

**ROLE OF MYCOBACTERIUM AVIUM PARATUBERCULOSIS (MAP)  
AND *TNFSF15* SNPS ON TL1A IN CD**

by

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## ABSTRACT

Tumor Necrosis Factor-Like Ligand 1a (TL1A) is a cytokine encoded by Tumor Necrosis Factor Super Family 15 gene (TNFSF15) gene mostly in endothelial cells which binds to T-cells and foments the production of pro-inflammatory cytokines including TNF- $\alpha$ , IL-6, IL-1b, IFN-  $\gamma$  and IL-13. TL1A level is elevated in inflammatory diseases including Crohn's Disease (CD). Although Single Nucleotide Polymorphisms (SNPs) in TNFSF15 have been reported in CD, no studies have investigated the effect of these SNPs on TL1A, inflammation, and susceptibility to Mycobacterium avium subspecies paratuberculosis (MAP) infection. MAP is a strong candidate in CD pathogenesis. This study is designed to elucidate the combined effect of MAP and SNPs in TNFSF15 (rs4263839, rs7848647, rs6478108, or rs6478109) on TL1A secretion and downstream effect on pro-inflammatory cytokines. Peripheral blood from CD and healthy subjects was analyzed for MAP DNA, TNFSF15 genotyping, circulating TL1A level, and IFN-  $\gamma$  and TNF- $\alpha$  gene expression. Our data is first to report that rs4263839, rs7848647, rs6478108, and rs6478109 in TNFSF15 resulted in increase in circulating TL1A level in healthy and CD samples. Specifically, in CD samples with rs7848647, the average TL1A level was 146.9 pg/mL  $\pm$  124.5 compared 62.4 pg/mL  $\pm$  82.8 in normal samples. Similarly, TL1A level in CD samples with rs6478109 was 141.9 pg/mL  $\pm$  127.7 compared to 71.5 pg/mL  $\pm$  88.4 in normal samples ( $p < 0.05$ ). All 4 SNPs resulted in significant elevation in TL1A level in healthy samples ( $p < 0.05$ ). Moreover, IFN- $\gamma$  expression was significantly higher, by approximately 1.6-fold in CD patients with SNPs relative to CD patients with no SNPs

( $p < 0.05$ ). Interestingly, SNPs in TNFSF15 had no significant effect on TNF- $\alpha$  expression. MAP was detected in the blood of 63% of CD compared to 6% healthy subjects ( $p < .001$ ). The data did not support a correlation between MAP presence and circulating TL1A levels, and no correlation between SNPs in TNFSF15 and MAP susceptibility. This study strongly suggests, that SNPs in TNFSF15 increase TL1A levels and may be a contributory factor to the inflammation experienced by CD patients. Over all, the study emphasizes the need for a pharmacogenomic approach in treatment delivery for patients with CD by using TNFSF15 SNPs to identify patients that would benefit from biologics targeting TL1A rather than TNF- $\alpha$  for more efficacious treatment regimens for CD patients.

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## LIST OF ABBREVIATIONS

<b>CD</b>	Crohn's Disease
<b>DR3</b>	Death Receptor 3
<b>ELISA</b>	Enzyme-linked immunosorbent assay
<b>GI</b>	Gastrointestinal
<b>IBS</b>	Irritable Bowel Syndrome
<b>IFN-<math>\gamma</math></b>	Interferon gamma
<b>IL-10</b>	Interleukin 10
<b>IL-1a</b>	Interleukin 1a
<b>IL-6</b>	Interleukin 6
<b>MAP</b>	<i>Mycobacterium avium Paratuberculosis</i>
<b>MAPK</b>	Mitogen-Activated Protein Kinase
<b>NF-<math>\kappa</math>B</b>	Nuclear Factor kappa-light-chain-enhancer of activated B cells
<b>RA</b>	Rheumatoid Arthritis
<b>SNPs</b>	Single Nucleotide Polymorphisms
<b>TB</b>	Tuberculosis
<b>TH1 Cells</b>	Type 1 Helper T-Cells
<b>TL1A</b>	TNF-like ligand 1A (TL1A)
<b>TNFSF15</b>	Tumor Necrosis Factor Superfamily 15
<b>TNF-<math>\alpha</math></b>	Tumor Necrosis Factor-Alpha

## CHAPTER 1: INTRODUCTION

### ***Crohn's Disease***

Crohn's Disease is chronic condition of the human gastrointestinal (GI) tract that can affect patients anywhere from mouth to anus. The disease is characterized by non-contiguous granulomatous lesions present in the GI tract, as well as general symptoms including, but not limited to, diarrhea, rectal bleeding, abdominal pain, weight loss, anal fistula formation, fatigue, and inflammation of the skin, eyes, joints, liver and/or bile ducts (Mayo Clinic). CD is a very physically and psychologically debilitating disease, but it is also associated with a significant financial burden of approximately \$8000 USD per patient annually, one third of which stems from hospitalization costs (Feagan et al 2000). The cause of CD is unknown, but research has indicated a significant correlation between the presence of *Mycobacterium avium subspecies paratuberculosis* (MAP) and CD and has led to the idea that MAP may play a causative or contributory role in CD pathogenesis (Naser et al 2004, Scanu et al 2007). MAP DNA has been identified in a majority of CD patients' intestinal biopsies, up to 87%, but has only been found in a small subset of healthy samples of intestinal biopsies, up to 15%, of healthy samples (Naser et al 2010, Scanu et al 2007, Bull et al 2003). It has also been long hypothesized that MAP may play a role as an infectious trigger of the immune response typically seen in CD (Chamberlin et al 2001, Naser et al 2014). Current approved treatments for CD are limited to symptom alleviation and thusly must be taken continuously such as corticosteroids, biologics, and analgesics (Dignass et al 2010).

### ***TNF-like ligand 1A (TL1A) in Inflammation***

The role *Tumor Necrosis Factor Superfamily 15* (TNFSF15) gene in CD-associated inflammation has recently come into the spotlight due to the correlation of single nucleotide polymorphisms (SNPs) within *TNFSF15* with many inflammatory diseases including rheumatoid arthritis (RA), CD, and Irritable Bowel Syndrome (IBS) (Yamakazi et al 2005). *TNFSF15* encodes for TNF-like ligand 1A (TL1A) which is a cytokine mainly secreted by endothelial cells and Lamina Propria macrophages in response to the presence of TNF- $\alpha$  and IL-1 $\alpha$  and binds to its cognate receptor, death receptor 3 (DR3) (Migone et al 2002, Kim et al 2005, Kamada et al 2009). DR3 is mainly located on the external surface of lymphocytes and, once conjugated to TL1A, leads to the activation of the pro-inflammatory P38 MAPK and the NF- $\kappa$ B pathways (Shih et al 2009). The stimulation of naïve T-cell DR3 receptors with TL1A leads to Th1 polarization of the naïve T-cells and induces the secretion of various pro-inflammatory cytokines such as IFN- $\gamma$ , TNF- $\alpha$ , IL-5, and IL-13 (IL-13 has specifically been found to play a direct role in the induction of intestinal inflammation) (Kamada et al 2009, Shih et al 2009, Meylan et al 2011).

It has been noted that TL1A levels and DR3 levels are both higher in intestinal tissue samples from CD patients (Bamias et al 2003). It was also demonstrated that elevated levels of TL1A and DR3 are present in murine models of intestinal inflammation (Bamias 2006). This data suggests a possible role for TL1A in the pathogenesis of intestinal inflammation as a potential local mediator of inflammation. While the use of murine models is not sufficient to infer pathophysiological processes in humans, it

demonstrates the potential for TL1A's behavior in humans. To date, no one has investigated the effect of SNPs in *TNFSF15* on circulating TL1A levels in CD.

The presence of MAP bacterial DNA has also been found to be significantly elevated in the blood, milk, and intestinal tissue samples of CD patients (Naser et al 2000). Recent work in our lab has demonstrated the ability of MAP to induce TNF- $\alpha$  secretion (which induces the secretion of TL1A by endothelial cells) by macrophages (data not published). The effect of MAP infection on TL1A levels in CD patients has not been investigated.

### ***Limitations of Approved CD Therapies***

While TL1A has been implicated in the inflammatory response as well as the severity of inflammation in CD, limited studies have been designed to analyze the effects of MAP infection on TL1A (Bamias et al 2003). Caprioli et al have reported that over one-third of patients taking TNF- $\alpha$  inhibitors have developed a resistance to the drug due to neutralizing antibodies targeting the TNF- $\alpha$  inhibitors (Antoni et al 2002). TNF- $\alpha$  inhibitors, such as infliximab, certolizumab, and adalimumab have also shown to be associated with elevated levels of bacterial infections, especially granulomatous infections such as Tuberculosis (Coussens et al 2004). Clinical data and research clearly show the deleterious effects of knocking out an integral and ubiquitous cytokine such as TNF- $\alpha$ , especially when patients regularly do not respond (approximately 35% of patients are non-responders to anti-TNF- $\alpha$  therapy) and approximately 61% of patients develop antibodies that diminish the effects of the anti-TNF- $\alpha$  therapies after approximately 10 months of treatment (Targan et al 1997, Baert et al 2003). Furthermore, MAP is a

Mycobacterium that foments granulomatous infection (much like *Mycobacterium tuberculosis* (TB)) in ruminants that greatly resembles Crohn's Disease (CD) and is thought to be a contributing factor to CD pathogenesis (Coussens et al 2004). Due to the similarities between TB pathogenesis and MAP pathogenesis in ruminants, treating patients with TNF- $\alpha$  inhibitors may lead to an exacerbation of the MAP infection present in CD patients, which may further aggravate the disease.

### ***Elucidating the Effect of MAP and TNFSF15 SNP's on TL1A***

This study is designed to determine if MAP is involved in the elevation of TL1A levels in CD patients, in an attempt to discover possible future treatments for CD-associated inflammation. Moreover, by determining what effect the SNP's in *TNFSF15* have on TL1A levels, we can determine a pharmacogenomic approach in which patients with *TNFSF15* mutations are given medications that target TL1A rather than TNF- $\alpha$ , which may be able to reduce intestinal inflammation without the elevated risk of infection that is present with TNF- $\alpha$  inhibitors. The pharmacogenomic approach may also be able to yield a more effective reduction in inflammation as it targets the specific cytokine implicated in the inflammation, rather than a ubiquitous cytokine that has many downstream effectors, such as TNF- $\alpha$ . Demonstrating the effects of MAP on patient TL1A levels would also aid in elucidating the mechanism by which MAP might contribute to inflammation in the intestines of CD patients and yield a better understanding of the pathogenic behavior of MAP.

Yamazaki et al/ also demonstrated a correlation between single nucleotide polymorphisms (SNPs) in *TNFSF15* and CD (Yamakazi et al 2005). While these

mutations are all present in intragenic regions, it has recently been demonstrated that mutations in non-coding regions can exert a physiological influence (Lue et al 2015). Intragenic mutations have been found in diseases ranging from the hepatitis C virus to non-small cell lung carcinoma and may exert their pathogenic influence via the modulation of transcription factor binding affinity, modification of gene splice sites and by causing mRNA degradation by causing reduced stability in the untranslated regions of mRNA (Lue et al 2015, Chin et al 2008). A recent study has found that mutations in TNFSF15 may contribute to elevated TL1A secretion by immune complex-stimulated monocytes in the Jewish population, but no research has been performed to examine the circulating TL1A levels of patients with SNPs in TNFSF15 in the general CD population (Michelson et al 2009). It is possible that these mutations may contribute to higher baseline levels of circulating TL1A which may contribute to the overzealous immune response present in the diseases associated with the presence of these mutations.

In light of the current literature gaps, we aimed to determine how genetic variations in TNFSF15 with and without MAP presence may alter TL1A levels in CD patients. The current study will focus on 4 different SNPs, all found in the intronic region of TNFSF15 gene (Table 1). The overall goal is an attempt to discover possible future treatments for CD-associated inflammation. Moreover, by determining what effect, if any, the SNP's in TNFSF15 have on TL1A levels, we can determine a pharmacogenomic approach in which patients with TNFSF15 mutations are given medications that target TL1A rather than TNF- $\alpha$  which may be able to reduce intestinal inflammation without the elevated risk of infection that is present with TNF- $\alpha$  inhibitors. The pharmacogenomic approach may also be able to yield a more effective reduction in inflammation as it targets the specific



cytokine implicated in the inflammation, rather than a ubiquitous cytokine that has many downstream effectors, such as TNF- $\alpha$ . Demonstrating the effects of MAP on patient TL1A levels would also aid in elucidating the mechanism by which MAP might contribute to exacerbated inflammation in the intestines of CD patients which we hope will yield a better understanding of the pathogenic behavior of MAP.

### ***Hypothesis***

In this study, we hypothesize that:

SNPs in TNFSF15 cause elevated circulating levels of TL1A which increases susceptibility to inflammation and MAP infection

## CHAPTER 2: MATERIALS AND METHODS

### ***Clinical Samples***

Peripheral blood was obtained from a total of 21 healthy volunteers and 34 CD volunteers by our clinical collaborators, the Digestive and Liver Center of Florida, as well as from Dr. Naser's lab, as approved by the UCF IRB #IRB00001138. All volunteers provided informed, written consent prior to donation. The volunteers donated a total of two 4.0-mL K2-EDTA coded blood tubes. One tube was used to determine the presence of MAP DNA. The other tube was used to derive plasma for TL1A ELISA analysis, DNA for SNP genotyping, and RNA for gene expression analysis via real time RT-PCR.

### ***DNA Extraction and MAP Detection via Nested PCR***

Whole blood was centrifuged at 3000 rpm for 15 minutes at room temperature to stratify the blood and isolate the white blood cell layer. Isolated white blood cells were then cultured in BD Bactec™ MGIT™ Para-TB medium for 6 months at 37 °C. Mycobacterial growth was first determined by UV illumination. After the UV illumination, mycobacterial presence was measured via nPCR as described earlier (Qasem et al 2016). One milliliter was used for DNA extraction and nested PCR (nPCR) analysis also as described previously (Qasem et al 2016). Briefly, MAP presence was determined via a nested (two-round) PCR utilizing primers specific for a conserved MAP-insertional element as previously discussed (Naser et al 2004). The first round of amplification used the P90 (GTTCGGGGCCGTCGCTTAGG) and P91 (GAGGTCGATCGCCCACGTGA) primers which resulted in the amplification of a 398-base pair (bp) fragment of the IS900 MAP-specific insertional element. The second nested PCR round consisted of the AV1

(ATGTGGTTGCTGTGTTGGATGG) and the AV2 (CCGCCGCAATCAACTCCAG) primers which generated a 298 bp fragment of the IS900 MAP-specific insertional element. The PCR samples were then run on a 2% agarose gel to visualize the presence of a 298 bp band. If a 298 bp band was present, then it was inferred that MAP DNA was also present in the patient sample.

### ***Quantitation of Circulating TL1A Levels via ELISA***

Whole blood samples were centrifuged at 30,000 rpm for 15 minutes to separate the plasma layer from the erythrocyte layer of the samples. Human TL1A ELISA Kit from LS Bio™ was used to determine the plasma protein levels according to manufacturer's protocol. Briefly, patient plasma samples were placed in ELISA kit wells in duplicates. The plasma was then incubated and washed as instructed by manufacturer's protocol (LSBio). After the incubation and wash steps were completed, the plate was read via a colorimetric plate reader using a SkanIt™ Microplate reader from Thermo Scientific™ at 450 nm wavelength.

### ***TNFSF15 SNP Presence Determination via TaqMan Genotyping***

DNA was isolated and purified from peripheral blood leukocytes via the QIAamp® DNA Blood Mini Kit (Qiagen™) following the manufacturer's instructions. TaqMan™ genotyping assays (Applied Biosystems™) for TNFSF15 SNPs rs6478108, rs6478109, rs7848647, and rs4263839 were performed on the isolated and purified DNA according to manufacturer's protocol at the University of Florida Pharmacotherapy and Translational Research Department (Gainesville, FL). Briefly, the reaction utilizes anti-parallel probes that hybridize at precise SNP locations on the specific gene. Fluorophores are attached

to the probes as well as a quencher. If a probe hybridizes, then the 5' to 3' exonuclease activity of Taq polymerase then cleaves the quencher and allows for the generation of a fluorescent signal thus allowing visual confirmation of the nucleotide present at the SNP location.

**Table 1:** Description of the studied SNPs.

<b>SNP Tested</b>	<b>Gene</b>	<b>SNP Type</b>	<b>Disease Association</b>
<b>rs6478108</b>	TNFSF15 Gene Superfamily (Chromosome 9)	Intron	CD, Leprosy, and IBS-D
<b>rs6478109</b>	TNFSF15 Gene Superfamily (Chromosome 9)	Intron	Arthritis, CD, Ulcerative Colitis (UC), and IBS-D
<b>rs7848647</b>	TNFSF15 Gene Superfamily (Chromosome 9)	Intron	CD, IBS-D, Arthritis
<b>rs4263839</b>	TNFSF15 Gene Superfamily (Chromosome 9)	Intron	IBS-C, CD, and UC

### ***Gene Expression Analysis of TNFSF15, TNF- $\alpha$ , and IFN- $\gamma$***

RNA was isolated and purified from whole blood samples using the TRIzol® reagent (Invitrogen) as recommended by the manufacturer. The isolated and purified RNA was then used for cDNA synthesis. The cDNA was then used to determine gene expression of TNF- $\alpha$ , and IFN- $\gamma$  via real time RT-PCR. Briefly, whole blood samples were centrifuged at 1000 rpm for 5 minutes and the resulting pellet (containing leukocytes) was suspended in TRIzol®. After, chloroform was added (to precipitate proteins). The sample was then centrifuged and the aqueous phase (containing RNA) was isolated. The isolated aqueous phase was then incubated with 100% isopropanol (to precipitate the RNA) and centrifuged. The supernatant was removed, and ethanol was added to wash the RNA and the sample was centrifuged once again. The RNA pellet was then left to air dry for 15-30 minutes and finally heated in RNase-free water at 60°C for 10 minutes.

cDNA synthesis was performed by 600 ng of RNA to 0.2 mL PCR reaction, 4 uL of iScript™ Reverse Transcription (Bio-Rad®), and 20 uL of RNase free water. The reaction tubes were then placed in the thermal cycler (MyGene™ Series Pelteir Thermal Cycler), which were run at 25°C for 5 minutes, then at 46°C for 20 minutes, and then at 95°C for 1 minute. After cDNA synthesis, 30 ng of cDNA (approximately 1 uL) are mixed with 10 uL of Fast SYBR Green Mastermix (Thermofisher Scientific®), 1 uL of either *TNFA* or *IFNG* PrimePCR SYBER Green Assay mix (Bio-Rad®), and 8 uL of biological grade sterile water. The controls consisted of a 18s rRNA gene (forward primer: 5'-GTA ACC CGT TGA ACC CCA TT-3'; reverse primer: 5'-CCA TCC AAT CGG TAG TAG CG-3'). The controls were used to obtain baseline readings to determine the relative mRNA expression of the target genes. The system used for the real time RT-PCR was the 7500 Fast Real-Time PCR System (Applied biosystems®). The formula used to determine relative mRNA expression is  $(2^{-\Delta CT}) \times 1000$  where  $\Delta CT = \text{sample reading} - \text{control reading}$  (18s rRNA).

### ***Statistical Analysis***

Statistical analysis was performed using GraphPad Prism® 7.02. Data was analyzed for significance using the Students T-Test. P-values less than 0.05 were considered statistically significant, unless otherwise noted.

## CHAPTER 3: RESULTS

### ***Disease State and TNFSF15 SNPs***

Whole blood from participant samples was used for TaqMan™ genotyping (Applied Biosystems™) to determine the presence or absence of *TNFSF15* SNPs and then correlated to disease state. There appeared to be no significant association between the presence of the studied *TNFSF15* SNPs (rs4263839, rs7848647, rs6478108, rs6478109) and disease state. Approximately 45% of healthy samples had rs4263839 and 45% of healthy samples had rs7848647, while approximately 42% and 51% of CD samples had rs4263839 and rs7848647, respectively. Furthermore, approximately 45% of healthy samples had rs6478108 and 45% had rs6478109, while approximately 44% and 47% of CD samples had rs6478108 and rs6478109, respectively (figure1).

### ***Disease State and TL1A Levels***

Plasma was isolated from participant samples and used for Human TL1A ELISA analysis to determine circulating TL1A levels. Circulating TL1A levels were then correlated to disease state. Overall, TL1A levels in CD samples were 103.26 pg/mL,  $\pm 111.8$  compared to 64.2 pg/mL  $\pm 123.7$  in healthy controls ( $p < 0.05$ ). TL1A levels in CD samples with no SNPs was  $54.3 \pm 79.02$  pg/mL compared to  $4.7 \pm 14.0$  pg/mL, in healthy samples with no SNPs (figure 2).

### ***Effect of TNFSF15 SNPs on TL1A Levels***

Circulating TL1A levels and presence, or absence, of SNPs were then analyzed to determine the presence of a correlation. In each sample group, healthy and CD, TL1A levels were elevated in the presence of SNPs in *TNFSF15* when compared to the lack of SNPs in *TNFSF15* (figure 3). TL1A levels in healthy samples with at least one SNP was  $108.81 \pm 150.3$  pg/mL, compared to  $4.7 \pm 14.0$  pg/mL healthy samples with no SNPs (figure 4). When examining each SNP individually, the results were exactly the same throughout and there appears to be a linkage between the 4 examined SNPs (rs4263839, rs7848647, rs6478108, rs6478109) (figure 5).

Patients with CD also demonstrated significantly elevated levels of TL1A in the presence of SNPs in *TNFSF15*. Patients with at least one of the studied SNPs (rs4263839, rs7848647, rs6478108, rs6478109) had circulating TL1A levels of  $149.1 \pm 120.6$  pg/mL while CD samples with no SNPs demonstrated TL1A levels of  $54.3 \pm 79.01$  pg/mL (figure 6). Furthermore, CD samples demonstrated significantly elevated TL1A levels in concurrence with the presence of individual SNPs rs7848647 and rs6478109. In the presence of SNP rs7848647, CD samples had average TL1A levels of  $146.9 \pm 124.5$  pg/mL, while CD samples without rs7848647 demonstrated average TL1A levels of  $62.4 \pm 82.8$  pg/mL. In the presence of rs6478109 average circulating TL1A levels were  $141.9 \pm 127.7$  pg/mL while CD samples without rs6478109 demonstrated average TL1A levels of  $71.5 \pm 88.4$  pg/mL (figure 7).

### ***Effect of SNPs in TNFSF15 on MAP Infection Presence***

DNA was isolated from participant whole blood and used for nPCR determination of MAP presence. The presence of MAP in samples was then correlated to disease state. The presence of MAP in CD patients was approximately 63% while the presence of MAP in healthy samples was approximately 6%, which yields a very significant association between MAP presence and CD ( $p < .001$ ) (figure 8).

The presence and absence of SNPs was then compared to the presence and absence of MAP DNA in all patient samples, regardless of disease. Analysis revealed that approximately 50% of overall samples without SNPs were MAP-positive while approximately 30% of samples with at least one SNP were MAP-positive (figure 9). While it appears that samples without SNPs, demonstrated elevated presence of MAP DNA in samples, there was no statistically significant association between the presence or absence of SNPs and the presence or absence of MAP DNA in patient samples.

Further analysis was conducted by examining MAP presence and SNP presence in CD samples only. It was revealed that about 55% of CD samples with SNPs were MAP-positive while approximately 35% of CD samples with SNPs were MAP-negative (figure 10). This data supports an association between the presence of SNPs in *TNFSF15* and MAP presence in CD samples.

Analysis to examine the association between SNPs in *TNFSF15* and MAP presence in healthy samples, was also performed. It was noted that no samples healthy had both, SNPs in *TNFSF15* and MAP, concurrently (figure10).



### ***Effect of MAP on circulating TL1A levels***

While analyzing all samples, regardless of disease state, samples with MAP DNA demonstrated average TL1A levels of  $87.04 \pm 116.1$  pg/mL while samples without MAP DNA demonstrated average TL1A levels of  $90.4 \pm 118.8$  pg/mL (figure 11). There was no significant association present between the presence of MAP DNA and TL1A levels.

Further analysis was done to eliminate the confounding variable of disease state. Average TL1A levels in CD samples that are MAP-negative were  $118.9 \pm 101.2$  pg/mL while average TL1A levels of MAP-positive CD samples were  $91.9 \pm 120.5$  pg/mL (figure 11).

Analysis examining the effect of MAP presence on circulating TL1A levels in healthy samples yielded interesting results. The levels of TL1A in MAP-negative healthy samples was  $70.9 \pm 128.4$  pg/mL, while the average TL1A levels in MAP-positive healthy samples were  $0 \pm 0$  pg/mL. There were only 2 samples that had MAP DNA within the healthy samples, which may have contributed to the current results (figure 11).

### ***The Effect of Concurrent SNP and MAP on TL1A***

The concurrent presence of SNPs and MAP was then correlated to circulating TL1A levels, in all samples, regardless of disease state. The analysis revealed that samples with both, MAP and SNP present had significantly elevated circulating TL1A levels. Specifically, TL1A levels were  $148.23 \pm 138.1$  pg/mL which is almost 5-fold higher compared to normal and MAP-free samples which had TL1A levels of  $30.28 \pm 63.3$  pg/mL. TL1A levels of CD samples with SNP and MAP were  $145.2 \pm 147.3$  pg/mL which is

approximately 2-fold higher than TL1A level in CD samples with no SNPs or MAP, which were  $64.3 \pm 90.4$  pg/mL (figure 12).

However, when examining all samples (healthy and CD) without SNPs but with MAP, TL1A levels were  $41.1 \pm 72.6$  pg/mL, while SNP only samples (MAP-negative, but SNP positive) had TL1A levels of  $126.5 \pm 130.6$  pg/mL.

When analyzing all samples, regardless of disease state, it was demonstrated that SNP-only samples had significantly elevated circulating levels of TL1A that were about 3 times as high as MAP only samples. When comparing MAP-only CD samples, which had average TL1A levels of  $49.4 \pm 77.4$  pg/mL, to CD samples with SNP only, which had average TL1A levels of  $153.1 \pm 97.1$  pg/mL (figure 12).

When analyzing healthy samples only, it was observed that there was a statistically significant elevation in average circulating TL1A levels when comparing the presence of SNP-only samples, which had TL1A levels of  $108.8 \pm 150.3$  pg/mL, to samples with no SNP and no MAP, which had TL1A levels of  $6 \pm 15.9$  pg/mL. There were no samples that had both, SNP and MAP, concurrently present and there were only two samples in the MAP-only group (figure 12).

### ***Effect of SNPs on IFNG and TNFA***

To further corroborate these findings, additional whole blood was obtained from participants with CD and used to determine relative mRNA levels of *IFNG* in buffy coat isolates. CD samples with any of the examined SNPs in *TNFSF15* had average mRNA levels of *IFNG* that were significantly elevated in relation to CD samples with no SNPs (1.7-fold increase in CD samples with SNP vs CD samples without SNP) (Figure 13).

Surprisingly, CD samples with SNPs demonstrated a 1.1-fold decrease in *TNFA* expression, when compared to CD samples with no SNPs which yielded no significant difference (Figure 14).

## CHAPTER 4: DISCUSSION

Crohn's Disease is a debilitating chronic condition with a high financial burden. Patients pay approximately \$8,000 USD, approximately one third which is attributable to hospitalization costs and approximately another third is attributable to pharmaceutical costs (Michelsen et al 2009). This comes as no surprise given that 4 of the top 15 most expensive medications, according to searchrx.com (a prescription price search site), are CD treatments. These medications can cost as much as \$4,700 USD a month without insurance. With insurance, the average out-of-pocket cost for biologics used for CD treatment is approximately \$4,300 (Karaca-Mandic et al 2010). Another issue with the treatment of CD using biologics is the amount of non-response that occurs, in fact, almost half of initial therapy responders develop an auto-immune resistance to biologic treatment (Caprioli et al 2011). It is believed that specific SNPs may contribute to lack of response to biologics by modulating the efficiency of the TNF- $\alpha$  pathways, the main target of CD biologics (Suryaprasaad et al 2003).

Our research aims to elucidate the presence of additional SNPs that may play a contributory role in non-response to TNF- $\alpha$  inhibitors in CD patients. SNPs in TNFSF15 have recently been demonstrated to be correlated to the IBD however, the underlying mechanism of the correlation has not been elucidated (Yamakazi et al 2005). Our research has indicated that the presence of any of our four studied SNPs (rs4263839, rs7848647, rs6478108, rs6478109) is correlated to elevated levels of TL1A. Overall, CD samples with no SNPs demonstrated lower levels of TL1A (54.3 pg/mL) compared to CD samples with at least one of the four tested SNPs (149.1 pg/mL) ( $P < 0.05$ ). Further analysis was conducted to determine the impact individual SNPs had on circulating TL1A

levels. It was found that rs7848647 is correlated to higher levels of TL1A in CD samples (146.9 pg/mL) when compared to CD samples without rs7848647 (62.4 pg/mL) ( $P < 0.05$ ). Likewise, it was demonstrated that CD samples with rs6478109 had elevated levels of circulating TL1A (141.9 pg/mL) compared to samples without rs6478109 (71.5 pg/mL) ( $P < 0.05$ ). This data indicates that SNPs in TNFSF15 are linked to elevated levels of the pro-inflammatory cytokine TL1A.

Further analysis was conducted examining the effects of SNPs on healthy samples. Our research revealed a similar pattern to CD samples in that healthy samples with at least one of the four tested SNPs also had elevated circulating levels of TL1A (108.81 pg/mL) compared to healthy samples that did not have any of the tested SNPs (4.7pg/mL) ( $P < 0.05$ ). Next, we analyzed the effect of each individual SNP on circulating TL1A levels which revealed an interesting piece of information; there was the same exact significant correlation between the presence of any one SNP (rs4263839, rs7848647, rs6478108, or rs6478109) and elevated TL1A levels. This appears to be the case because within the healthy population each sample either had none of the tested SNPs or all four of the tested SNPs. In fact, within our CD samples approximately 85% (50% had 0 SNPs while 35% had 4 SNPs) of our samples had either none of the tested SNPs or all four of the tested SNPs concurrently. Sun et al. was able to demonstrate linkage disequilibrium between rs6478108 and rs6478109 while recent research by Zucchelli et al demonstrated a linkage disequilibrium between rs4263839 and rs6478109 (Sun et al 2016, Zucchelli et al 2011).

Next, we analyzed the effect SNPs have on MAP presence by correlating the presence of the studied SNPs to the presence of MAP DNA. Our results indicated that

there was no correlation between the presence of any of the four studied SNPs and MAP presence, even when correcting for disease. This data appears to imply that our studied SNPs have no correlation with MAP infection in the overall population. However, further analysis revealed an association between SNPs in *TNFSF15* and increased MAP presence in CD samples. Furthermore, none of the healthy samples had both the SNPs in *TNFSF15* and MAP, which corroborates the theory that CD is a multi-factorial disease in which having just one factor (MAP or genetic susceptibility in this case) would not be sufficient to foment CD, hence none of the healthy samples have both factors (Sharp et al 2018).

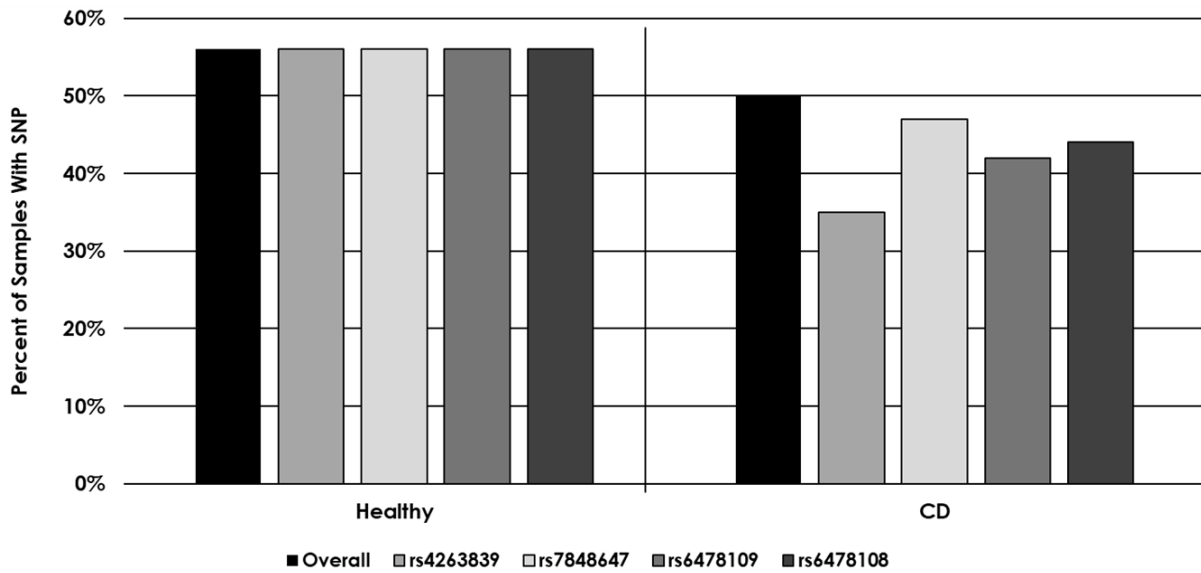
To further corroborate the presence of elevated TL1A levels, we analyzed the transcription levels of two major downstream cytokines; TNF- $\alpha$  and IFN- $\gamma$ . There were significantly elevated levels of IFN- $\gamma$  mRNA in CD samples that had at least one SNP (1.6-fold change) ( $P < 0.05$ ). This data corroborates the elevated levels of TL1A present, as elevated TL1A levels would yield elevated levels of IFN- $\gamma$ . When examining the levels of TNF- $\alpha$  mRNA in correlation to the examined SNPs on the other hand, surprisingly, no correlation was demonstrated. This is most likely due to the nature of CD treatment regimens in which many patients are given corticosteroids. These treatments are known inhibitors of NF- $\kappa$ B, AP-1, and P38MAPK, all of which are transcription factors that foment the transcription of TNF- $\alpha$  (Means et al 2000, Falyo et al 2010). Elevated levels of pro-inflammatory cytokines TL1A and IFN- $\gamma$  even while patients are undergoing corticosteroid treatments further clarify why a significant subset of CD patients do not respond to TNF- $\alpha$  inhibitors. In light of this last piece of data, one may assume that presence of SNPs on TL1A encoding gene may confer resistance or diminished response to conventional anti

TNF- $\alpha$  treatments, such as biologics and corticosteroids, and patients may benefit from undergoing genetic testing to determine if such treatments are appropriate for them. Using the pharmacogenomic approach, it may be possible to determine better suited medications and treatment options for patients with these SNPs and save them from the considerable financial burden associated with taking biologics.

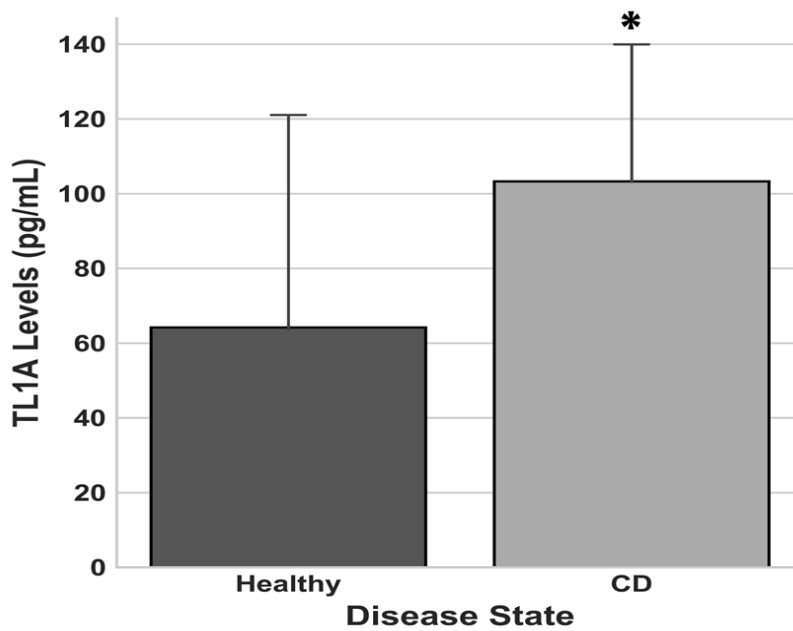
Conclusion: We found that the presence at least one of the four studied TNFSF15 SNPs (rs4263839, rs7848647, rs6478108, or rs6478109) is correlated to elevated levels of circulating TL1A in CD and healthy patients, while no correlation was found with the presence of MAP. Furthermore, the presence of rs7848647 or rs6478109 individually was correlated to elevated levels of TL1A in CD samples. The presence of SNPs in CD samples was also correlated to elevated levels of IFN- $\gamma$  but there was no correlation with elevated TNF- $\alpha$  levels implying that the pro-inflammatory cytokines TL1A and IFN- $\gamma$  can be elevated and thereby effect downstream factors without requiring elevated levels of TNF- $\alpha$ . Most importantly, this data implies that treating TNF- $\alpha$  would have less to no efficacy in controlling inflammation in the subset of patients that have these SNPs. A pharmacogenomic approach in which patients are screened for the presence of these SNPs can prove very efficient in providing a more suited treatment regimen to these patients while canceling the need for the inefficacious yet financially daunting anti-TNF- $\alpha$  treatments that are currently ubiquitously offered to all CD patients.

## APPENDIX: GRAPHS AND FIGURES

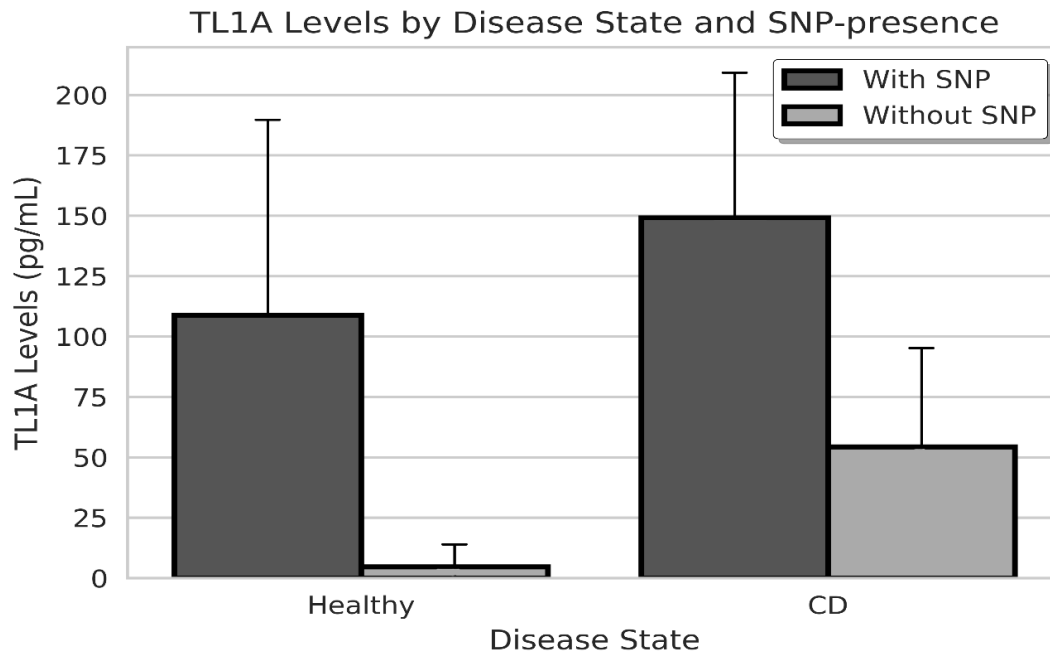




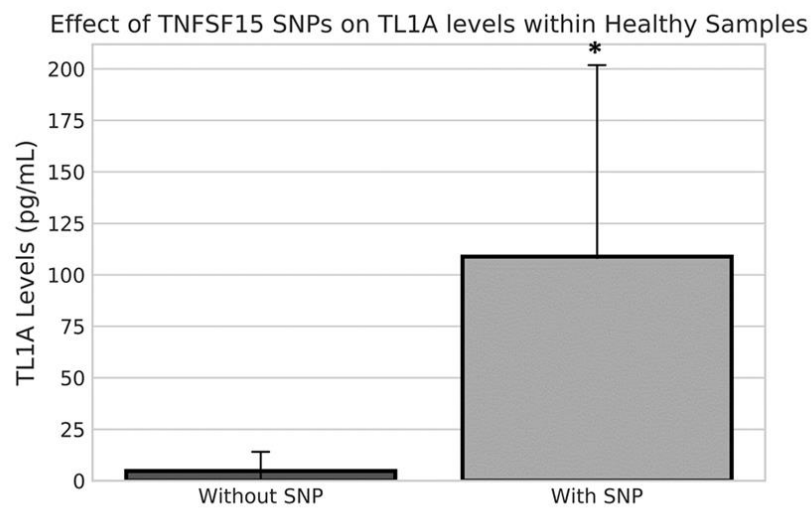
**Figure 1:** Percent of samples with individual *TNFSF15* SNPs in healthy and CD samples.



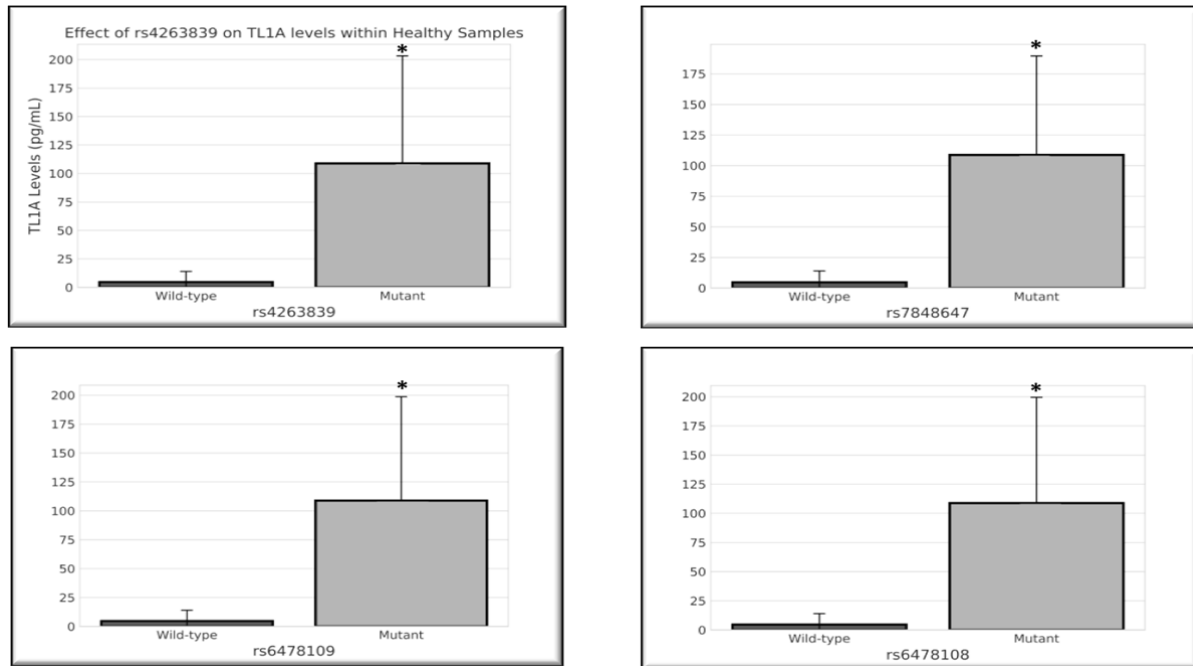
**Figure 2:** ELISA analysis of TL1A levels in association to disease state. \* indicates  $p < 0.05$ .



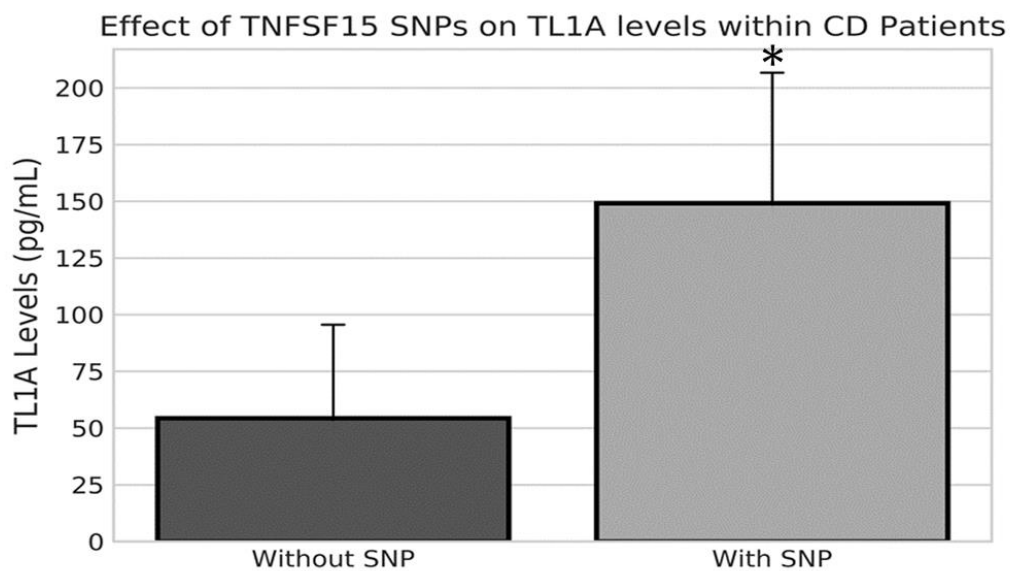
**Figure 3:** ELISA analysis of circulating TL1A levels in CD and healthy samples, without and without SNPs in *TNFSF15*.



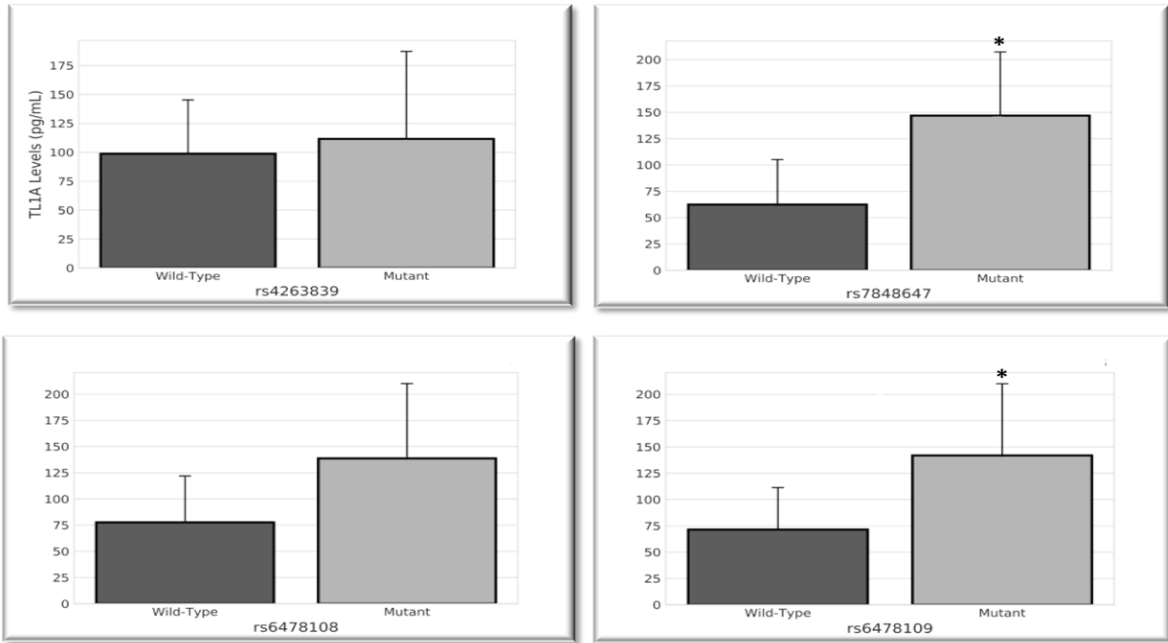
**Figure 4:** ELISA analysis of the effect of *TNFSF15* SNP on circulating TL1A Levels in healthy samples. \* indicates a P-value < 0.05



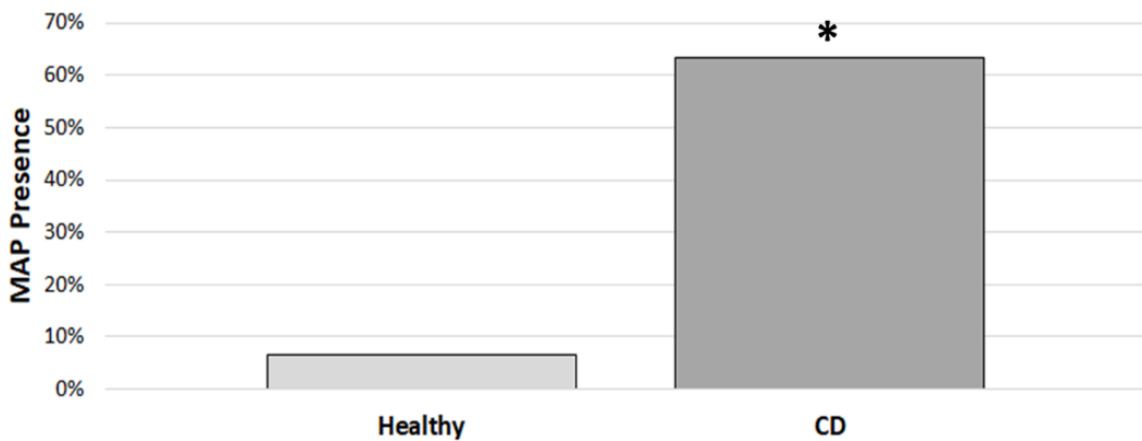
**Figure 5:** ELISA analysis of the effect of individual *TNFSF15* SNPs on circulating TL1A Levels in healthy samples. \* indicates a P-value <0.05



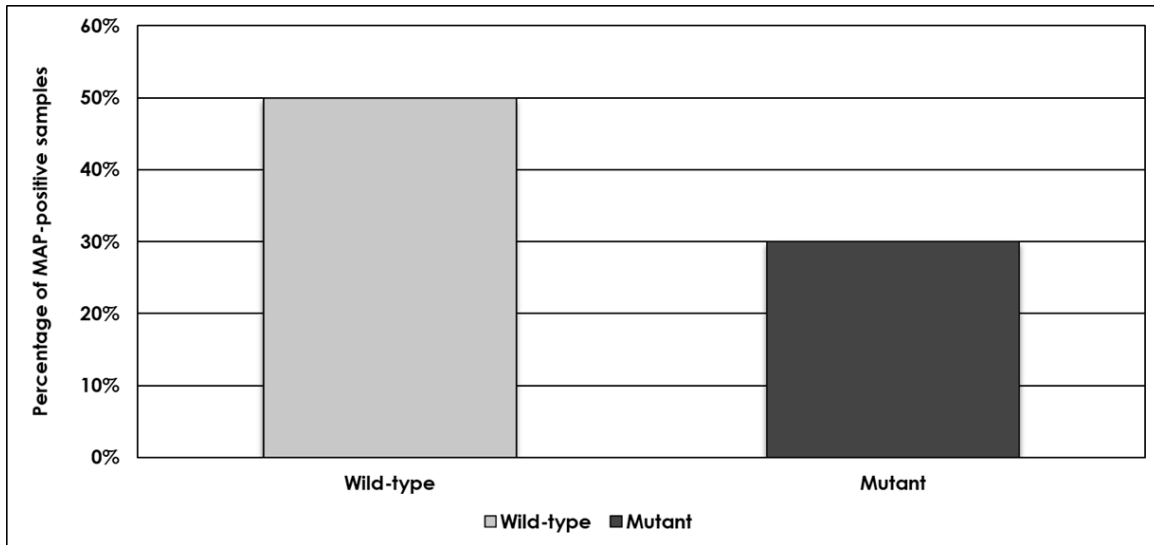
**Figure 6:** ELISA analysis of the effect of overall *TNFSF15* SNPs on circulating TL1A levels in CD samples. \* indicates a P-value <0.05



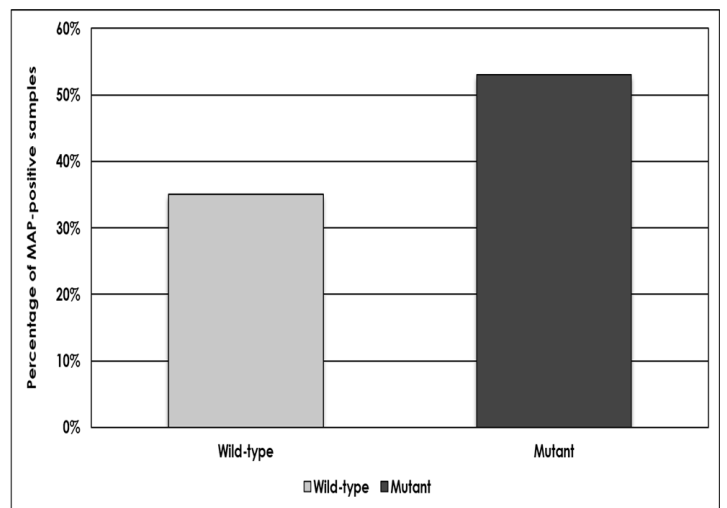
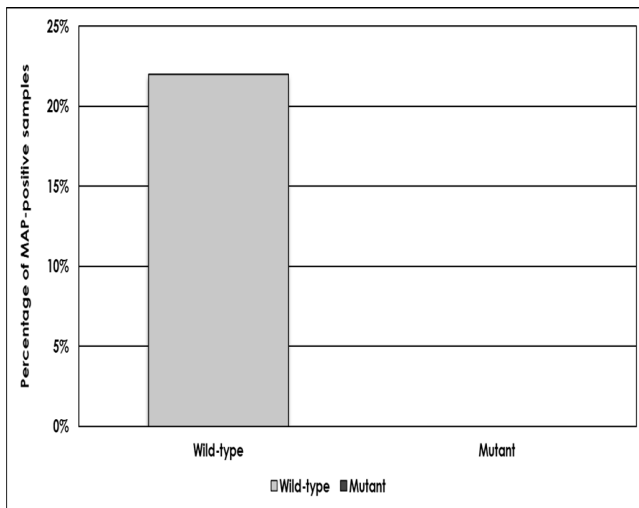
**Figure 7:** ELISA analysis of the effect of individual *TNFSF15* SNPs on circulating TL1A levels in CD samples. \* indicates a P-value < 0.05.



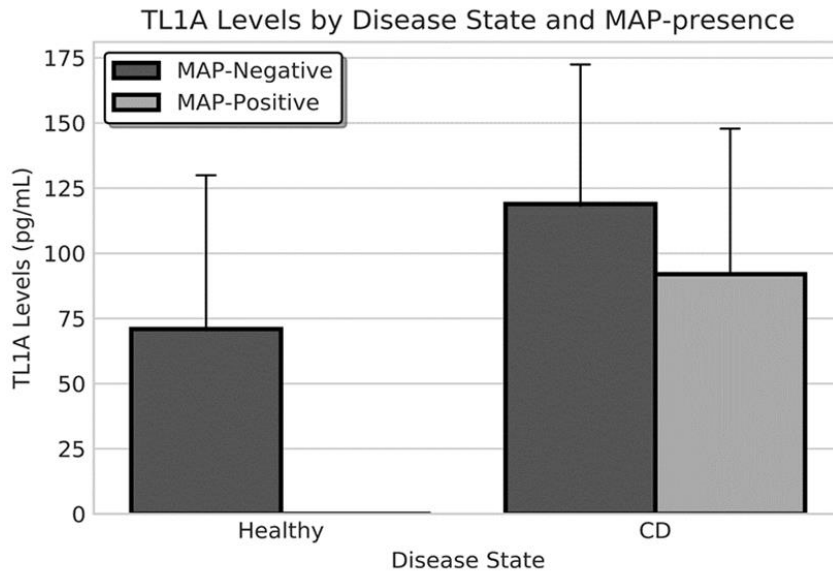
**Figure 8:** nPCR analysis of MAP association with CD. \* indicates a P-value < 0.01 in this figure.



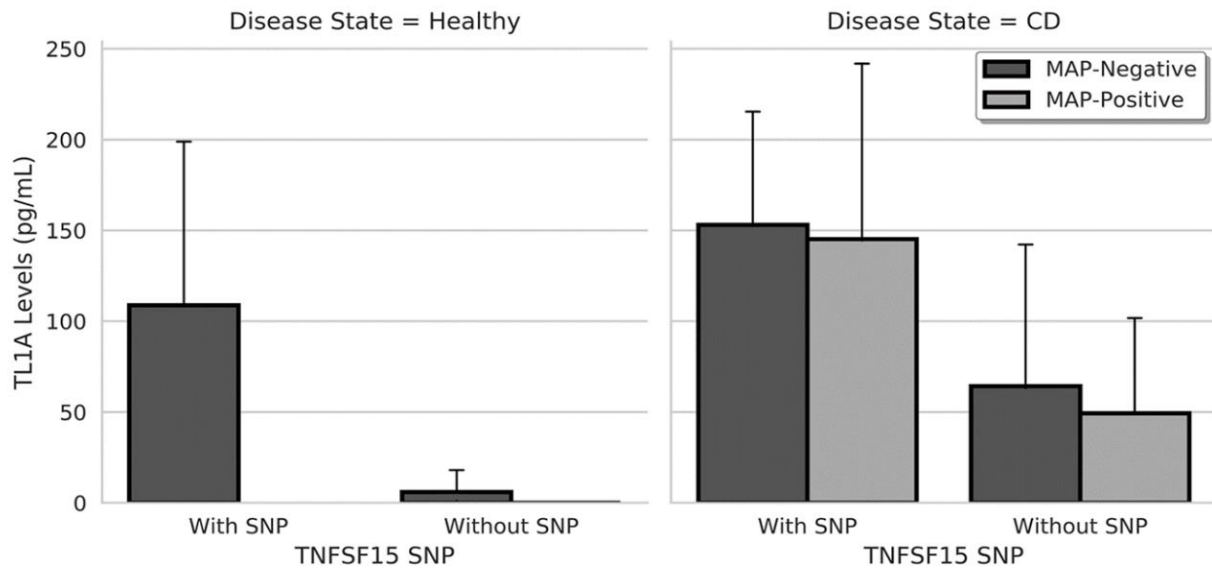
**Figure 9:** nPCR analysis of the effect of *TNFSF15* SNP presence on MAP presence, in overall sample population.



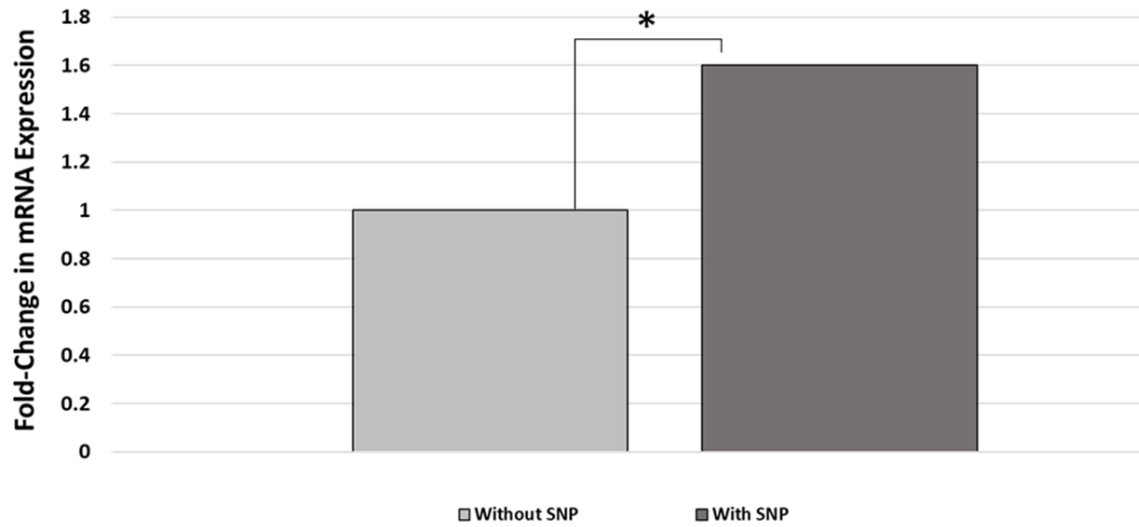
**Figure 10:** nPCR analysis of the effect of SNPs in *TNFSF15* on MAP presence in healthy and CD samples, individually



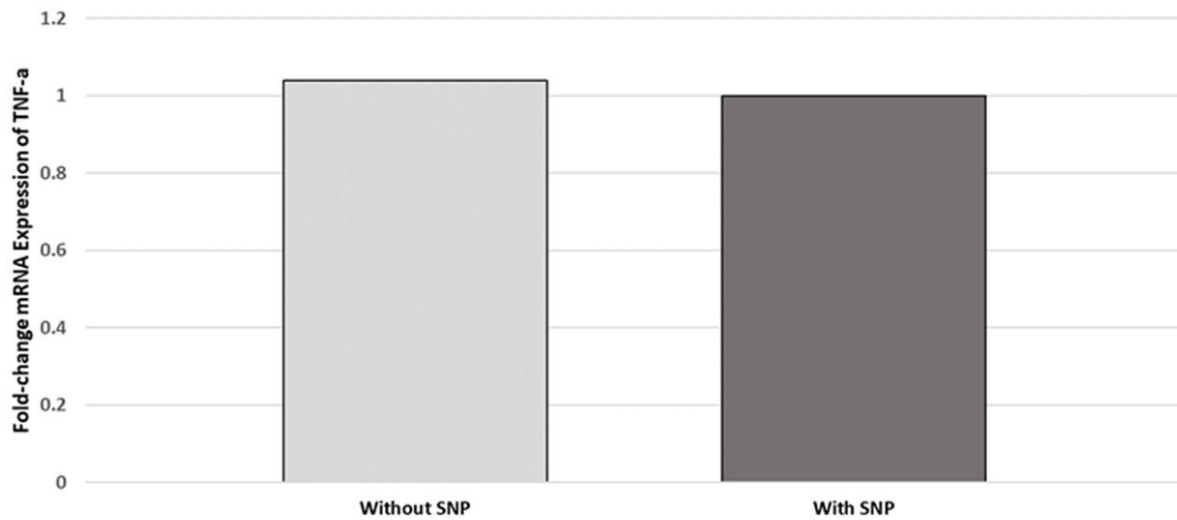
**Figure 11:** ELISA analysis of the effect of MAP presence on circulating TL1A levels. There appears to be no significant association.



**Figure 12:** ELISA analysis of the concurrent effect of TNFSF15 SNPs and MAP presence on TL1A levels in healthy and CD samples



**Figure 13:** RT-PCR analysis of the effect of TNFSF15 SNPs on relative IFNG mRNA expression in CD samples. \* indicates a P-value <0.05.



**Figure 14:** RT-PCR analysis of the effect of TNFSF15 SNPs on relative TNFA mRNA expression in CD samples. \* indicates a P-value <0.05.

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