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Macrophage Migration Inhibitory Factor Polymorphisms
and Tuberculosis Disease Susceptibility in KwaZulu-Natal, South Africa

Max Wang
**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 CATT₅₋₈ 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 CATT₅₋₈ (p = 0.3316) and -173 G/C (p = 0.7452) loci. However, stratification by gender reveals a near significant difference in the frequency of CATT₅₋₅ (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-\(\gamma\)\(^3\), 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-\(\gamma\) dependent manner. These results have led to the use of IFN-\(\gamma\) 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-1\(\beta\), TGF-\(\alpha\), 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.  

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. Epidemiological studies in Gambia have also correlated disease susceptibility to polymorphisms in the natural resistance-associated macrophage protein (NRAMP1) as well as interleukin-1. 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. 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. 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. In contrast, MIF also has been linked to enhanced immunity to intracellular microbes, including Leishmania, Salmonella, Toxoplasma, and Mycobacteria. 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. In vitro studies have demonstrated that MIF inhibits the growth of virulent M. tuberculosis in human macrophages.
Four polymorphisms have been identified in the human MIF gene \((MIF)\). In the promoter region, these include a functional -794 tetranucleotide repeat (CATT\textsubscript{5:8}) and a -173 G/C single nucleotide polymorphism (SNP) that is in linkage disequilibrium with CATT\textsuperscript{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\textsuperscript{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\textsuperscript{17}. Susceptibility to meningococcal disease\textsuperscript{18} and community-acquired pneumonia\textsuperscript{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\textsuperscript{20}. It is hypothesized that this trend evolved as a protection against malaria, given that severe malarial anemia is associated with high-expression alleles\textsuperscript{17}. However, patients with low expression \(MIF\) alleles have been shown to be at significantly greater risk for high TB bacteremias and are more likely to develop sepsis\textsuperscript{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). 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.

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.

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

Table 1 Clinical and demographic characteristics of subjects.

Genotype Distributions of MIF Polymorphisms

The genotype distribution of MIF-794 CATT₅₋₈ 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).
Table 2 Distribution of MIF -794 CATT<sub>5/5</sub> polymorphisms and chi-squared analysis. Outcome: C = control, HIV+ No Active TB; I = case, HIV+ Active Tuberculosis.

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.

Table 4 Logistical regression. Multivariate analysis adjusting for CATT<sub>5/5</sub> 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<sub>5/5</sub> 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).

<table>
<thead>
<tr>
<th>Sex (1=female)</th>
<th>Table of Outcome by CATT</th>
<th>Frequency</th>
<th>Percent</th>
<th>Row Pct</th>
<th>Col Pct</th>
<th>C</th>
<th>55</th>
<th>56</th>
<th>57</th>
<th>66</th>
<th>67</th>
<th>77</th>
<th>Total</th>
</tr>
</thead>
<tbody>
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<td>Female</td>
<td></td>
<td>14</td>
<td>12</td>
<td>3</td>
<td>5</td>
<td>6</td>
<td>0</td>
<td>49</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>17.96</td>
<td>15.30</td>
<td>3.85</td>
<td>6.41</td>
<td>7.65</td>
<td>0.60</td>
<td></td>
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<td></td>
<td></td>
<td>66.67</td>
<td>38.71</td>
<td>42.86</td>
<td>55.56</td>
<td>76.90</td>
<td>0.60</td>
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<td>26.92</td>
<td>39.74</td>
<td>9.37</td>
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<td>16.26</td>
<td>2.56</td>
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<td>6</td>
<td>8</td>
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<td>12.79</td>
<td>29.97</td>
<td>10.47</td>
<td>6.98</td>
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<td>18.33</td>
<td>41.67</td>
<td>15.00</td>
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<td>55.00</td>
<td>25.76</td>
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<td>23.25</td>
<td>36.37</td>
<td>11.62</td>
<td>10.47</td>
<td>12.79</td>
<td>2.49</td>
<td>100.00</td>
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</tbody>
</table>

Table 5 Distribution of MIF-794 CATT<sub>5-8</sub> polymorphisms and chi-squared analysis by gender (left: female; right: male). Outcome: C = control, HIV+ No Active TB; I = case, HIV+ Active Tuberculosis.
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<sub>5/5</sub> Low-Expresser Genotype in Aggregate and by Gender

The MIF CATT<sub>5/5</sub> genotype has been previously correlated with low MIF expression levels<sup>13,15</sup>. Aggregate distribution of CATT<sub>5/5</sub> 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<sub>5/5</sub> 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<sub>5/5</sub> genotype, although it too was not yet significant (Table 7; chi-squared p-value = 0.100).
Table 6 Distribution of MIF -794 CATT<sub>55</sub> genotype and chi-squared analysis. Outcome: C = control, HIV+ No Active TB; I = case, HIV+ Active Tuberculosis.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>0</th>
<th>1</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>49</td>
<td>16</td>
<td>65</td>
</tr>
<tr>
<td>I</td>
<td>75</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>Total</td>
<td>124</td>
<td>41</td>
<td>165</td>
</tr>
</tbody>
</table>

Table 7 Distribution of MIF -794 CATT<sub>55</sub> genotype and chi-squared analysis by gender (left: female; right: male). Outcome: C = control, HIV+ No Active TB; I = case, HIV+ Active Tuberculosis.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Table of Outcome by CATT55</th>
<th>Statistics for Table of Outcome by CATT55</th>
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<tbody>
<tr>
<td></td>
<td>Frequency Percent</td>
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</table>
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\textsuperscript{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).

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.
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.

Figure 3 MIF cytokine levels (ng/ml) by -794 CATT 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 ethnic groups\(^{23}\) complicate generalization, and further research is needed to elucidate the effects of \textit{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 \textit{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 \textit{MIF} genotypes likewise showed no statistical predictive value \textit{(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 \textit{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 CATT5–8 and the -173 G/C promoter sites (Table 2, Table 3). Stratification by gender showed that the low-expressor CATT5/5 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 CATT5/5 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-expressor -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 effects24,25, which may partially
explain the results of this study. Additional research is needed to establish whether this relationship persists in other populations.
References


