January 2016

Macrophage Migration Inhibitory Factor Polymorphisms And Tuberculosis Disease Susceptibility In Kwazulu-Natal, South Africa

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Background: TB is a leading cause of mortality, infecting over one-third of the world's population. Difficulties combating the disease are compounded by the fact that a majority of TB infections remain in an asymptomatic latent state. Macrophage migration inhibitory factor (MIF) is an innate cytokine that is encoded in a functionally polymorphic genetic locus with characterized allelic variants that correlate to TB progression. Understanding the genetic factors that regulate host immune responses to TB will help to identify individuals who are at higher risk of severe infection.

Methods: A case-control study of HIV$^+$ active pulmonary TB cases and HIV$^+$ controls with no history of active TB was conducted on a South African cohort. With informed consent, we obtained demographics, clinical information, and blood samples for determination of MIF promoter polymorphisms: a functional -794 CATT$_{5-8}$ microsatellite, and a closely associated -173 G/C SNP. Serum cytokine levels were quantified using ELISA.

Results: Among 165 enrolled patients (100 cases, 65 controls), 79 were female (40 cases, 39 controls). Aggregate polymorphism assessment revealed non-significant distribution differences between cases and controls for both the 794 CATT$_{5-8}$ (p = 0.3316) and -173 G/C (p = 0.7452) loci. However, stratification by gender reveals a near significant difference in the frequency of CATT$_{5/5}$ (p-value = 0.0863) and -173 G/G (p-value = 0.0949) low expresser genotypes in female cases versus controls but not in males. ELISA showed a significant difference (P = 0.0056) in serum cytokine levels between cases and controls but not between different polymorphisms.

Conclusions: The results from this study suggest that MIF polymorphisms might contribute to susceptibility to TB in a sex-dependent manner and that MIF low-expresser genotypes might confer higher risk for active TB. However, additional studies will need to be done to establish this relationship.

Introduction

Tuberculosis (TB) is a highly contagious disease that is transmitted by *Mycobacterium tuberculosis* carried in airborne particles generated when individuals who have active untreated pulmonary or laryngeal disease cough, sneeze, shout, or sing. Only about 10 percent of subsequently exposed individuals go on to develop an active, symptomatic infection[1]. Over one-third of the world's population is infected with *M. tuberculosis* at any given time. A quarter of these cases occur in Africa, where an estimated 250,000 individuals die of TB every year. More than half of new cases occur in individuals already infected with HIV/AIDS[1]. This is of particular concern because immune compromised individuals have much higher rates of active infection, mortality, and increased susceptibility to drug-resistant strains[2].

Both the innate and adaptive immune responses play a crucial role in controlling the development and severity of infection. In healthy individuals, 90% of TB infections remain in a controlled latent state compared to approximately 60% in immune compromised individuals[2]. Therefore, better characterization of the host immune response to TB infection will be necessary for development of more effective treatment options. Additionally, elements of the immune response may offer predictive value for disease susceptibility and progression. Animal and epidemiological studies have identified several immune factors that are implicated in suppressing TB infections. Some noticeable examples include IFN-γ[3], which plays a pivotal and essential role in protective cellular immunity to tuberculosis infection, and IL-12[4], whose main role is activation of antigen-specific lymphocytes in an IFN-γ dependent manner. These results have led to the use of IFN-γ as a biomarker for tuberculosis diagnosis and treatment monitoring, however, its use has had limited predictive value[5]. Additional biomarkers, such as IP-10, MIP-1β, TGF-α, and VEGF have been proposed for prediction of development of active TB and differentiation of

active vs. latent infection, but so far none have shown high accuracy for diagnosing infection in HIV-infected individuals[5].

A second line of approach for identifying immune factors important in the host immune response to TB has been to search for genetic variants in humans that are associated with active infection. For example, one study showed specific HLA alleles in an Indonesian population were correlated with progression to active TB infection[6]. Epidemiological studies in Gambia have also correlated disease susceptibility to polymorphisms in the natural resistance-associated macrophage protein (NRAMP1) as well as interleukin-1[7]. In this paper, we will utilize a similar approach to examine the effects of Macrophage Migration Inhibitory Factor (MIF) gene polymorphisms on disease susceptibility.

MIF is a cytokine that plays an important role in regulating innate immune and inflammatory responses in humans and has been linked to both autoimmune and infectious disease responses. MIF was definitively cloned and recombinant MIF characterized in 1993 by the Bucala group[9]. A single gene located on chromosome 22 encodes MIF, which is a 12.5 kD protein that is highly conserved across species. MIF is produced by T-cells and macrophages, activates cytokine production, upregulates TLR-4 expression, and suppresses activation-induced apoptosis of inflammatory cells[10]. MIF has been shown to have roles both in pathogenic inflammation and enhanced immunity. Elevated levels of MIF have been linked to sepsis syndromes, malarial infection, and autoimmune diseases[10]. In contrast, MIF also has been linked to enhanced immunity to intracellular microbes, including *Leishmania*, *Salmonella*, *Toxoplasma*, and *Mycobacteria*[11]. The Bucala lab has shown that MIF-deficient mice have lowered cytokine production and impaired ability to control mycobacterial infection, resulting in a higher pulmonary bacterial burden and decreased survival[12,13]. *In vitro* studies have demonstrated that MIF inhibits the growth of virulent *M. tuberculosis* in human macrophages[14].

Four polymorphisms have been identified in the human MIF gene (*MIF*). In the promoter region, these include a functional -794 tetranucleotide repeat ($CATT_{5-8}$) and a -173 G/C single nucleotide polymorphism (SNP) that is in linkage disequilibrium with CATT[15,16]. These polymorphisms can be used to characterize an individual's *MIF* expression as either high, medium, or low. MIF expression genotype also has been correlated with plasma cytokine levels, with low expression allele patients having lower plasma levels of MIF [13]. Studies have shown an association between *MIF* polymorphisms and susceptibility to different infectious states. For example, susceptibility to severe malarial anemia was shown to be partially mediated by *MIF* polymorphisms. An association was found between increasing CATT repeats at -794, the -173 CC haplotype, and severity of malarial anemia[17]. Susceptibility to meningococcal disease[18] and community-acquired pneumonia[19] also have been associated with polymorphisms in *MIF*.

In epidemiological studies carried out by Dr. Bucala's group, some African and Asian populations were shown to have a significantly higher prevalence of low-expression *MIF* alleles compared to other geographical areas[20]. It is hypothesized that this trend evolved as a protection against malaria, given that severe malarial anemia is associated with high-expression alleles[17]. However, patients with low expression *MIF* alleles have been shown to be at significantly greater risk for high TB bacteremia and are more likely to develop sepsis[13]. Our present study was undertaken in South Africa, which has one of the highest incidence rates of tuberculosis in the world, including both multi-drug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) strains. As such, understanding how TB spreads in the context of South Africa is critical to combating the disease. Studying how *MIF* polymorphisms affect susceptibility to TB in this high incidence area will contribute to the understanding of TB pathogenesis as well as help inform prevention and treatment programs, allowing for identification of individuals and groups who are at higher risk of active TB infection.

__Methods__

Patient Recruitment

A case-control study design was used to compare *MIF* polymorphisms between cases who had microbiologically confirmed TB and controls who had no active or history of active TB. Patients were enrolled from two different treatment centers in KwaZulu Natal, South Africa: the Church of Scotland Hospital/Philanjalo NGO in Tugela Ferry and the Greytown MDR-TB Hospital in Greytown from 10 February 2015 to 5 August 2015. This region of the KZN province of South Africa is home to approximately 180,000 traditional Zulu people. The population suffers from high TB incidence (1100/100,000) as well as high HIV prevalence (30% of antenatal patients). The medical infrastructure that serves this area includes a 350-bed Church of Scotland Hospital (COSH) and satellite primary health care clinics. Greytown specialized MDR-TB Hospital is a nearby referral hospital which treats cases of confirmed MDR-TB.

Cases were selected based on the presence of active pulmonary TB, as confirmed by sputum smear, Gene Xpert, or culture. TB cases were further separated into drug susceptible (DS-TB) or multi-drug resistant (MDR-TB). In contrast, controls were individuals who had neither signs of active TB nor history of being on TB treatment. All individuals enrolled were HIV positive and on or beginning Antiretroviral therapy (ART). All patients provided informed consent and the studies were approved by the institutional review boards (IRB) at the collecting institutions and at Yale. Demographic information (age, gender, race, etc.), HIV status and treatment regimen, CD4 counts, history of TB and treatment, as well as laboratory values and image results were collected from each patient. In addition, blood samples were collected by a trained nurse directly into vacutainer tubes, a portion from which serum was separated and transported to K-RITH to be stored at -80° C. All specimens collected were labeled with study ID codes only. Names or identifiers were not used on laboratory specimens.

Genotyping and Serum Analysis

The acquisition and transfer of patient samples to Yale was approved by the providing institution and the Yale human investigation committee (HIC). DNA was extracted from the red blood cell fraction using Invitrogen's Easy DNA extraction kit. Analysis of the *MIF* promoter polymorphism, -794 $CATT_{5-8}$ microsatellite repeat [rs5844572], was carried out by PCR using a forward primer (5'-TGCAGGAACCAATACCCATAGG-3') and a fluorescence-labeled reverse primer (5'-AATGGTAAACTCGGGGGAC-3'). Automated capillary electrophoresis on a DNA sequencer was performed on the PCR products, and the CATT alleles were identified using Genotyper version 3.7 software (Applied Biosystems)[13]. Analysis of the -173 G/C single nucleotide polymorphism (SNP, rs755622) was carried out using a pre-developed TaqMan assay for allelic discrimination and analyzed on a Roche 480 Lightcycler real time PCR machine. Serum MIF levels were measured by sandwich ELISA using specific antibodies[13].

Statistical Analysis

Differences in demographic characteristics were analyzed using the Student t test. Multivariate odds ratios were calculated using logistic regression, controlling for age and sex. The proportion of *MIF* genotypic low expressers ($CATT_{5/5}$ and -173 G/G) as well as the different allelic combinations of the two polymorphisms in the cases and controls were compared by χ2 analysis. Subsequent χ2 analyses were conducted split by gender. SAS (Statistical Analysis Software) was used for all statistical calculations.

Results

Patient Demographics

A total of 165 patients (100 active TB cases, 65 no history of TB controls) were enrolled. Important clinical and demographic characteristics are listed in Table 1. The median age was 36 years with an IQR of 31-46 for the active cases compared to a median age of 37 with an IQR of 28.5-46 for controls. A significantly higher proportion of controls 39/65 (60%) were female compared to cases 40/100 (40%), 2-sample z-test; $p < 0.05$. The median $CD4^+$ T-cell count was 78 for cases (IQR 30.75-163.75) and 207 for controls (IQR 73-480.25). Average $CD4^+$ T-cell counts were 157.2 cells/µl for cases and 274.5 cells/µl for controls.

Characteristic	HIV+ Active TB	HIV+ No TB	P-value
N=	100	65	
Age, median years (IQR)	36 (31-46)	37 (28.5-46)	
Female Sex	40 (40.0%)	39 (60.0%)	**< 0.05***
CD4 T-cell count, median cells/µl (IQR)	78 (30.75-163.75)	207 (73-480.25)	
Average (SD)	157.2 (237.3)	274.5 (233.5)	

Table 1 Clinical and demographic characteristics of subjects.

Genotype Distributions of *MIF* Polymorphisms

The genotype distribution of *MIF*-794 $CATT_{5-8}$ and -173 G/C between the active TB cases and the no TB controls were found to be non-statistically different, as calculated by chi-squared tests for CATT distributions (Table 2, $p = 0.3316$) and for SNP (Table 3, $p = 0.745$).

Frequency Percent Row Pct Col Pct	Table of Outcome by CATT						
				CATT			
Outcome(Outcome)	55	56	57	66	67	77	Total
C	16	27	5	7	5	4	64
	9.76	16.46	3.05	4.27	3.05	2.44	39.02
	25.00	42.19	7.81	10.94	7.81	6.25	
	39.02	42.19	29.41	38.89	26.32	80.00	
I	25	37	12	11	14	1	100
	15.24	22.56	7.32	6.71	8.54	0.61	60.98
	25.00	37.00	12.00	11.00	14.00	1.00	
	60.98	57.81	70.59	61.11	73.68	20.00	
Total	41	64	17	18	19	5	164
	25.00	39.02	10.37	10.98	11.59	3.05	100.00

Frequency Missing = 1

Statistics for Table of Outcome by CATT

Statistic	DF	Value	Prob
Chi-Square	5	5.7470	0.3316
Likelihood Ratio Chi-Square	5	5.8261	0.3235
Mantel-Haenszel Chi-Square	1	0.0067	0.9347
Phi Coefficient		0.1872	
Contingency Coefficient		0.1840	
Cramer's V		0.1872	

Effective Sample Size = 164
Frequency Missing = 1

Table 2 Distribution of *MIF* -794 CATT$_{5-8}$ polymorphisms and chi-squared analysis. Outcome: C = control, HIV+ No Active TB; I = case, HIV+ Active Tuberculosis.

Frequency Percent Row Pct Col Pct	Table of Outcome by SNP			
			SNP(SNP)	
Outcome(Outcome)	CC	GC	GG	Total
C	23	33	9	65
	13.94	20.00	5.45	39.39
	35.38	50.77	13.85	
	38.98	37.93	47.37	
I	36	54	10	100
	21.82	32.73	6.06	60.61
	36.00	54.00	10.00	
	61.02	62.07	52.63	
Total	59	87	19	165
	35.76	52.73	11.52	100.00

Statistics for Table of Outcome by SNP

Statistic	DF	Value	Prob
Chi-Square	2	0.5882	0.7452
Likelihood Ratio Chi-Square	2	0.5791	0.7486
Mantel-Haenszel Chi-Square	1	0.1883	0.6644
Phi Coefficient		0.0597	
Contingency Coefficient		0.0596	
Cramer's V		0.0597	

Sample Size = 165

Table 3 Distribution of *MIF* -173 G/C single nucleotide polymorphism and chi-squared analysis. Outcome: C = control, HIV+ No Active TB; I = case, HIV+ Active Tuberculosis.

Analysis of Maximum Likelihood Estimates

Parameter	DF	Estimate	Standard Error	Wald Chi-Square	Pr > ChiSq
Intercept	1	0.7750	0.6445	1.4461	0.2292
CATT55	1	0.0299	0.3806	0.0062	0.9373
SNPGG	1	-0.2871	0.5037	0.3248	0.5687
AGE	1	0.00221	0.0160	0.0190	0.8904
SEX	1	-0.8033	0.3273	6.0239	0.0141

Odds Ratio Estimates

Effect	Point Estimate	95% Wald Confidence Limits	
CATT55	1.030	0.489	2.172
SNPGG	0.750	0.280	2.014
AGE	1.002	0.971	1.034
SEX	0.448	0.236	0.851

Table 4 Logistical regression. Multivariate analysis adjusting for CATT$_{5/5}$ genotype (CATT55), -173 G/G genotype (SNPGG), age, and sex. Gender was found to significantly predict development of TB (p = 0.0141) with females being less likely to develop disease (OR 0.448 (0.236-0.851)).

Logistic Regression

Logistic regression was conducted to predict development of TB from CATT$_{5/5}$ genotype, -173 G/G genotype, age, and sex (Table 4). Only

the independent variable of sex was determined to significantly predict development of TB (p = 0.0141) with females being at a lower risk of developing disease (OR = 0.448 (0.236-0.851)).

MIF Polymorphisms Distributions by Gender

Due to the significant predictive value of sex in the logistic regression, we next examined the distribution and chi-squared statistic for the different polymorphisms separated by gender (Table 5, Table 6). When separated by gender, the CATT chi-squared p-value drops to 0.1496 for females and 0.2568 for males (Table 5). For SNP distribution, the chi-squared p-value becomes 0.2379 for females and 0.5099 for males (Table 6).

Sex (1=female)=1

Table of Outcome by CATT

Frequency / Percent / Row Pct / Col Pct — Outcome(Outcome)	CATT 55	56	57	66	67	77	Total
C	7	19	4	4	2	2	38
	8.97	24.36	5.13	5.13	2.56	2.56	48.72
	18.42	50.00	10.53	10.53	5.26	5.26	
	33.33	61.29	57.14	44.44	25.00	100.00	
I	14	12	3	5	6	0	40
	17.95	15.38	3.85	6.41	7.69	0.00	51.28
	35.00	30.00	7.50	12.50	15.00	0.00	
	66.67	38.71	42.86	55.56	75.00	0.00	
Total	21	31	7	9	8	2	78
	26.92	39.74	8.97	11.54	10.26	2.56	100.00

Frequency Missing = 1

Statistics for Table of Outcome by CATT

Statistic	DF	Value	Prob
Chi-Square	5	8.1220	0.1496
Likelihood Ratio Chi-Square	5	9.0419	0.1074
Mantel-Haenszel Chi-Square	1	0.0558	0.8133
Phi Coefficient		0.3227	
Contingency Coefficient		0.3071	
Cramer's V		0.3227	

WARNING: 67% of the cells have expected counts less than 5. Chi-Square may not be a valid test.

Sex (1=female)=0

Table of Outcome by CATT

Frequency / Percent / Row Pct / Col Pct — Outcome(Outcome)	CATT 55	56	57	66	67	77	Total
C	9	8	1	3	3	2	26
	10.47	9.30	1.16	3.49	3.49	2.33	30.23
	34.62	30.77	3.85	11.54	11.54	7.69	
	45.00	24.24	10.00	33.33	27.27	66.67	
I	11	25	9	6	8	1	60
	12.79	29.07	10.47	6.98	9.30	1.16	69.77
	18.33	41.67	15.00	10.00	13.33	1.67	
	55.00	75.76	90.00	66.67	72.73	33.33	
Total	20	33	10	9	11	3	86
	23.26	38.37	11.63	10.47	12.79	3.49	100.00

Statistics for Table of Outcome by CATT

Statistic	DF	Value	Prob
Chi-Square	5	6.5447	0.2568
Likelihood Ratio Chi-Square	5	6.6562	0.2475
Mantel-Haenszel Chi-Square	1	0.0272	0.8690
Phi Coefficient		0.2759	
Contingency Coefficient		0.2659	
Cramer's V		0.2759	

WARNING: 42% of the cells have expected counts less than 5. Chi-Square may not be a valid test.

Table 5 Distribution of *MIF* -794 $CATT_{5-8}$ polymorphisms and chi-squared analysis by gender (left: female; right: male). Outcome: C = control, HIV+ No Active TB; I = case, HIV+ Active Tuberculosis.

Sex (1=female)=1

Frequency Percent Row Pct Col Pct	Table of Outcome by SNP			
	SNP(SNP)			
Outcome(Outcome)	CC	GC	GG	Total
C	14	17	8	39
	17.72	21.52	10.13	49.37
	35.90	43.59	20.51	
	43.75	47.22	72.73	
I	18	19	3	40
	22.78	24.05	3.80	50.63
	45.00	47.50	7.50	
	56.25	52.78	27.27	
Total	32	36	11	79
	40.51	45.57	13.92	100.00

Statistics for Table of Outcome by SNP

Statistic	DF	Value	Prob
Chi-Square	2	2.8716	0.2379
Likelihood Ratio Chi-Square	2	2.9581	0.2279
Mantel-Haenszel Chi-Square	1	2.0133	0.1559
Phi Coefficient		0.1907	
Contingency Coefficient		0.1873	
Cramer's V		0.1907	

Sex (1=female)=0

Frequency Percent Row Pct Col Pct	Table of Outcome by SNP			
	SNP(SNP)			
Outcome(Outcome)	CC	GC	GG	Total
C	9	16	1	26
	10.47	18.60	1.16	30.23
	34.62	61.54	3.85	
	33.33	31.37	12.50	
I	18	35	7	60
	20.93	40.70	8.14	69.77
	30.00	58.33	11.67	
	66.67	68.63	87.50	
Total	27	51	8	86
	31.40	59.30	9.30	100.00

Statistics for Table of Outcome by SNP

Statistic	DF	Value	Prob
Chi-Square	2	1.3471	0.5099
Likelihood Ratio Chi-Square	2	1.5563	0.4593
Mantel-Haenszel Chi-Square	1	0.7741	0.3789
Phi Coefficient		0.1252	
Contingency Coefficient		0.1242	
Cramer's V		0.1252	

Table 5 Distribution of *MIF* -173 G/C single nucleotide polymorphism and chi-squared analysis by gender (left: female; right: male). Outcome: C = control, HIV+ No Active TB; I = case, HIV+ Active Tuberculosis.

Distribution of CATT$_{5/5}$ Low-Expresser Genotype in Aggregate and by Gender

The *MIF* CATT$_{5/5}$ genotype has been previously correlated with low MIF expression levels[13,15]. Aggregate distribution of CATT$_{5/5}$ showed no statistical significance ($p = 0.955$) between cases and controls, as compared using a chi-squared test (Table 6). However, separating by gender suggested that the frequency of the CATT$_{5/5}$ low producer genotype is over expressed in female cases versus controls in an almost significant manner. (Table 7; chi-squared p-value = 0.086). In males, the relationship was inversed with a higher proportion of controls having the CATT$_{5/5}$ genotype, although it too was not yet significant (Table 7; chi-squared p-value = 0.100).

Statistics for Table of Outcome by CATT55

Frequency Percent Row Pct Col Pct	Table of Outcome by CATT55		
		CATT55	
Outcome(Outcome)	0	1	Total
C	49	16	65
	29.70	9.70	39.39
	75.38	24.62	
	39.52	39.02	
I	75	25	100
	45.45	15.15	60.61
	75.00	25.00	
	60.48	60.98	
Total	124	41	165
	75.15	24.85	100.00

Statistics for Table of Outcome by CATT55

Statistic	DF	Value	Prob
Chi-Square	1	0.0031	0.9555
Likelihood Ratio Chi-Square	1	0.0031	0.9554
Continuity Adj. Chi-Square	1	0.0000	1.0000
Mantel-Haenszel Chi-Square	1	0.0031	0.9556
Phi Coefficient		0.0043	
Contingency Coefficient		0.0043	
Cramer's V		0.0043	

Table 6 Distribution of *MIF* -794 CATT$_{5/5}$ genotype and chi-squared analysis. Outcome: C = control, HIV+ No Active TB; I = case, HIV+ Active Tuberculosis.

Sex (1=female)=1

Frequency Percent Row Pct Col Pct	Table of Outcome by CATT55		
		CATT55	
Outcome(Outcome)	0	1	Total
C	32	7	39
	40.51	8.86	49.37
	82.05	17.95	
	55.17	33.33	
I	26	14	40
	32.91	17.72	50.63
	65.00	35.00	
	44.83	66.67	
Total	58	21	79
	73.42	26.58	100.00

Statistics for Table of Outcome by CATT55

Statistic	DF	Value	Prob
Chi-Square	1	2.9418	0.0863
Likelihood Ratio Chi-Square	1	2.9877	0.0839
Continuity Adj. Chi-Square	1	2.1330	0.1442
Mantel-Haenszel Chi-Square	1	2.9046	0.0883
Phi Coefficient		0.1930	
Contingency Coefficient		0.1895	
Cramer's V		0.1930	

Sex (1=female)=0

Frequency Percent Row Pct Col Pct	Table of Outcome by CATT55		
		CATT55	
Outcome(Outcome)	0	1	Total
C	17	9	26
	19.77	10.47	30.23
	65.38	34.62	
	25.76	45.00	
I	49	11	60
	56.98	12.79	69.77
	81.67	18.33	
	74.24	55.00	
Total	66	20	86
	76.74	23.26	100.00

Statistics for Table of Outcome by CATT55

Statistic	DF	Value	Prob
Chi-Square	1	2.6944	0.1007
Likelihood Ratio Chi-Square	1	2.5730	0.1087
Continuity Adj. Chi-Square	1	1.8594	0.1727
Mantel-Haenszel Chi-Square	1	2.6631	0.1027
Phi Coefficient		-0.1770	
Contingency Coefficient		0.1743	
Cramer's V		-0.1770	

Table 7 Distribution of *MIF* -794 CATT$_{5/5}$ genotype and chi-squared analysis by gender (left: female; right: male). Outcome: C = control, HIV+ No Active TB; I = case, HIV+ Active Tuberculosis.

Distribution of -173 G/G Low-Expresser Genotype in Aggregate and by Gender

The -173 G/G genotype has likewise been associated with low *MIF* expression[21].

Aggregate distribution of -173 G/G also showed no statistical significance (p = 0.4495) between

cases and controls (Table 8; chi-squared p-value = 0.4495). Separating by gender suggested that

female cases had a higher proportion of the -173 G/G genotype, however, it did not reach

statistical significance (Table 9; chi-squared p-value = 0.0949). The male distribution also

returned a non-significant result (Table 9; chi-squared p-value = 0.2515).

Frequency Percent Row Pct Col Pct	Table of Outcome by SNPGG		
		SNPGG	
Outcome(Outcome)	0	1	Total
C	56 33.94 86.15 38.36	9 5.45 13.85 47.37	65 39.39
I	90 54.55 90.00 61.64	10 6.06 10.00 52.63	100 60.61
Total	146 88.48	19 11.52	165 100.00

Statistics for Table of Outcome by SNPGG

Statistic	DF	Value	Prob
Chi-Square	1	0.5719	0.4495
Likelihood Ratio Chi-Square	1	0.5627	0.4532
Continuity Adj. Chi-Square	1	0.2567	0.6124
Mantel-Haenszel Chi-Square	1	0.5685	0.4509
Phi Coefficient		-0.0589	
Contingency Coefficient		0.0588	
Cramer's V		-0.0589	

Table 8 Distribution of *MIF* -173 G/G genotype and chi-squared analysis. Outcome: C = control, HIV+ No Active TB; I = case, HIV+ Active Tuberculosis.

Sex (1=female)=1

Frequency Percent Row Pct Col Pct	Table of Outcome by SNPGG		
		SNPGG	
Outcome(Outcome)	0	1	Total
C	31 39.24 79.49 45.59	8 10.13 20.51 72.73	39 49.37
I	37 46.84 92.50 54.41	3 3.80 7.50 27.27	40 50.63
Total	68 86.08	11 13.92	79 100.00

Statistics for Table of Outcome by SNPGG

Statistic	DF	Value	Prob
Chi-Square	1	2.7899	0.0949
Likelihood Ratio Chi-Square	1	2.8757	0.0899
Continuity Adj. Chi-Square	1	1.8098	0.1785
Mantel-Haenszel Chi-Square	1	2.7546	0.0970
Phi Coefficient		-0.1879	
Contingency Coefficient		0.1847	
Cramer's V		-0.1879	

Sex (1=female)=0

Frequency Percent Row Pct Col Pct	Table of Outcome by SNPGG		
		SNPGG	
Outcome(Outcome)	0	1	To
C	25 29.07 96.15 32.05	1 1.16 3.85 12.50	30
I	53 61.63 88.33 67.95	7 8.14 11.67 87.50	69
Total	78 90.70	8 9.30	100

Statistics for Table of Outcome by SNPGG

Statistic	DF	Value	Pr
Chi-Square	1	1.3149	0.25
Likelihood Ratio Chi-Square	1	1.5252	0.21
Continuity Adj. Chi-Square	1	0.5514	0.45
Mantel-Haenszel Chi-Square	1	1.2997	0.25
Phi Coefficient		0.1237	
Contingency Coefficient		0.1227	
Cramer's V		0.1237	

WARNING: 25% of the cells have expected counts le
than 5. Chi-Square may not be a valid test.

MIF Polymorphisms and Serum Cytokine Levels

MIF cytokine levels in serum were measured by sandwich ELISA using specific antibodies and showed a statistically significant difference between TB cases and non-infected controls with cases having a higher serum level (Figure 1, 31.9 ng/ml vs 20.9 ng/ml, P = 0.0056). Neither -794 CATT$_{5-8}$ nor -173 G/C polymorphisms were found to correlate with MIF cytokine expression (Figure 2, Figure 4) even after being separated by gender (Figure 3, Figure 5).
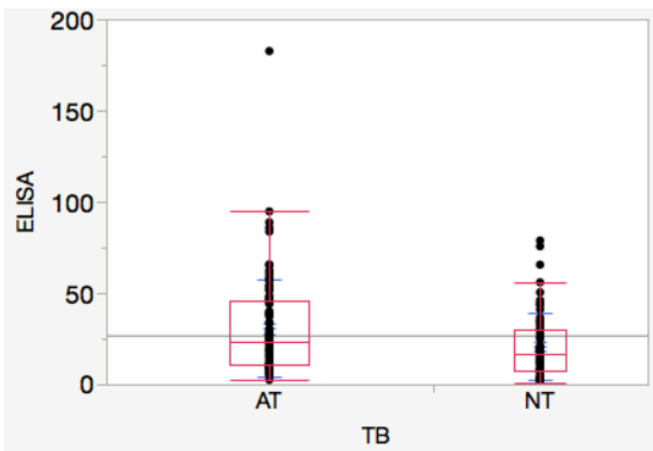

Figure 1 MIF cytokine levels (ng/ml) measured using sandwich ELISA. AT = TB-infected cases. NT = no-TB controls. (Mean (SD): AT = 31.9 (27.4), NT = 20.9 (18.4))

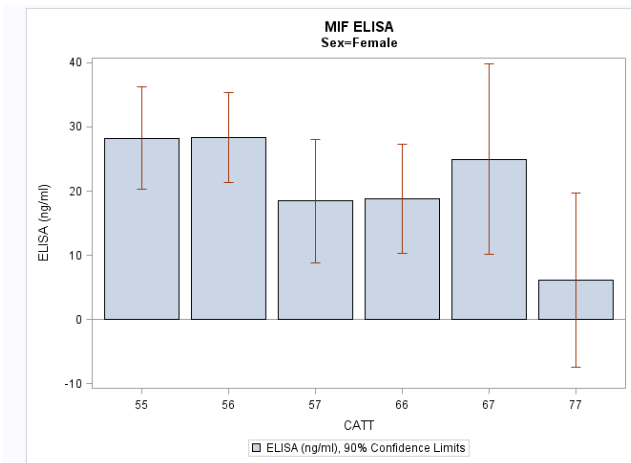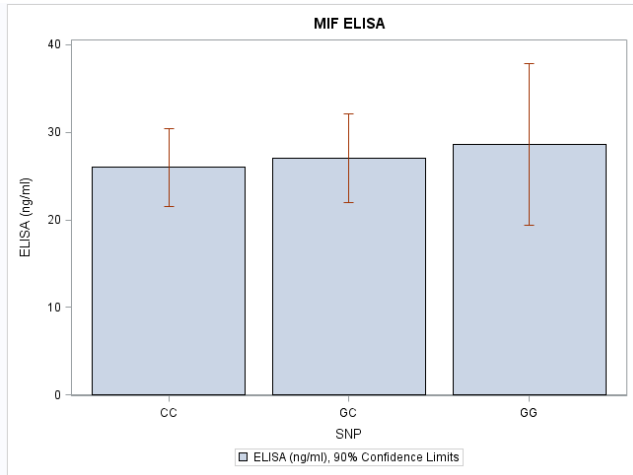**Figure 2** MIF cytokine levels (ng/ml) by -794 CATT genotype and corresponding values.

CATT=55

Analysis Variable : ELISA2 ELISA (ng/ml)

N	N Miss	Mean	Std Dev	Std Error	Lower 90% CL for Mean	Upper 90% CL for Mean
18	2	26.6066827	24.8295667	5.8523850	16.4258344	36.7875310

CATT=56

Analysis Variable : ELISA2 ELISA (ng/ml)

N	N Miss	Mean	Std Dev	Std Error	Lower 90% CL for Mean	Upper 90% CL for Mean
32	1	31.2641937	34.4320771	6.0867888	20.9439289	41.5844584

CATT=57

Analysis Variable : ELISA2 ELISA (ng/ml)

N	N Miss	Mean	Std Dev	Std Error	Lower 90% CL for Mean	Upper 90% CL for Mean
10	0	31.1029337	22.7001639	7.1784221	17.9440753	44.2617922

CATT=66

Analysis Variable : ELISA2 ELISA (ng/ml)

N	N Miss	Mean	Std Dev	Std Error	Lower 90% CL for Mean	Upper 90% CL for Mean
9	0	25.1699081	28.7097141	9.5699047	7.3742106	42.9656055

CATT=67

Analysis Variable : ELISA2 ELISA (ng/ml)

N	N Miss	Mean	Std Dev	Std Error	Lower 90% CL for Mean	Upper 90% CL for Mean
9	2	24.3436118	15.2224802	5.0741601	14.9079674	33.7792562

Figure 3 MIF cytokine levels (ng/ml) by -794 CATT genotype and separated by gender.

Figure 4 MIF cytokine levels (ng/ml) by -173 G/C genotype and and corresponding values.
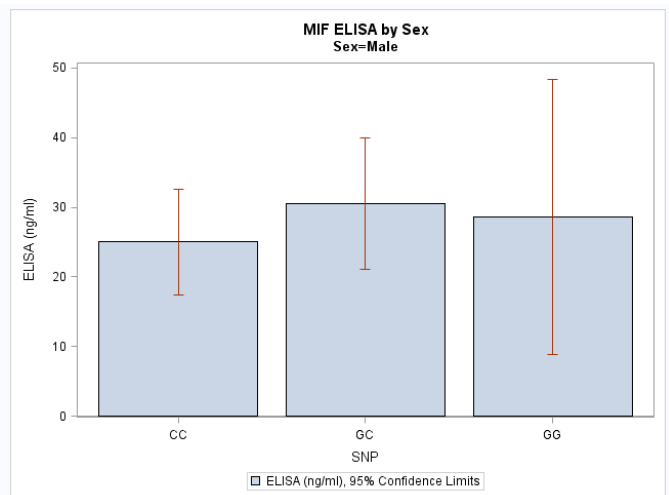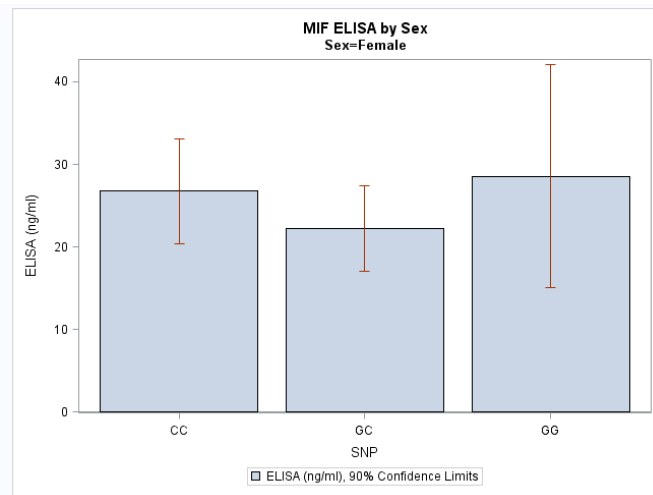

Figure 5 MIF cytokine levels (ng/ml) by -173 G/C genotype and separated by gender.

Discussion

Infection with *M. tuberculosis* results in a wide range of outcomes from asymptomatic clearance to latent infection to clinical disease and mortality. Understanding how the immune system influences clinical outcome is crucial for developing new therapeutics and preventative strategies. Here we utilized a case-control study to examine the effects of functional polymorphisms in the human gene for MIF on tuberculosis disease susceptibility. We enrolled cases who were HIV[+] and had microbiologically confirmed TB together with controls who were

HIV$^+$ but with no history of TB or TB treatment. South Africa has one of the world's most severe tuberculosis epidemics, which is compounded by rising drug resistance and HIV co-infection[22]. Limiting our study to individuals who were HIV$^+$ allowed us to control for immune-deficiency status. However, this approach in turn limits the ability of our results to be extrapolated to other population groups that are not HIV$^+$ or outside of Kwazulu-Natal, South Africa. Even within South Africa, large genetic differences between ethic groups[23] complicate generalization, and further research is needed to elucidate the effects of *MIF* polymorphisms on TB susceptibility in other populations.

Among the patients we recruited, there was a significantly higher proportion of female controls compared to cases (Table 1). How this sample difference affects the calculated results is difficult to determine; however, the subsequent analysis with stratification by gender should have accounted for the discrepancy. The different gender distributions between the cases and controls might partially explain the non-significant results when the data was taken in aggregate.

When we used logistical regression to model the data, the predictor variables we selected were age, sex, and the low *MIF* genotypes CATT$_{5/5}$ and -173 G/G (Table 4). Besides sex, none of the other variables were good predictors for development of TB. Other models with differing combinations of *MIF* genotypes likewise showed no statistical predictive value (*data not shown*). Variables such as CD4 T cell count or HAART treatment history were not included in the model due to missing or otherwise unreliable data points. These results again highlight the important of gender when examining the results of our data set. Whether the significance of gender is due to the distribution of the patients we enrolled or if there is an underlying interaction between gender and *MIF* in the context of HIV and TB is a difficult question to answer.

Another limitation of the study that might explain the non-significant results is the small sample size. Initial calculations gave a conservative minimum required sample size of 210 to

have enough power to find a significant difference between cases and controls. Our sample size of 165 limited the power of our study. Additional recruitment or combining with previous cohorts of patient samples might result in a more significant difference between the two groups due to an increased sample size.

In summary, genetic analysis of *MIF* polymorphisms in this population of HIV^+ individuals in KwaZulu-Natal, South Africa show no statistical difference in the distribution of functional *MIF* polymorphisms between cases who had microbiologically confirmed TB compared to controls who had no history of TB, both at the -794 $CATT_{5-8}$ and the -173 G/C promoter sites (Table 2, Table 3). Stratification by gender showed that the low-expresser $CATT_{5/5}$ genotype was overexpressed in female cases vs. controls in a near significant manner (p = 0.0863, Table 7). In males, the relationship was inversed with controls having a higher expression of $CATT_{5/5}$ genotype, however, the difference also was not statistically significant (p = 0.100, Table 7). Likewise, stratification of -173 G/C distribution by gender suggested that female controls had a higher proportion of the low-expresser -173 G/G genotype, although only at a near significant level (p = 0.0949, Table 9). ELISA showed a difference in serum MIF cytokine levels between cases and controls (Figure 1), which is likely due to TB treatment or response to infection as opposed to basal genetic expression. No difference in cytokine levels were found between the different polymorphisms (Figure 2, Figure 4) or by gender (Figure 3, Figure 5).

The data presented here suggest that there may be a gender-related difference in the relationship between *MIF* polymorphisms and TB disease susceptibility. Additional research is needed to establish whether this relationship persists in other populations or in a larger sample. Previous studies have shown MIF to have gender-dependent effects[24,25], which may partially

explain the results of this study. Additional research is needed to establish whether this relationship persists in other populations.

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