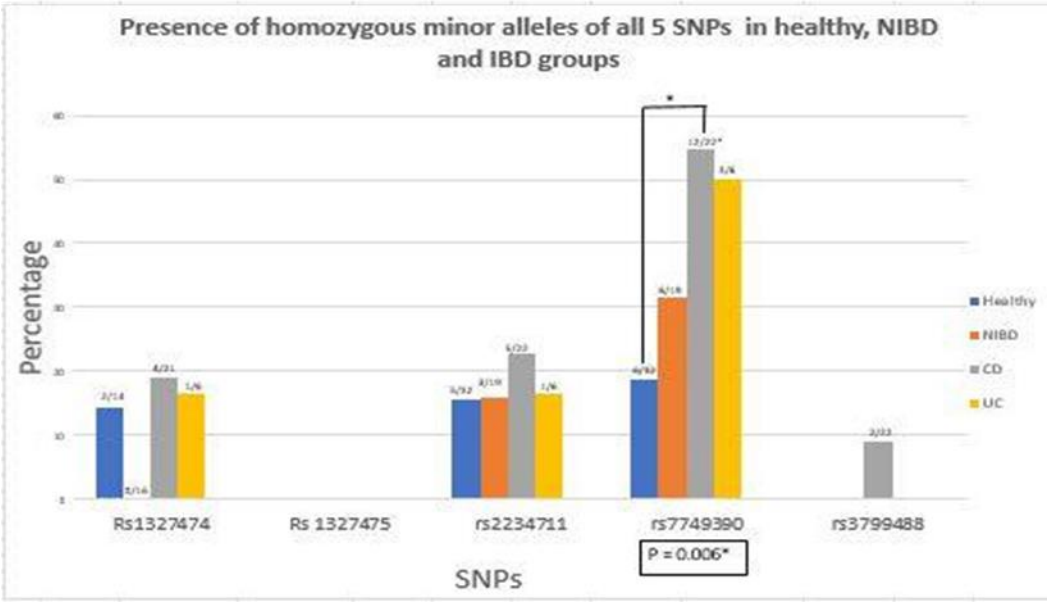

Figure 3: Prevalence of homozygous minor alleles in CD, UC, healthy and NIBD subject groups for various SNP's

CD patients have higher incidence in rs1327474, rs2234711, rs7749390. Rs1327475 is not detected in any of the samples. Rs3799488 is found only in CD patients. Rs7749390's higher incidence in CD is statistically significant ($P<0.05$)

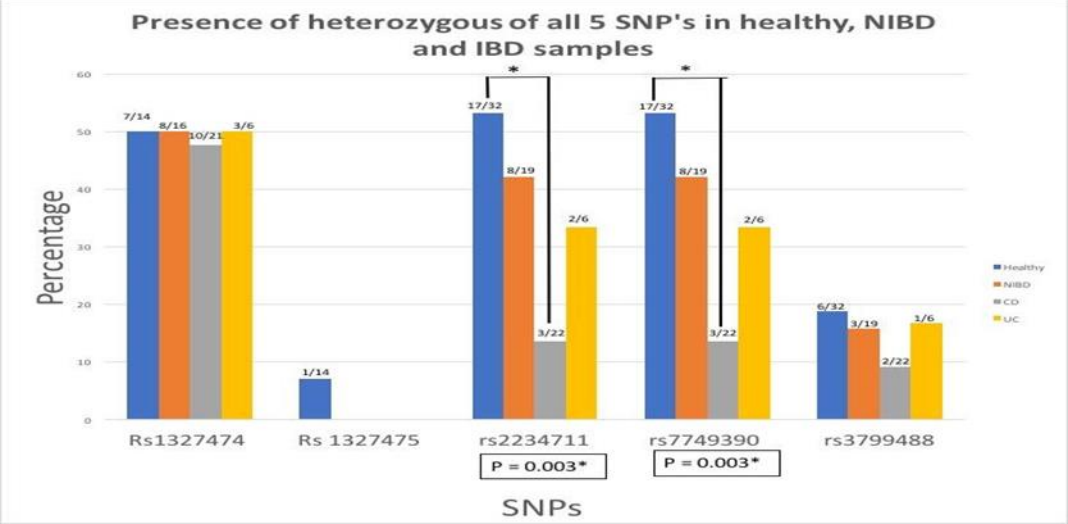

Figure 4: Prevalence of heterozygous alleles in CD, UC, healthy and NIBD subject groups for various SNP's

rs1327474 incidence is relatively similar in all groups. Rs1327475 is found only in healthy. Rs2234711 and rs7749390 are more common in healthy and NIBD ($P<0.05$), suggesting their protective role and recessive pattern of inheritance to take affect. Rs3799488 is of higher incidence in healthy and NIBD.

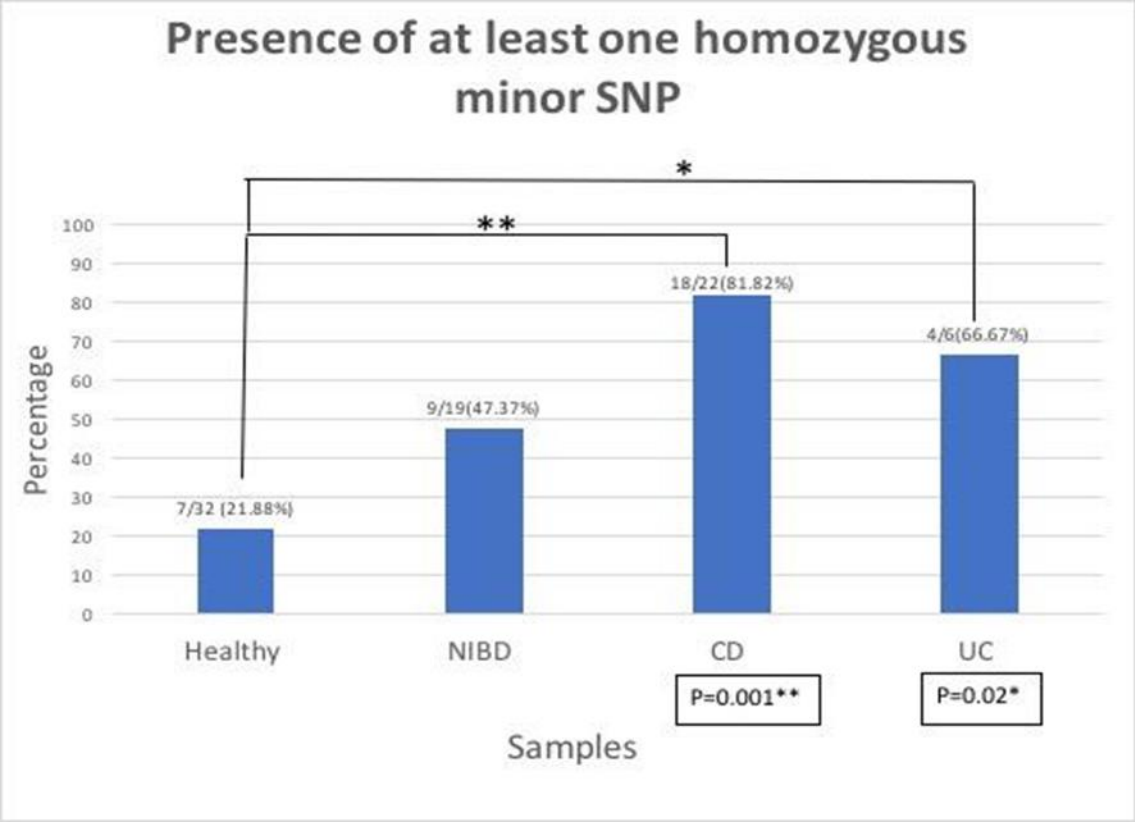

Figure 5: Prevalence of at least one minor homozygous SNP in patient groups

81.82% of CD patients and 66.67% of UC have at least one homozygous minor allele compared to 21.88% of healthy and 47.37% of NIBD ($P < 0.05$ in both CD and UC). This shows CD and UC patients are more prone to have homozygous minor *IFNGR1* SNPs, hence more susceptible to MAP infection.

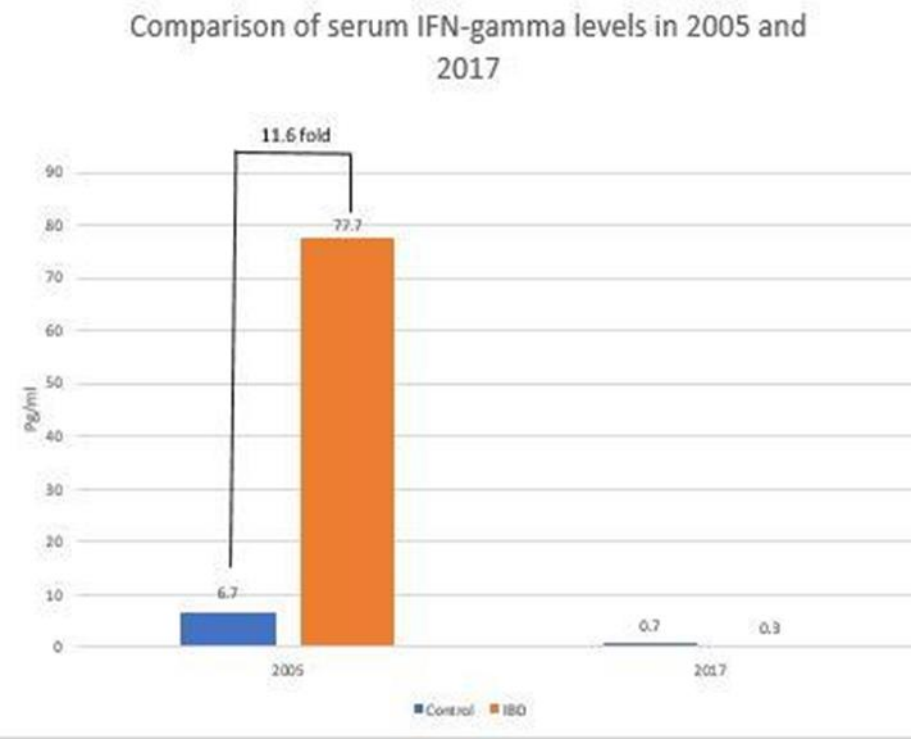

Figure 6: Comparison of 2005 and 2017 serum IFN-gamma levels in both control and IBD groups

In 2005, serum γ-IFN levels of IBD are 11.6-fold higher than control group. In 2017, serum γ-IFN levels of both IBD and control groups are not elevated anymore.

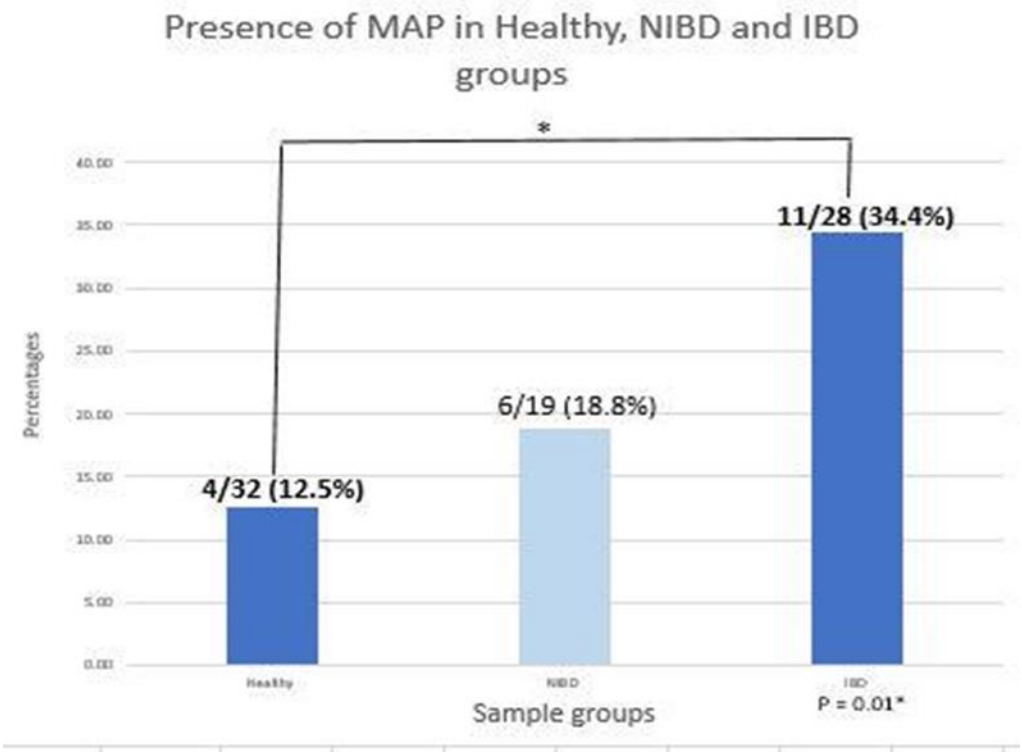

Figure 7: Detection of MAP in Healthy, NIBD, IBD groups

34.4% of IBD have MAP presence while only 12.5% of healthy and 18.8% of NIBD have MAP detected ($P<0.05$). MAP infection is more common in IBD.

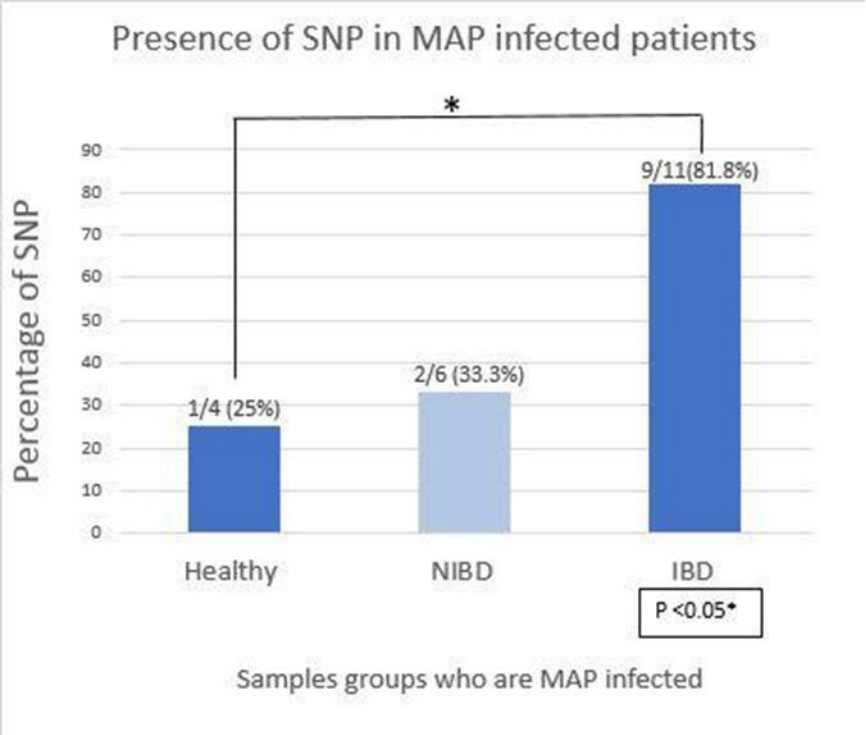

Figure 8: Detection of at least one homozygous minor SNP in MAP infected subjects

81.8% of MAP positive IBD patients have underlying IFNGR1 homozygous minor SNP while only 25% of MAP positive healthy and 33.3% of MAP positive NIBD have underlying SNP ($P<0.05$).

APPENDIX B: TABLES

Table 1: Genes differentially expressed in both CD and UC compared to healthy subjects:

A total of 7 genes' expressions were different, consisting of 6 downregulated genes and 1 upregulated gene.

No.	GeneBank Accession Number	Gene Product	Variable expression
1	W79396	Zinc finger protein 211	Downregulated
2	AI262976	Zinc finger protein 211	Downregulated
3	W93370	NKG2-A and NKG2-B type II integral membrane protein	Downregulated
4	AA463248	CD 160 antigen	Downregulated
5	AA775616	Secreted phosphoprotein 1	Downregulated
6	AA682637	Carbohydrate sulfotransferase 2	Downregulated
7	AI371874	Toll-like receptor 4	Upregulated

Table 2: Genes that are differentially expressed in either CD or UC or both

8 genes are differentially expressed in either CD or UC

No	GeneBank Accession Number	Gene Product	Expression in CD	Expression in UC
1	AA083407	Tripartite motif-containing 22 (TRIM22)	No change	Downregulated
2	AA454646	Lymphotoxin-beta receptor	No change	Upregulated
3	AA476221	Zinc finger protein 558	Downregulated	No change
4	AI123732	EBV induced G protein coupled receptor 2 (EBI2)	Downregulated	No change
5	AA281497	Interferon gamma receptor 1 (IFNGR1)	Downregulated	No change
6	AI298976	Lymphotactin precursor (XCL1)	Downregulated	No change
7	AA495985	Macrophage inflammatory protein 1-alpha precursor (MIP-1α)	Downregulated	Upregulated
8	R47893	Macrophage inflammatory protein 1-alpha precursor (MIP-1α)	Downregulated	Upregulated

Table 3: Five SNPs that are investigated in this study along with their location, microbes that they cause susceptibility to, Assay ID and context sequence

Due to proprietary reasons from Thermo Fisher Scientific, we cannot provide the forward and reverse primer sequences.

However, we can provide the assay ID number and context sequence for each SNP assay. rs# = reference SNP number

No.	SNP	Location on Gene	Associated condition and microbial infection that are found to be susceptible	Assay ID	Context sequence
1	rs1327474	**UTR 5 promotor**	• *Mycobacterium tuberculosis* • *Helicobacter pylori* • **Mendelian susceptablity**	C___2523634_10	GCAATTCAGTGTCAAATCAGT TTAT[C/T]AGGCAGCCTCTCAT GAAGAG GTCTG
2	rs1327475	**Intron**	• *Schistomsoma mansoni*	C___7578576_10	AGACTATTTTCTGGTGACTTCA AAA[A/G]CATCTGCTTCAGGT AATGTTT CCTG
3	rs2234711	**Transcription factor AP4 binding site**	• *Mycobacterium tuberculosis* • **Leishmaniasis**	C__11693991_10	CGAGCGCCTGCGGGACCAGC CCAGC[A/G]CTGCCCTCCAGC CCCGGCCT TACGT
4	rs7749390	**Extron/Intron splice site**	• *Mycobacterium tuberculosis* • **Leishmaniasis** • **Ezema herpeticum caused**	C__25647357_10	CGACCCGGCCGCAGCCCTGCC GCGA[A/G]CGACGGTACCTGA GGACGG CCCCAG
5	rs3799488	**Intron**	• *Mycobacterium tuberculosis* • **leishmaniasis** • **Ezema herpeticum caused**	C__25647358_10	TAGCACTTCTTACCACAGAGA TCTA**[C/T]**GGGGAGAAAAATTG ATTAAA GATAA

Table 4: Gene expression levels studies among healthy, NIBD and IBD groups

IBD patients are 1.4fold downregulated (P =0.07) while NIBD patients are 1.2-fold downregulated (P=0.32).

Samples	**Mean of RT-PCR 2^(-ΔCT)*1000 Mean ± 95% CI**	**Fold change**	**P values**
Healthy (N = 30)	5.3 ± 2.4		
NIBD (N=14)	4.5 ± 0.9	-1.2 fold	0.32
IBD (N = 54)	3.8 ± 0.9	-1.4 fold	0.07

Table 5: Gene expression level studies comparing MAP positive and MAP negative samples among the same group, and comparison of samples with both MAP and SNP with MAP negative group.

MAP positivity alone is associated with 1 7-fold downregulation (P =0.06) and MAP and SNP positivity together is also associated with 1.7-fold downregulation (P=0.08).

Groups	**MAP Positive (Mean ± 95% CI)**	**MAP Negative**	**Fold change**	**P value**
Healthy	1.4 ± 1.1 (N = 3)	3.6 ± 2.1 (N = 21)	-2.6fold	0.2
NIBD	3.8 ± 5.9 (N = 3)	4.7 ± 0.8 (N = 11)	-1.2 fold	0.2
IBD	3.3 ± 1.8 (N = 10)	5.8 ± 2.4 (N = 14)	-1.7 fold	0.06
IBD (MAP + SNP)	3.4 ± 1.9 (N = 8)		-1.7 fold	0.08

Table 6: Gene expression level studies comparing SNP positive and SNP negative samples among the same group.

SNP positivity causes downregulation of 1.5-fold in IBD group (P = 0.1)

	SNP positive	**SNP negative**	**Fold change**	**P value**
Healthy	7.6 ± 7.3 (N = 6)	4.8 ± 2.6 (N = 24)	1.6-fold	0.2
NIBD	5.0 ± 0.9 (N = 7)	4.1 ± 1.7 (N = 7)	1.2-fold	0.1
IBD	4.6 ± 1.6 (N = 19)	6.9 ± 7.7 (N = 4)	-1.5-fold	0.1

Table 7: Serum IFNGR1 protein levels in healthy, NIBD and IBD groups.

IBD and NIBD groups are 1.53-fold (P =0.15) and 1.61-fold (P =0.21) lower than normal.

Sample	**IFNGR1 serum (pg/ml) Mean ± 95% CI**	**Fold change**	**P value**
Healthy (N=6)	810.8 ± 1024.5		
NIBD (N=9)	502.7 ± 263.2	-1.61-fold	0.21
IBD (N=24)	530.3 ± 190.5	-1.53-fold	0.15

Table 8: Serum IFNGR1 protein levels compared in MAP positive and MAP negative patients

Sample	MAP positive Mean ± 95% CI	MAP negative	Fold change	P value
Healthy	520.6 (N =1)	365 ± 208.6 (N = 4)	-1.4	
NIBD	465.1 ± 466 (N = 3)	478.9 ± 358.9 (N =7)	-1.0	0.5
IBD	419.7± 141.8 (N =8)	573.5 ± 333.9 (N =13)	-1.4	0.5

.

Table 9: Serum IFNGR1 protein levels in patients with existence of MAP and SNP together

Sample	MAP + SNP Mean ± 95% CI	Fold change	P value
Healthy	520.6 (N = 1)	-1.42	
NIBD	223.2 (N = 1)	-2.14	
IBD	377.7 ± 112.5 (N = 8)	-1.51	0.2

Table 10: Serum IFNGR1 protein levels in SNP positive and SNP negative patients

Sample	SNP positive	SNP negative	Fold change	P value
Healthy	440.7 ± 369.9 (N=3)	329.2 ± 703.2 (N = 3)	1.3	0.2
NIBD	476.1 ± 360.7 (N= 7)	471.7 ± 440.8 (N = 3)	1.0	0.98
IBD	512.7 ± 213.6 (N=20)	454.4 (N =2)	1.1	0.4

Table 11: Presence of MAP in different types of *IFNGR1* homozygous minor SNP.

In rs1327474, 1 out of 5 IBD patients with SNP (20%)) has MAP infection, compared to 0 out of 1 in healthy (0%). Rs1327475 is not detected in any of samples. Among rs2234711 positive samples, 71.4% of IBD patients have MAP infection compared to 0% in healthy and NIBD. Rs7749390 SNP positive samples have 16.7% of healthy, 33.3% of NIBD and 26.7% of IBD having MAP detected. Rs3799488 is detected only in IBD and 50% of them has MAP presence.

SNP	**Healthy (N = 32)**	**NIBD (N = 19)**	**IBD (N = 28)**	**P value**
rs1327474	0/1 (0%)	----	1/5 (20%)	0.62
rs1327475	---	----	---	---
rs2234711	0/5 (0%)	0/3 (0%)	5/7 (71.4%)	0.013*
rs7749390	1/6 (16.7%)	2/6 (33.3%)	4/15 (26.7%)	0.62
rs3799488	---	---	1/2 (50%)	---

LIST OF REFERENCES

1. Economou, M., E. Zambeli, and S. Michopoulos, *Incidence and prevalence of Crohn's disease and its etiological influences.* Annals of gastroenterology, 2009. **22**(3): p. 158-167.
2. Mekhjian, H.S., et al., *Clinical features and natural history of Crohn's disease.* Gastroenterology, 1979. **77**(4 Pt 2): p. 898-906.
3. Hoffmann, J.C. and M. Zeitz, *Treatment of Crohn's disease.* Hepato-gastroenterology, 1999. **47**(31): p. 90-100.
4. Liverani, E., et al., *Mycobacterium avium subspecies paratuberculosis in the etiology of Crohn's disease, cause or epiphenomenon.* World J Gastroenterol, 2014. **20**(36): p. 13060-13070.
5. Brand, S., *Crohn's disease: Th1, Th17 or both? The change of a paradigm: new immunological and genetic insights implicate Th17 cells in the pathogenesis of Crohn's disease.* Gut, 2009. **58**(8): p. 1152-1167.
6. Cummings, J., et al., *Confirmation of the role of ATG16L1 as a Crohn's disease susceptibility gene.* Inflammatory bowel diseases, 2007. **13**(8): p. 941-946.
7. Parkes, M., et al., *Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility.* Nature genetics, 2007. **39**(7): p. 830-832.
8. Van Limbergen, J., D.C. Wilson, and J. Satsangi, *The genetics of Crohn's disease.* Annual review of genomics and human genetics, 2009. **10**: p. 89-116.

9. Sharp, R.C., et al., *Genetic Variations of PTPN2 and PTPN22: Role in the Pathogenesis of Type 1 Diabetes and Crohn's Disease.* Frontiers in cellular and infection microbiology, 2015. **5**.

10. Ng, S.C., et al., *Environmental risk factors in inflammatory bowel disease: a population-based case-control study in Asia-Pacific.* Gut, 2014: p. gutjnl-2014-307410.

11. Kamada, N., et al., *Role of the gut microbiota in immunity and inflammatory disease.* Nat Rev Immunol, 2013. **13**(5): p. 321-335.

12. Greenstein, R.J., *Is Crohn's disease caused by a mycobacterium? Comparisons with leprosy, tuberculosis, and Johne's disease.* The Lancet infectious diseases, 2003. **3**(8): p. 507-514.

13. Ellingson, J.L., et al., *Detection of viable Mycobacterium avium subsp. paratuberculosis in retail pasteurized whole milk by two culture methods and PCR.* Journal of food protection, 2005. **68**(5): p. 966-972.

14. Naser, S.A., D. Schwartz, and I. Shafran, *Isolation of Mycobacterium avium subsp paratuberculosis from breast milk of Crohn's disease patients.* The American journal of gastroenterology, 2000. **95**(4): p. 1094.

15. Naser, S.A., et al., *Culture of Mycobacterium avium subspecies paratuberculosis from the blood of patients with Crohn's disease.* The Lancet, 2004. **364**(9439): p. 1039-1044.

16. Feller, M., et al., *Mycobacterium avium subspecies paratuberculosis and Crohn's disease: a systematic review and meta-analysis.* The Lancet Infectious Diseases, 2007. **7**(9): p. 607-613.

17. Sartor, R.B., *Does Mycobacterium avium subspecies paratuberculosis cause Crohn's disease?* Gut, 2005. **54**(7): p. 896-898.

18. Sechi, L.A., et al., *Detection and isolation of Mycobacterium avium subspecies paratuberculosis from intestinal mucosal biopsies of patients with and without Crohn's disease in Sardinia.* The American journal of gastroenterology, 2005. **100**(7): p. 1529-1536.

19. Romero, C., et al., *Evaluation of surgical tissue from patients with Crohn's disease for the presence of Mycobacterium avium subspecies paratuberculosis DNA by in situ hybridization and nested polymerase chain reaction.* Inflammatory bowel diseases, 2005. **11**(2): p. 116-125.

20. El-Zaatari, F.A., et al., *Characterization of Mycobacterium paratuberculosis p36 antigen and its seroreactivities in Crohn's disease.* Current microbiology, 1999. **39**(2): p. 115-119.

21. Naser, S.A., et al., *Mycobacterium avium subspecies paratuberculosis causes Crohn's disease in some inflammatory bowel disease patients.* World J Gastroenterol, 2014. **20**(23): p. 7403-15.

22. Qasem, A., et al., *Oxidative stress due to Mycobacterium avium subspecies paratuberculosis (MAP) infection upregulates selenium-dependent GPx activity.* Gut pathogens, 2016. **8**(1): p. 12.

23. Qasem, A., M. Safavikhasraghi, and S.A. Naser, *A single capsule formulation of RHB-104 demonstrates higher anti-microbial growth potency for effective treatment of Crohn's disease associated with Mycobacterium avium subspecies paratuberculosis.* Gut Pathogens, 2016. **8**(1): p. 45.

24. Mendoza, J.L., R. Lana, and M. Díaz-Rubio, *Mycobacterium avium subspecies paratuberculosis and its relationship with Crohn's disease.* World J Gastroenterol, 2009. **15**(4): p. 417-22.

25. Fais, S., et al., *Spontaneous release of interferon gamma by intestinal lamina propria lymphocytes in Crohn's disease. Kinetics of in vitro response to interferon gamma inducers.* Gut, 1991. **32**(4): p. 403-407.

26. Jouanguy, E., et al., *A human IFNGR1 small deletion hotspot associated with dominant susceptibility to mycobacterial infection.* Nature genetics, 1999. **21**(4): p. 370-378.

27. Koch, O., et al., *IFNGR1 gene promoter polymorphisms and susceptibility to cerebral malaria.* Journal of Infectious Diseases, 2002. **185**(11): p. 1684-1687.

28. Mohamed, H., et al., *Genetic susceptibility to visceral leishmaniasis in The Sudan: linkage and association with IL4 and IFNGR1.* Genes and immunity, 2003. **4**(5): p. 351.

29. Naka, I., et al., *IFNGR1 polymorphisms in Thai malaria patients.* Infection, Genetics and Evolution, 2009. **9**(6): p. 1406-1409.

30. de Silva, K., et al., *Lymphoproliferative and Gamma Interferon.* 2014.

31. Schroder, K., et al., *Interferon-γ: an overview of signals, mechanisms and functions.* Journal of leukocyte biology, 2004. **75**(2): p. 163-189.

32. Le Coniat, M., et al., *Human interferon gamma receptor 1 (IFNGR1) gene maps to chromosome region 6q23–6q24.* Human Genetics, 1989. **84**(1): p. 92-94.

33. Huang, S., et al., *Immune response in mice that lack the interferon-gamma receptor.* Science, 1993. **259**(5102): p. 1742-1746.

34. Altare, F., et al., *A causative relationship between mutant IFNgR1 alleles and impaired cellular response to IFNgamma in a compound heterozygous child.* American journal of human genetics, 1998. **62**(3): p. 723.

35. Naderi, M., et al., *Association of genetic polymorphisms of IFNGR1 with the risk of pulmonary tuberculosis in Zahedan, Southeast Iran.* Tuberculosis research and treatment, 2015. **2015**.

36. Salih, M.A., et al., *IFNG and IFNGR1 gene polymorphisms and susceptibility to post-kala-azar dermal leishmaniasis in Sudan.* Genes and immunity, 2007. **8**(1): p. 75-78.

37. Lü, J., et al., *Genetic polymorphisms of IFNG and IFNGR1 in association with the risk of pulmonary tuberculosis.* Gene, 2014. **543**(1): p. 140-144.

38. He, J., et al., *Analysis of functional SNP in ifng/ifngr1 in Chinese Han population with tuberculosis.* Scandinavian journal of immunology, 2010. **71**(6): p. 452-458.

39. Gao, P., et al., *Interferon-gamma (IFNG) and IFNGR1 Gene Variants are Associated with Atopic Dermatitis Complicated by Eczema Herpeticum (ADEH) and IFN Production.* Journal of Allergy and Clinical Immunology, 2011. **127**(2): p. AB137.

40. Francisco, M., et al., *Assessment of two flexible and compatible SNP genotyping platforms: TaqMan® SNP Genotyping Assays and the SNPlex™ Genotyping System.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2005. **573**(1): p. 111-135.

41. Cannom, R.R., et al., *Inflammatory bowel disease in the United States from 1998 to 2005: has infliximab affected surgical rates?* The American surgeon, 2009. **75**(10): p. 976-980.

42. Amiot, A. and L. Peyrin-Biroulet, *Current, new and future biological agents on the horizon for the treatment of inflammatory bowel diseases.* Therapeutic Advances in Gastroenterology, 2014. **8**(2): p. 66-82.