Genetic polymorphisms in tumour necrosis factor receptors (*TNFRSF1A/1B*) illustrate differential treatment response to TNF α inhibitors in patients with Crohn's disease

Ahmad Qasem,¹ Seela Ramesh,² Saleh A Naser¹

To cite: Qasem A, Ramesh S, Naser SA. Genetic polymorphisms in tumour necrosis factor receptors (*TNFRSF1A/1B*) illustrate differential treatment response to TNF α inhibitors in patients with Crohn's disease. *BMJ Open Gastro* 2019;6:e000246. doi:10.1136/bmjgast-2018-000246

Received 10 September 2018
Revised 26 November 2018
Accepted 6 December 2018

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¹Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, Orlando, Florida, USA

²Digestive and Liver Center of Florida, Orlando, Florida, USA

Correspondence to

Dr Saleh A Naser;
Saleh.Naser@ucf.edu

ABSTRACT

Background Monoclonal antibodies inhibiting tumour necrosis factor- α (TNF α) signalling pathway (anti-TNF α) have been widely used in Crohn's disease (CD). However, treatment response varies among patients with CD and the clinical outcome is dependent on single nucleotide polymorphisms (SNP) in TNF α receptor superfamily 1A and 1B (*TNFRSF1A/1B*).

Methods We tested nine SNPs in *TNF α* , *TNFRSF1A* and *TNFRSF1B* by TaqMan genotyping from peripheral blood samples of 104 subjects. Additionally, we quantified the effects of these SNPs on their corresponding gene expression by RT-PCR and susceptibility to *Mycobacterium avium* subsp *paratuberculosis* (MAP) infection by IS900 nested PCR.

Results Four SNPs (*TNF α* :rs1800629, *TNFRSF1A*:rs767455, *TNFRSF1B*:rs1061624 and *TNFRSF1B*:rs3397) were over-represented significantly ($p < 0.05$) among patients with CD compared with healthy controls. The *TNFRSF1A*:rs767455 GG genotype was found in 15/54 patients with CD (28%), while it was only found in 2/50 healthy controls (4%) (OR 9.2, 95% CI 1.98 to 42.83). The *TNFRSF1B*:rs3397TT genotype was found in 15/54 patients with CD (28%) compared with (4/50) healthy controls (8%) (OR 4.4, 95% CI 1.36 to 14.14). Furthermore, the SNPs *TNFRSF1A*:rs767455 and *TNFRSF1B*:rs3397 were associated with downregulating their corresponding genes significantly ($p < 0.05$). MAP infection was predominantly found among patients with CD in comparison to healthy controls (57% vs 8%, respectively), which was also dependent on the SNPs *TNFRSF1A*:rs767455 and *TNFRSF1B*:rs3397. Our SNP haplotype analysis of *TNFRSF1A*:rs767455 and *TNFRSF1B*:rs3397 indicates that the G-T haplotype is significantly distributed among patients with CD (46%) and MAP infection susceptibility is also associated with this specific haplotype (31%).

Conclusion The SNPs *TNFRSF1A*:rs767455 and *TNFRSF1B*:rs3397, which are known to affect anti-TNF α clinical outcome in CD, were associated with lower corresponding gene expression and higher MAP infection susceptibility.

Summary box

What is already known about this subject?

- ▶ There is a significant variable response among patients with Crohn's disease (CD) receiving anti-tumour necrosis factor- α (TNF α).
- ▶ About 10%–30% of patients with inflammatory bowel disease have no initial response to anti-TNF α treatment.
- ▶ Over 50% of the anti-TNF α initial responders have lost response to treatment over time.
- ▶ Genetic polymorphisms in *TNFRSF1A* and *TNFRSF1B* have been shown to affect anti-TNF α treatment response significantly among patients with CD.
- ▶ The risk for tuberculosis infection has substantially increased in patients receiving anti-TNF α .
- ▶ *Mycobacterium avium* subsp *paratuberculosis* (MAP) is the most investigated suspect to be a causative pathogen in a subset of patients with CD.

What are the new findings?

- ▶ The single nucleotide polymorphisms (SNP) *TNFRSF1A*:rs767455 and *TNFRSF1B*:rs3397 were both associated with lower expression of their corresponding genes.
- ▶ MAP infection susceptibility is associated with the SNPs *TNFRSF1A*:rs767455 and *TNFRSF1B*:rs3397.
- ▶ SNP haplotype analysis of *TNFRSF1A*:rs767455 and *TNFRSF1B*:rs3397 indicates that the G-T haplotype has a significant difference in frequency among patients with CD, and MAP infection susceptibility is also associated with this specific haplotype.

How might it impact on clinical practice in the foreseeable future?

- ▶ Genetic testing for *TNFRSF1A*:rs767455 and *TNFRSF1B*:rs3397, in addition to MAP infection screening methods, should be considered before initiation of anti-TNF α treatment in CD for a better clinical outcome.

INTRODUCTION

Tumour necrosis factor- α (TNF α) is a proinflammatory cytokine that has been found to be dysregulated in numerous inflammatory disorders including rheumatoid arthritis, psoriasis and Crohn's disease (CD).^{1,2} It is secreted primarily by macrophages, but it can also be produced by lymphocytes, natural killer cells and mast cells.³ In order to exert its biological activity, TNF α binds to two different cell-surface receptors: TNF α receptor superfamily 1A (*TNFRSF1A*), which is expressed in most tissues, and TNF α receptor superfamily 1B (*TNFRSF1B*), which is typically found in immune cells.⁴

Targeting TNF α by monoclonal antibodies (anti-TNF α) such as adalimumab (Humira) and infliximab (Remicade) has shown that blocking this cytokine signalling pathway may control the symptoms of hyperactive immune response in CD initially.⁵ However, there is a significant variable response to anti-TNF α therapeutics among patients receiving this treatment.⁶ Additionally, clinical observations have reported that 10%–30% of patients with inflammatory bowel disease have no initial response to anti-TNF α treatment, and over 50% of the initial responders have lost response to treatment over time.⁷

Clinical studies have shown that anti-TNF α therapeutics have many deleterious side effects, such as malignancy, neurologic disorders, heart failure and, more importantly, multiple infections.⁸ Since TNF α plays a critical role in the immune defence against infections, it was unsurprising to notice that TNF α -deficient animal models are more susceptible to develop mycobacterial infections compared with wild-type controls.⁹ On the other hand, patients receiving anti-TNF α treatment are at higher risk for meningitis, sepsis, histoplasmosis, and pneumonia.^{10–12} Moreover, the risk for tuberculosis development has substantially increased in patients receiving anti-TNF α , which might raise a question about their effect on *Mycobacterium avium* subsp *paratuberculosis* (MAP) infection in a subset of patients with CD.^{13–16} Thus, prescribing anti-TNF α to patients with CD without considering MAP infection could worsen disease condition eventually.

Genetic polymorphisms are associated with the overall risk of developing an inflammatory disorder, and they play a role in the treatment outcome. For instance, polymorphisms in *TNFRSF1A* and *TNFRSF1B* have been shown to affect anti-TNF α treatment response significantly among patients with CD.^{17–19} However, the mechanism of those effects remains unknown. Predicting the efficacy of anti-TNF α treatment based on patient's genetics and MAP infection status would be more effective and beneficial to patients by reducing the risk for adverse effects and treatment cost.

In this study, we investigated the frequency of single nucleotide polymorphisms (SNP) in *TNF α* , *TNFRSF1A* and *TNFRSF1B* among patients with CD in comparison to healthy controls, in addition to their effect on gene expression and susceptibility to MAP intracellular

Table 1 Demographics of study participants

Diagnosis	n	Age range	Average age	Gender ratio (Male:female %)
All subjects	104	20–66	35	53:47
Crohn's disease	54	21–66	39	48:52
Healthy controls	50	20–63	31	58:42

infection. Finally, we further linked the effect of SNPs in these genes and presence of MAP to anti-TNF α treatment response in patients with CD.

MATERIALS AND METHODS

Clinical samples

Peripheral blood from a total of 104 subjects (54 patients with CD and 50 healthy controls) was included in this study. All participants provided written informed consent prior to enrolment.

Each subject provided two 4.0 mL K₂-EDTA coded blood tubes, where one tube was processed for detection of MAP infection, and the second tube was processed for gene expression analysis and genotyping of nine SNPs in *TNF α* , *TNFRSF1A* and *TNFRSF1B*. There was no significant difference in average age or gender between the two groups (CD vs healthy controls). All subject demographics are listed in [table 1](#).

Extraction of DNA and detection of MAP by *IS900* nested PCR

Purified white blood cells separated from blood samples were cultured in BD Bactec MGIT Para-TB Medium for 6 months of incubation at 37°C. Mycobacterial growth was measured initially using the ultraviolet illuminator and 1.0 mL was used for DNA extraction and nested PCR (nPCR) analysis as described earlier.^{15,20} Briefly, MAP infection was detected using *MAP-specific IS900* derived oligonucleotide primers. Round 1 of amplification was performed using P90 (GTTCGGGGCCGTCGCTTAGG) and P91 (GAGGTCGATCGCCACGTGA) primers following these conditions: 95°C for 5 min, then 34 cycles of 95°C for 1 min, 58°C for 1.5 min, 72°C for 1.5 min. Final extension of 10 min at 72°C. The amplified product from this round was 398 bp.

A second round of amplification involved using AV1 (ATGTGGTTGCTGTGTTGGATGG) and AV2 (CCGC-CGCAATCAACTCCAG) primers, which was performed following these conditions: 95°C for 5 min, then 34 cycles of 95°C for 1 min, 58°C for 1.5 min, 72°C for 1.5 min. Final extension of 10 min at 72°C. The final product following this round was 298 bp, which was identified on 2% agarose gel.

Table 2 Gene mutations, locations and mutation phenotypes of SNPs selected for this study

Gene	Reference SNP	Gene mutation*	Location and AA change*	Mutation phenotype	Reference
<i>TNF</i>	rs1800629	G→A	Promoter	Higher susceptibility to CD	22
	rs1799964	T→C	Promoter	Associated with IBD in general	23
	rs1799724	C→T	Promoter	Linked to ankylosing spondylitis	24
<i>TNFRSF1A</i>	rs4149584	C→T	Exon 4 (R→Q)	Higher susceptibility to MS	25
	rs767455	A→G	Exon 1: No AA change	Used to predict anti-TNF α response in CD	17
	rs4149570	G→T	320 bp upstream of gene	Used to predict anti-TNF α response in RA	26
<i>TNFRSF1B</i>	rs1061622	T→G	Exon 6 (M→R)	Higher susceptibility to IBD	27
	rs1061624	G→A	Exon 10	Higher susceptibility to IBD	27
	rs3397	C→T	Exon 10	Used to predict anti-TNF α response in CD	18

*Gene mutation and location data were obtained from the National Center for Biotechnology Information (NCBI).²¹

AA, amino acid; CD, Crohn's disease; IBD, inflammatory bowel disease; MS, multiple sclerosis; Q, glutamine; R, arginine; RA, rheumatoid arthritis; SNP, single nucleotide polymorphism; TNF α , tumour necrosis factor- α .

Identification of SNPs in *TNF α* , *TNFRSF1A* and *TNFRSF1B* genes

Genomic DNA was purified from peripheral blood leucocytes using QIAamp DNA Blood Mini Kit (Qiagen) following manufacturer's instructions. TaqMan genotyping assays (Applied Biosystems) for *TNF α* (rs1800629, rs1799964, and rs1799724), *TNFRSF1A* (rs4149584, rs767455, and rs4149570) and *TNFRSF1B* (rs1061624, rs1061622, and rs3397) were performed on isolated DNA samples following manufacturer protocol at the University of Florida Pharmacotherapy and Translational Research Department (Gainesville, FL). Briefly, the reaction mixture had 2 $\times$ TaqMan master mix and 20 $\times$ assay working stock (primers with VIC and FAM fluorophore attachment). Isolated DNA samples along with reaction mixture and controls were added into a 384-well microtitre plate, following RT-PCR assay (one cycle at 95°C for 10 min, 92°C for 15 s and 50 cycles at 58°C for 1 min) using Applied Biosystems QuantStudio RT-PCR System. The plate was analysed for VIC and FAM fluorophores at 551 and 517 nm, respectively. Fluorescence of VIC or FAM alone determined that the sample had allele 1 or allele 2, while VIC and FAM together determined that the sample was heterozygous for each allele. Gene mutation, location and phenotype for SNPs used in this study are summarised in table 2.^{21–27}

Gene expression of *TNF α* , *TNFRSF1A*, and *TNFRSF1B* by RT-PCR

RNA was isolated from 1.0 mL of whole blood samples, and used for cDNA synthesis in order to analyse gene expression of *TNF α* , *TNFRSF1A*, and *TNFRSF1B* via RT-PCR. Briefly, 1.0 mL of blood was transferred into a 2.0 mL RNase-free microcentrifuge tube, and then centrifuged at 1000 rpm for 5 min. Pellets containing leucocytes were collected and suspended in 1.0 mL of TRIzol reagent (Invitrogen) for 15 min of gentle shaking. A total volume of 0.2 mL of chloroform was added to each tube,

and the mixture was then incubated at room temperature for 3 min. All tubes were then centrifuged at 11 400 RPM for 15 min at 4°C. The upper aqueous colourless phase containing RNA was transferred into a new 2.0 mL RNase-free microcentrifuge tube. A total volume of 0.5 mL of 100% isopropanol was then added following 10 min of incubation at room temperature. Each tube was then centrifuged at 11 400 RPM for 10 min at 4°C. A total volume of 1.0 mL of 75% ethanol was used to wash RNA pellets and then centrifuged at 8700 RPM for 5 min at 4°C. RNA pellets were air-dried for 15–30 min, suspended in 20 μ L of RNase-free H₂O, and finally heated at 60°C for 10 min.

For cDNA synthesis, 600 ng of RNA from each sample was added to 0.25 mL tubes containing 0.2 mL of PCR reaction, 4 μ L of iScript Reverse Transcription (Bio-Rad), and up to 20 μ L RNase-free H₂O. All tubes were then transferred to thermal cycler (MyGene Series Peltier Thermal Cycler) and ran for 5 min at 25°C, 20 min at 46°C and 1 min at 95°C. The final concentration of cDNA synthesised for each sample was 30 ng/ μ L. A total volume of 1 μ L of cDNA was mixed with 10 μ L of Fast SYBR Green Mastermix (Thermo Fisher Scientific), 1 μ L of either *TNF α* , *TNFRSF1A*, or *TNFRSF1B* PrimePCR SYBER Green Assay mix (Bio-Rad) and 8 μ L of molecular biological grade sterile H₂O in a 96-well microamp RT-PCR reaction plate. Controls of 18s RNA gene oligonucleotide primers (forward primer: 5'-GTA ACC CGT TGA ACC CCA TT-3'; reverse primer: 5'-CCA TCC AAT CGG TAG TAG CG-3') were used in order to obtain baseline CT readings. The 7500 Fast Real-Time PCR System (Applied Biosystems) was used to perform the RT-PCR reaction. Relative mRNA expression levels were calculated using the equation ($2^{-(\Delta\Delta CT)} \times 1000$), where ΔCT =Sample RT-PCR CT reading – 18s CT baseline.

Table 3 Genotype frequencies of selected SNPs for patients with CD and healthy controls

Genotype	Patients with CD (n=54)	Healthy controls (n=50)	P value* OR (95% CI)
TNFα rs1800629			
GG (reference allele)	34 (64%)	41 (82%)	0.03
GA	20 (37%)	7 (14%)	0.01 3.6 (1.37 to 9.54)
AA	0 (0%)	2 (4%)	0.13
GA+AA	20 (37%)	9 (18%)	0.03 2.7 (1.08 to 6.64)
TNFα rs1799964			
TT (reference allele)	33 (61%)	34 (68%)	0.75
TC	18 (33%)	16 (32%)	0.89
CC	3 (6%)	0 (0%)	1.2 (0.51 to 2.64)
TC+CC	21 (39%)	16 (32%)	0.09 7.2 (0.35 to 144.9)
TNFα rs1799724			
CC (reference allele)	45 (83%)	43 (86%)	0.7
CT	9 (16%)	7 (14%)	0.38
TT	0 (0%)	0 (0%)	1.2 (0.42 to 3.59)
CT+TT	9 (16%)	7 (14%)	NA 0.70 1.2 (0.42 to 3.59)
TNFRSF1A rs4149584			
CC (reference allele)	52 (96%)	49 (98%)	0.6
CT	2 (4%)	1 (2%)	0.60
TT	0 (0%)	0 (0%)	1.9 (0.17 to 21.40)
CT+TT	2 (4%)	1 (0%)	NA 0.60 1.9 (0.17 to 21.40)
TNFRSF1A rs767455			
AA (reference allele)	17 (31%)	29 (58%)	0.01
AG	22 (41%)	19 (38%)	0.29
GG	15 (28%)	2 (4%)	0.01 9.2 (1.98 to 42.83)
AG+GG	37 (68%)	21 (42%)	0.01 3.0 (1.34 to 6.71)
TNFRSF1A rs4149570			
GG (reference allele)	25 (46%)	26 (52%)	0.56
GT	13 (24%)	18 (36%)	0.18
TT	16 (30%)	7 (14%)	0.75 (0.31 to 1.84)
GT+TT	29 (54%)	25 (50%)	0.06 2.4 (0.83 to 6.75)
TNFRSF1B rs1061624			
AA (reference allele)	13 (24%)	27 (54%)	0.70 1.2 (0.56 to 2.59)

Continued

Table 3 Continued

Genotype	Patients with CD (n=54)	Healthy controls (n=50)	P value* OR (95% CI)
AG	27 (50%)	15 (30%)	0.04 2.3 (1.04 to 5.22)
GG	14 (26%)	8 (16%)	0.02 3.6 (1.21 to 10.83)
AG+GG	41 (76%)	23 (46%)	0.01 3.7 (1.61 to 8.53)
<i>TNFRSF1B</i> rs1061622			
TT (reference allele)	24 (44%)	31 (62%)	0.07
TG	22 (41%)	16 (32%)	0.36 1.8 (0.77 to 4.1)
GG	8 (15%)	3 (6%)	0.14 3.44 (0.82 to 14.4)
TG+GG	30 (%)	19 (38%)	0.07 2.03 (0.93 to 4.47)
<i>TNFRSF1B</i> rs3397			
CC (reference allele)	12 (22%)	31 (62%)	0.01
CT	31 (57%)	15 (30%)	0.01 3.1 (1.4 to 7.07)
TT	15 (28%)	4 (8%)	0.01 4.4 (1.36 to 14.14)
CT+TT	46 (85%)	19 (38%)	0.01 6.25 (2.66 to 14.69)

Two-tailed Z test and OR analysis were used to compare between the presence of SNPs in patients with CD versus healthy controls.

*P<0.05 was considered as significance threshold.

CD, Crohn's disease; NA, unknown residue change; SNP, single nucleotide polymorphism; TNF α , tumour necrosis factor- α .

Statistical analysis

All statistical tests were performed using GraphPad Prism V.7.02. MAP infection incidence was used to estimate the power of this study. Since patients with CD have MAP infection incidence proportion of nearly 40%, while in healthy controls MAP infection incidence proportion is about 10%,^{13 14} at 90% power and alpha (type I error) of 0.05, our calculated sample size was 84 samples (42 CD and 42 healthy controls). We also aimed for a similar number of Matsukura *et al*'s anti-TNF α treatment response study which included 81 participants.¹⁷ For genotype frequency, we used two-tailed Z test and OR analysis to compare between the presence of SNPs in patients with CD versus healthy controls. At each locus examined, SNP genotypes were subcategorised into four groups (major, heterozygous, homozygous and both heterozygous+homozygous), then tested for significance within each subcategory at p<0.05 and a 95% CI. For haplotype analysis, we used Fisher's exact test since we had a smaller number of samples. Patients with CD were subcategorised into four haplotype groups and then tested for significance in-between MAP infection groups. A p value <0.05 was considered significant. For gene expression analysis, first, we compared the average gene expression in CD versus healthy control for each gene regardless of their genotype using unpaired two-tailed t-test at p<0.05

and a 95% CI, then we compared individuals who carried two major alleles with others for each SNP tested by using one-way analysis of variance, where Newman-Keuls post-test was selected for multiple comparisons. For MAP infection susceptibility, we compared infection proportions between SNP genotypes and major alleles in CD group and healthy controls separately using two-tailed Z test at p<0.05. Age and gender were not included as covariates as for all data sets no age or gender effects were observed.

RESULTS

Frequency of SNPs in *TNF α* , *TNFRSF1A*, and *TNFRSF1B* among patients with CD

We have assessed 104 subjects (54 patients with CD and 50 healthy controls) for three SNPs in *TNF α* (rs1800629, rs1799964, and rs1799724), three SNPs in *TNFRSF1A* (rs4149584, rs767455, and rs4149570) and three SNPs in *TNFRSF1B* (rs1061624, rs1061622, and rs3397). Genotype distribution of these SNPs conveyed the Hardy-Weinberg equilibrium.

We identified one SNP in *TNF α* (*TNF α :rs1800629*) with a significant difference in frequency in patients with CD compared with healthy controls (p<0.05). The *TNF α :rs1800629* GA genotype was found in 20/54 patients with CD (37%) compared with 7/50 healthy controls (14%) (OR 3.6, 95% CI 1.37 to 9.54). As shown

in table 3, the other two SNPs (*TNFα*:rs1799964 and *TNFα*:rs1799724) have shown no significance among patients with CD compared with healthy controls ($p>0.05$). Similarly, there was a significant difference in frequency of one SNP in *TNFRSF1A* (*TNFRSF1A*:rs767455) among patients with CD compared with healthy controls ($p<0.05$). The *TNFRSF1A*:rs767455 GG genotype was found in 15/54 patients with CD (28%), while it was only found in 2/50 healthy controls (4%) (OR 9.2, 95% CI 1.98 to 42.83). The other two SNPs (*TNFRSF1A*:rs4149584 and *TNFRSF1A*:rs4149570) have shown no significance ($p>0.05$).

Additionally, two SNPs in *TNFRSF1B* (*TNFRSF1B*:rs1061624 and *TNFRSF1B*:rs3397) were found to be significant in patients with CD compared with healthy controls ($p<0.05$). The *TNFRSF1B*:rs1061624 AG genotype was found in 27/54 patients with CD (50%) compared with 15/50 healthy controls (30%) (OR 2.3, 95% CI 1.04 to 5.22). The *TNFRSF1B*:rs3397 CT genotype was found in 31/54 patients with CD (57%) compared with (15/50) healthy controls (30%) (OR 3.1, 95% CI 1.40 to 7.07). Besides, the *TNFRSF1B*:rs3397 TT genotype was also significantly found in 15/54 patients with CD (28%) compared with (4/50) healthy controls (8%) (OR 4.4, 95% CI 1.36 to 14.14). However, *TNFRSF1B*:rs1061622

was not found to be significant among patients with CD in comparison to healthy controls ($p>0.05$). A complete list of SNPs and genotyping frequency is shown in table 3.

SNPs downregulate *TNFRSF1A* and *TNFRSF1B* gene expression in CD

We quantified relative gene expression level of *TNFα*, *TNFRSF1A*, and *TNFRSF1B* in all 104 study participants. Then, we further analysed the data according to each SNP present, in order to find out if SNPs are associated with gene expression level. In general, the relative expression of *TNFα* was more than three times higher in patients with CD (2.44 ± 0.30), in comparison to healthy subjects (0.72 ± 0.20), regardless of SNPs involved ($p<0.5$). However, none of *TNFα* SNPs (rs1800629, rs1799964, and rs1799724) were associated with gene expression level significantly ($p>0.05$) (figure 1A–C).

Overall, the expression level of *TNFRSF1A* and *TNFRSF1B* was significantly downregulated in patients with CD (0.38 ± 0.15 ; $p<0.5$ and 0.34 ± 0.13 ; $p<0.05$, respectively), compared with healthy subjects (0.79 ± 0.24 and 0.66 ± 0.17 , respectively) (figure 1D–I). Nevertheless, patients with CD with the SNP *TNFRSF1A*:rs767455 who had GG genotype, had a significantly lower relative gene expression level compared with patients without SNP

Figure 1 Gene expression level of *TNFα*, *TNFRSF1A* and *TNFRSF1B* according to each allele type in selected single nucleotide polymorphisms (SNP) (A–I) among patients with Crohn's disease (CD) (n=54) and healthy controls (n=50). Unpaired two-tailed t-test at $p<0.05$ and a 95% CI was used to test gene expression significance in patients with CD versus healthy controls, then one-way analysis of variance (ANOVA), where Newman-Keuls post-test was selected for multiple comparisons, was used to test individuals who carried two major alleles with others for each SNP. *TNFα*, tumour necrosis factor.

Figure 2 Influence of (A) *TNFRSF1A* (rs767455) and (B) *TNFRSF1B* (rs3397) single nucleotide polymorphisms (SNP) on MAP infection susceptibility in patients with CD (n=54) and healthy subjects (n=50). Infection proportions were compared between SNP genotypes and major alleles in patients with CD and healthy controls separately using two-tailed Z test at $p < 0.05$. CD, Crohn's disease; MAP, *Mycobacterium avium* subsp *paratuberculosis*.

who had AA genotype (0.26 ± 0.09 vs 0.49 ± 0.12 ; $p < 0.5$) (figure 1E).

Similarly, the expression level of *TNFRSF1B* was significantly lower in patients with CD with the SNP *TNFRSF1B:rs3397* who had either CT (0.32 ± 0.11) or TT (0.2 ± 0.09) genotype, compared with patients without SNP (0.59 ± 0.10) who had CC genotype ($p < 0.5$) (figure 1I).

SNPs in *TNFRSF1A* and *TNFRSF1B* induce susceptibility to MAP infection

We evaluated MAP infection status in all 104 study participants by *IS900* nPCR analysis, which is very sensitive and specific for MAP. Overall, 31/54 patients with CD were infected with MAP compared with only 4/50 healthy controls (OR 15.5, 95% CI 4.88 to 49.22, $p < 0.05$).

We further looked for any association between a specific genotype in all SNPs we tested and the presence of MAP infection. Interestingly, patients with CD with the SNP *TNFRSF1A:rs767455* who had either AG or GG genotype were more susceptible to MAP infection (63% and 66.6%, respectively), while patients with CD without SNP (AA genotype) were 41% infected with MAP. However, this result was not statistically significant ($p > 0.05$) (figure 2A). On the other hand, patients with CD with the SNP *TNFRSF1B:rs3397* who had either CT or TT genotype were significantly susceptible to MAP infection (58% and 70.5%, respectively), compared with patients with CD without SNP (CC genotype), who were only 17% infected with MAP ($p < 0.05$) (figure 2B). None of the additional SNPs we tested (rs1800629, rs1799964, and rs1799724 of *TNFA*, rs4149584 and rs4149570 of *TNFRSF1A*, rs1061624 and rs1061624 of *TNFRSF1B*) were found to be significantly associated with higher susceptibility to MAP infection.

SNP haplotypes distribution and MAP infection susceptibility

After we have identified *TNFRSF1A:rs767455* and *TNFRSF1B:rs3397* as SNPs associated with lower gene expression and higher MAP infection susceptibility in patients with CD, we further performed haplotype analysis of these two SNPs. The haplotype frequencies among patients with CD

Figure 3 Haplotypes inferred from rs767455 (A/G) and rs3397 (C/T), and their distributions in patients with Crohn's disease (CD) (A) and healthy subjects (B).

and healthy controls showed a significant difference for the G-T haplotype ($p < 0.05$), which was found in 25/54 patients with CD (46%), while in healthy controls it was 7/50 (14%) (figure 3). Consequently, MAP infection was significantly higher among patients with CD who had the G-T haplotype (17/25), which contributes to 31% of patients with CD overall, while none of the healthy controls had the G-T haplotype and MAP infection at the same time. As described in table 4, none of the other SNP haplotypes inferred from *TNFRSF1A* and *TNFRSF1B* (A-C, G-C and A-T) were found to be associated with susceptibility to MAP infection significantly ($p > 0.05$).

DISCUSSION

Increased level of $\text{TNF}\alpha$ has been found in the inflamed intestinal mucosa of patients with CD, which is considered an essential mediator of immunologic response for this inflammatory disease.²⁸ Consequently, $\text{TNF}\alpha$ plays a role in increasing intestinal permeability, granuloma formation and coagulation pathways.³ Most recently, we elucidated the relationship between MAP infection and the upregulation of $\text{TNF}\alpha$ expression in vitro, which has shown that both MAP and *M. tuberculosis* induce gene expression significantly in comparison to other mycobacteria and non-mycobacteria strains.²⁹ Although inhibition of $\text{TNF}\alpha$ has shown a positive clinical outcome in some patients with CD, poor response was also found in others, which leaves those patients with severe side effects and higher susceptibility to infections.¹³ Therefore, several

Table 4 Haplotypes inferred from *TNFRSF1A* rs767455 (A/G)–*TNFRSF1B* rs3397 (C/T) and MAP infection distributions among patients with CD

Haplotype* (rs767455– rs3397)	CD MAP (+) (n=31)	CD MAP (–) (n=23)	Overall	P value
A–C	2 (4%)	6 (11%)	8 (15%)	0.14
G–C	3 (5%)	2 (4%)	5 (9%)	0.64
A–T	9 (17%)	7 (13%)	16 (30%)	0.59
G–T	17 (31%)	8 (15%)	25 (46%)	0.01

Fisher's exact test was used to test group significance at $p < 0.05$. CD, Crohn's disease; MAP, *Mycobacterium avium* subsp *paratuberculosis*.

Figure 4 Recommended Crohn's disease (CD) treatment algorithm based on haplotypes inferred from *TNFRSF1A* rs767455 (A/G)–*TNFRSF1B* rs3397 (C/T). MAP, *Mycobacterium avium* subsp *paratuberculosis*; SNP, single nucleotide polymorphism; TNFα, tumour necrosis factor.

pharmacogenetic studies have evaluated the variation of anti-TNFα treatment response among patients with CD in order to predict treatment response ultimately.^{17–19}

Genetic polymorphisms in *TNFRSF1A* and *TNFRSF1B* were found to be correlated with anti-TNFα treatment response in patients with CD. Specifically, the *TNFRSF1A*:rs767455 AG and GG genotype has a significant difference in frequency among non-responders to anti-TNFα treatment compared with the majority of drug responders who had the AA genotype.¹⁶ Similarly, the *TNFRSF1B*:rs3397 CT and TT also have a significant difference in frequency among anti-TNFα non-responders in comparison to patients with CD who were classified as drug responders (CC genotype).^{18, 30} However, these observations were limited to drug response only, while the molecular effects of these SNPs were not discussed.

Since MAP is a suspected microbial causative agent of CD,^{14 15 31 32} it was worthy to find out if genetic polymorphisms are inducing susceptibility to MAP infection in patients with CD, which will ultimately influence anti-TNFα treatment outcome. Therefore, we tested 104 subjects (54 patients with CD and 50 healthy controls) for selective SNPs in *TNFα* (rs1800629, rs1799964, and rs1799724), *TNFRSF1A* (rs4149584, rs767455, and rs4149570) and *TNFRSF1B* (rs1061624, rs1061622, and rs3397). First, four out of these nine SNPs were found to have a significant difference in frequency among patients with CD compared with healthy controls (*TNFα*:rs1800629, *TNFRSF1A*:rs767455, *TNFRSF1B*:rs1061624 and *TNFRSF1B*:rs3397), which also correlates with previous reports.^{33–35} Second, we quantified gene expression level of *TNFα*, *TNFRSF1A* and *TNFRSF1B* in all study participants. We found that *TNFα* relative expression level is about 3.4-fold higher in patients with CD compared with healthy controls overall. However, none of *TNFα*-associated SNPs had a significant association with *TNFα*

gene expression level. The expression of *TNFRSF1A* and *TNFRSF1B* was significantly downregulated (~2-fold) in patients with CD compared with healthy controls regardless of SNPs involved. Furthermore, the SNPs *TNFRSF1A*:rs767455 and *TNFRSF1B*:rs3397 were both found to be associated with a significant lower gene expression. Additionally, MAP infection was predominantly found among patients with CD in comparison to healthy controls (57% vs 8%, respectively). Interestingly, MAP infection was also associated with the SNPs *TNFRSF1A*:rs767455 and *TNFRSF1B*:rs3397. Furthermore, our SNP haplotype analysis of *TNFRSF1A*:rs767455 and *TNFRSF1B*:rs3397 indicates that the G–T haplotype has a significant difference in frequency among patients with CD (46%) and MAP infection susceptibility is also associated with this specific haplotype (31%).

It is relevant to compare between MAP and *M. tuberculosis* infection since *mycobacterial protein tyrosine phosphatase* (*PtpA*) in MAP shares 90% homology to *PtpA* in *M. tuberculosis*.³⁶ This protein inhibits phagosome–lysosome fusion in the macrophage by dephosphorylating the host vacuolar protein sorting 33B. Consequently, the pathogen will be able to avoid containment eradication and it establishes a successful intracellular infection by preventing fusion of lysosome and phagosome into the phagolysosomal complex.^{37 38} Therefore, the primary mechanism for MAP eradication is by inducing apoptosis of the infected macrophage through TNFα-dependent mechanism.^{39 40} This is a critical point when we consider anti-TNFα treatment for patients with CD who are infected with MAP. In addition to genetic testing of selective SNPs we discussed, MAP infection screening method should be implemented before initiation of therapy. Finally, in figure 4, we suggest following a treatment algorithm for CD, based on patient's haplotype inferred from *TNFRSF1A* rs767455 (A/G) and *TNFRSF1B* rs3397 (C/T).

Acknowledgements Our thanks are due to all members of Dr Naser's lab and to Brent Cao for his valuable assistance in the statistical analysis. A sincere acknowledgement to all the subjects whose clinical samples were included in this study.

Contributors AQ is the primary author who performed all experiments, collected data and participated in writing the manuscript. SR is our collaborator gastroenterologist at the Digestive and Liver Center of Florida, who provided us with clinical samples. SAN is the leading investigator in the lab and has supervised all aspects of the study including writing and editing of the manuscript.

Funding This study was funded, in part, by the Florida Legislative Grant and UCF Doctoral Research Support Award.

Competing interests None declared.

Patient consent for publication Obtained.

Ethics approval University of Central Florida Institutional Review Board (IRB00001138).

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement The data sets supporting the conclusions of this article are included in the article.

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REFERENCES

- Victor FC, Gottlieb AB. TNF-alpha and apoptosis: implications for the pathogenesis and treatment of psoriasis. *J Drugs Dermatol* 2002;1:264-75.
- Brynskov J, Foegh P, Pedersen G, et al. Tumour necrosis factor alpha converting enzyme (TACE) activity in the colonic mucosa of patients with inflammatory bowel disease. *Gut* 2002;51:37-43.
- Furst DE, Wallis R, Broder M, et al. Tumour necrosis factor antagonists: different kinetics and/or mechanisms of action may explain differences in the risk for developing granulomatous infection. *Semin Arthritis Rheum* 2006;36:159-67.
- Papadakis KA, Targan SR. Tumour necrosis factor: biology and therapeutic inhibitors. *Gastroenterology* 2000;119:1148-57.
- Hyrich KL, Watson KD, Silman AJ, et al. Predictors of response to anti-TNF-alpha therapy among patients with rheumatoid arthritis: results from the British Society for Rheumatology Biologics Register. *Rheumatology* 2006;45:1558-65.
- Tracey D, Klareskog L, Sasso EH, et al. Tumour necrosis factor antagonist mechanisms of action: a comprehensive review. *Pharmacol Ther* 2008;117:244-79.
- Roda G, Jharap B, Neeraj N, et al. Loss of Response to Anti-TNFs: Definition, Epidemiology, and Management. *Clin Transl Gastroenterol* 2016;7:e135.
- Antoni C, Braun J. Side effects of anti-TNF therapy: current knowledge. *Clin Exp Rheumatol* 2002;20(6 Suppl 28):S152-7.
- Botha T, Ryffel B. Reactivation of latent tuberculosis infection in TNF-deficient mice. *J Immunol* 2003;171:3110-8.
- Marotte H, Charrin JE, Miossec P. Infliximab-induced aseptic meningitis. *The Lancet* 2001;358:1784.
- Baghai M, Osmon DR, Wolk DM, et al. Fatal sepsis in a patient with rheumatoid arthritis treated with etanercept. *Mayo Clin Proc* 2001;76:653-6.
- Ritz MA, Jost R. Severe pneumococcal pneumonia following treatment with infliximab for Crohn's disease. *Inflamm Bowel Dis* 2001;7:327.
- Qasem A, Naser AE, Naser SA. The alternate effects of anti-TNFα therapeutics and their role in mycobacterial granulomatous infection in Crohn's disease. *Expert Rev Anti Infect Ther* 2017;15:637-43.
- Chamberlin WM, Naser SA. Integrating theories of the etiology of Crohn's disease. On the etiology of Crohn's disease: questioning the hypotheses. *Med Sci Monit* 2006;12:RA27-RA33.
- Qasem A, Abdel-Aty A, Abu-Suwa H, et al. Oxidative stress due to Mycobacterium avium subspecies paratuberculosis (MAP) infection upregulates selenium-dependent GPx activity. *Gut Pathog* 2016;8:12.
- Cao BL, Qasem A, Sharp RC, et al. Systematic review and meta-analysis on the association of tuberculosis in Crohn's disease patients treated with tumor necrosis factor-α inhibitors (Anti-TNFα). *World J Gastroenterol* 2018;24:2764-75.
- Matsukura H, Ikeda S, Yoshimura N, et al. Genetic polymorphisms of tumour necrosis factor receptor superfamily 1A and 1B affect responses to infliximab in Japanese patients with Crohn's disease. *Aliment Pharmacol Ther* 2008;27:765-70.
- Medrano LM, Taxonera C, Márquez A, et al. Role of TNFRSF1B polymorphisms in the response of Crohn's disease patients to infliximab. *Hum Immunol* 2014;75:71-5.
- Steenholdt C, Enevold C, Ainsworth MA, et al. Genetic polymorphisms of tumour necrosis factor receptor superfamily 1b and fas ligand are associated with clinical efficacy and/or acute severe infusion reactions to infliximab in Crohn's disease. *Aliment Pharmacol Ther* 2012;36:650-9.
- Naser SA, Ghobrial G, Romero C, et al. Culture of Mycobacterium avium subspecies paratuberculosis from the blood of patients with Crohn's disease. *The Lancet* 2004;364:1039-44.
- NCBI, 2018. National Center for Biotechnology Information. Available from: ncbi.nlm.nih.gov
- Ferreira AC, Almeida S, Tavares M, et al. NOD2/CARD15 and TNFA, but not IL1B and IL1RN, are associated with Crohn's disease. *Inflamm Bowel Dis* 2005;11:331-9.
- Cooke J, Zhang H, Greger L, et al. Mucosal genome-wide methylation changes in inflammatory bowel disease. *Inflamm Bowel Dis* 2012;18:2128-37.
- Zhu X, Wang Y, Sun L, et al. A novel gene variation of TNFα associated with ankylosing spondylitis: a reconfirmed study. *Ann Rheum Dis* 2007;66:1419-22.
- Comabella M, Caminero AB, Malhotra S, et al. TNFRSF1A polymorphisms rs1800693 and rs4149584 in patients with multiple sclerosis. *Neurology* 2013;80:2010-6.
- Sode J, Vogel U, Bank S, et al. Anti-TNF treatment response in rheumatoid arthritis patients is associated with genetic variation in the NLRP3-inflammasome. *PLoS One* 2014;9:e100361.
- Ferguson LR, Han DY, Huebner C, et al. Tumour necrosis factor receptor superfamily, member 1B haplotypes increase or decrease the risk of inflammatory bowel diseases in a New Zealand caucasian population. *Gastroenterol Res Pract* 2009;2009:1-9.
- MacDonald TT, Hutchings P, Choy MY, et al. Tumour necrosis factor-alpha and interferon-gamma production measured at the single cell level in normal and inflamed human intestine. *Clin Exp Immunol* 1990;81:301-5.
- Qasem A, Naser SA. TNFα inhibitors exacerbate Mycobacterium paratuberculosis infection in tissue culture: a rationale for poor response of patients with Crohn's disease to current approved therapy. *BMJ Open Gastroenterol* 2018;5:e000216.
- Chen W, Xu H, Wang X, et al. The tumor necrosis factor receptor superfamily member 1B polymorphisms predict response to anti-TNF therapy in patients with autoimmune disease: A meta-analysis. *Int Immunopharmacol* 2015;28:146-53.
- Naser A, Qasem A, Naser SA. Mycobacterial infection influences bone biomarker levels in patients with Crohn's disease. *Can J Physiol Pharmacol* 2018;96:662-7.
- Qasem A, Safavikhasraghi M, Naser SA. 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 Pathog* 2016;8:45.
- Waschke KA, Villani AC, Vermeire S, et al. Tumour necrosis factor receptor gene polymorphisms in Crohn's disease: association with clinical phenotypes. *Am J Gastroenterol* 2005;100:1126-33.
- Kawasaki A, Tsuchiya N, Hagiwara K, et al. Independent contribution of HLA-DRB1 and TNF alpha promoter polymorphisms to the susceptibility to Crohn's disease. *Genes Immun* 2000;1:351-7.
- Bouma G, Xia B, Crusius JB, et al. Distribution of four polymorphisms in the tumour necrosis factor (TNF) genes in patients with inflammatory bowel disease (IBD). *Clin Exp Immunol* 1996;103:391-6.
- Bach H, Sun J, Hmama Z, et al. Mycobacterium avium subsp. paratuberculosis PtpA is an endogenous tyrosine phosphatase secreted during infection. *Infect Immun* 2006;74:6540-6.
- Crowle AJ, Dahl R, Ross E, et al. Evidence that vesicles containing living, virulent Mycobacterium tuberculosis or Mycobacterium avium in cultured human macrophages are not acidic. *Infect Immun* 1991;59:1823-31.
- Fréhel C, de Chastellier C, Lang T, et al. Evidence for inhibition of fusion of lysosomal and prelysosomal compartments with phagosomes in macrophages infected with pathogenic Mycobacterium avium. *Infect Immun* 1986;52:252-62.
- Fratazzi C, Arbeit RD, Carini C, et al. Macrophage apoptosis in mycobacterial infections. *J Leukoc Biol* 1999;66:763-4.

40. Fratazzi C, Arbeit RD, Carini C, *et al.* Programmed cell death of *Mycobacterium avium* serovar 4-infected human macrophages prevents the mycobacteria from spreading and induces

mycobacterial growth inhibition by freshly added, uninfected macrophages. *J Immunol* 1997;158:4320–7.