

## Low expression *Macrophage Migration Inhibitory Factor (MIF)* alleles and tuberculosis in HIV infected South Africans

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### ABSTRACT

Host immunity is crucial for controlling *M. tuberculosis* infection. Functional polymorphisms in the cytokine macrophage migration inhibitory factor (MIF) show global population stratification, with the highest prevalence of low expression *MIF* alleles found in sub-Saharan Africans, which is a population with the greatest confluence of both TB and HIV infection and disease. We investigated the association between *MIF* alleles and tuberculosis (TB) and HIV in South Africa. We acquired clinical information and determined the frequency of two *MIF* promoter variants: a functional  $-794$  CATT<sub>5,8</sub> microsatellite and an associated  $-173$  G/C SNP in two HIV-positive cohorts of patients with active laboratory-confirmed TB and in controls without active TB who were all HIV positive. We found a greater frequency of low expression *MIF* promoter variants ( $-794$  CATT<sub>5,6</sub>) among TB disease cases compared to controls (OR = 2.03,  $p = 0.023$ ), supporting a contribution of genetic low *MIF* expression to the high prevalence of TB in South Africa. Among those with HIV, circulating MIF levels also were associated with lower CD4 cell counts irrespective of TB status ( $p = 0.016$ ), suggesting an influence of HIV immunosuppression on *MIF* expression.

### 1. Introduction

An estimated one quarter of the world's population is infected by *Mycobacterium tuberculosis* (*M. tuberculosis*), with a significant proportion of these cases in Africa, where approximately 450,000 individuals died from tuberculosis (TB) in 2014 [1]. In South Africa, where TB is epidemic, most new cases occur in individuals co-infected with human immunodeficiency virus (HIV) [2]. These immune compromised individuals have a higher rate of TB disease, with both drug susceptible and drug resistant strains of *M. tuberculosis*, and higher mortality. While there is an estimated 10% lifetime risk for developing active TB from reactivation in healthy individuals, the reactivation rate increases to 10% per year in those co-infected with HIV [3,4] and mortality in these

patients is predicted by level of HIV immunosuppression [5–7].

A genetic component to TB infection and disease is recognized; for instance, increased susceptibility to infection occurs in children with rare defects in the IFN- $\gamma$ /IL-12/IL-23 axis [8,9]. The contribution of genetics to adult infection has been more difficult to define. Candidate genes that affect macrophage handling of *Mycobacteria*, including variants in the pattern recognition receptors DC-SIGN, TLRs, NOD2, and LTHA4H, or rare polymorphisms in *SLC11A1* that influence phagosome function have been reported [9–11]. Macrophage migration inhibitory factor (MIF) is an upstream regulator of innate immunity that inhibits activation-induced apoptosis and sustains monocyte/macrophage responses [12]. Whether MIF contributes meaningfully to TB acquisition, reactivation, or severity of disease in different human populations

**Abbreviations:** *MIF*, macrophage migration inhibitory factor gene; MIF, macrophage migration inhibitory factor protein; SNP, single nucleotide polymorphism

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95 °C for 30 s, 54 °C for 30 s, and 72 °C for 60 s, and a final extension at 72 °C for 10 min. The PCR products were purified, diluted to 1:10 in ddH<sub>2</sub>O, and sequenced on an ABI 3730 sequencer [13]. For analysis of the *MIF* -173 G/C SNP (*rs755622*), a TaqMan reaction was used with a realtime PCR machine (TaqMan Lightcycler 480) [13]. A 5 µl reaction mixture containing TaqMan Universal PCR Master Mix, No AmpErase UNG, 20X Assays-On-Demand SNP Genotyping Assay Mix, and 1.0 µl of 10 ng/µl DNA. DNA was amplified in a TaqMan Lightcycler 480 with a hold of 10 min at 95 °C, denaturing for 15 s at 92 °C, and annealing for 1 min at 60 °C. The denaturing and annealing steps were repeated for 50 cycles. The G allele is tagged with FAM (465–510 nm) while the C allele is tagged with VIC (533–580 nm). Four negative controls and four positive controls were included on each plate run.

Serum MIF levels were measured by sandwich ELISA as previously [13] using specific antibodies and a native sequence human MIF standard following our originally developed protocol [22]. The capture and detector anti-MIF monoclonal antibody clones were 3H2F and 10G8D, respectively. The sensitivity of the ELISA is  $\geq 0.834$  ng/ml (CV% = 3.7) and the range is 0–100 ng/ml.

CD4+ lymphocyte counts were measured with a Becton Dickinson LSRII flow cytometer using antibodies from Biolegend). The reference CD4+ T cell value in HIV+ South Africans without evidence of latent or active TB was  $283 \pm 113$  [23].

### 2.5. Statistical and genetic analysis

The sequencing results were analyzed by GeneMapper software. Data were analyzed by the JMP software version 12.1.0 (JMP, NC, USA) and Genepop genetics software [24,25]. Allelic frequencies were calculated in Genepop and an exact test was performed to identify departures from Hardy-Weinberg proportions. Odds ratios (OR) with 95% confidence interval (CI) were calculated for the different *MIF* alleles. In the analysis of the *MIF* -173G/C SNP, the homozygous GG genotype was used as the reference group (*i.e.* OR = 1), and a dominant genetic model employed as in previous studies [26]. To disentangle the independent effects of each *MIF* polymorphism, we used haplotypic analysis. Arlequin suite software (version 3.5.2.2.) was used for estimation of gametic phase based on Gibbs sampling strategy and the Excoffier-Laval-Balding (ELB) method [27,28]. P-values reported are for Mann Whitney U or Kruskal-Wallis tests cases of non-parametric data, or Student's *t*-test or one-way analysis of variance (ANOVA) for parametric data.

## 3. Results

### 3.1. Study population

Overall, the study population totaled 361 HIV infected patients. The Durban cohort consisted of 196 patients of whom 101 had TB disease and 95 who were controls (latent TB or negative for latent TB). The Tugela Ferry/Greytown cohort consisted of 165 patients of whom 100 had TB disease and 65 were controls without TB disease (presumed to contain subjects both with and without latent TB). Tables 1a and 1b provides the clinical and demographic characteristics of the studied subjects. Both cohorts were demographically similar, but the Tugela Ferry/Greytown patients with and without active TB disease had

**Table 1a**  
Demographics and CD4 cell levels of the Durban cohorts (iThimba and String Study).

	TB Disease, n (%) n = 101 (51.5)	No TB, n (%) n = 95 (48.5)	Total n = 196	p value
Age, median (IQR)	34 (29–38)	34 (29–42)	34 (29–39)	0.88
Female sex	55 (54%)	79 (83%)	134 (68%)	< 0.01
CD4 T-cell count, median (IQR)	131.5 (79.5–259.5)	431.5 (330.75–576.75)	302.0 (130.75–470.75)	< 0.01

**Table 1b**

Demographics and CD4 cell levels of the Tugela Ferry/Greytown Cohort.

	TB Disease, n (%) n = 100 (60.6)	No TB, n (%) n = 65 (39.4)	Total n = 165	p value
Age, median (IQR)	36 (31–46)	37 (28.5–46)	36 (30–46)	1.0
Female sex	40 (40%)	39 (60%)	79 (48%)	< 0.02
CD4 T-cell count, median (IQR)	78 (30.75–163.8)	207 (73.0–480.3)	91 (38.5–308.0)	< 0.02

significantly lower mean and median CD4 cell counts.

### 3.2. *MIF* allele, genotype, and haplotype frequencies.

We first investigated two commonly occurring *MIF* promoter polymorphisms in subjects from Durban cohorts (iThimba and String Study): a -173 G/C SNP and a -794 CATT<sub>5-8</sub> promoter microsatellite. The *MIF* allele and genotype frequencies of subjects with TB disease, latent TB, and negative latent TB were not found to be significantly different from those predicted by the Hardy-Weinberg equilibrium. Genetic stratification was not observed in the studied groups as measured by the fixation index ( $F_{ST} = 0.004$  for both loci), indicating that the patients and controls were from a similar genetic background. Statistically significant levels of linkage disequilibrium were found between the two studied *MIF* loci across all study groups ( $D' = 0.76$ ), as reported previously in a southern African population [16].

The *MIF* genotype distributions at the -173 G/C and -794 CATT<sub>5-8</sub> promoter polymorphisms did not demonstrate statistically significant differences in the frequencies of these allelic variants for the latent TB versus negative latent TB groups (*data not shown*). The latent TB and negative latent TB groups were combined as a single control group for comparison with TB disease. The frequency of the low expression *MIF* -794 CATT<sub>5,6</sub> (5/5 + 5/6 + 6/6) genotypes were significantly higher in subjects with TB disease (82.7%) when compared to controls (68.1%) (OR = 2.23, 95% CI = 1.08–4.71,  $p = 0.019$ ; Table 2a). We also observed a similar increase in low expression *MIF* -794 CATT<sub>5,6</sub> (5 + 6) alleles in subjects with TB disease compared with controls (90.8% vs. 83.0%, OR = 2.03, 95% CI = 1.05–3.99,  $p = 0.023$ ; Table 2a).

*MIF* promoter haplotypes were re-constructed computationally and their estimated frequencies and relative odds ratios were calculated. The G6 haplotype was the most prevalent, constituting 30.3% of all haplotypes across the TB subject and control groups; this is in accord with the high prevalence of these two allelic variants and the known linkage disequilibrium between these two promoter polymorphisms [16]. The most significant comparison was observed in active TB subjects versus in controls for the C7 haplotype - which incorporates the functional high expression -794 CATT<sub>7</sub> allele that has been associated with high MIF production in prior studies of pneumococcal infection [31]. A greater proportion of C7 haplotypes were found in the control group compared to the TB disease group (C7-active TB: 8.9% vs. controls: 16.0%; OR = 0.51, 95% CI = 0.27–0.96,  $p = 0.038$ ).

*MIF* genotype distributions for the combined Tugela Ferry and Greytown cohorts are shown in Table 2b. In contrast to the observation in the Durban patients, an association between low expression *MIF*

**Table 2a**

Durban Cohorts. Genotypic and allelic frequencies of *MIF* CATT –794 subjects with TB vs controls, with uncorrected OR. “X” represents any allele, so 7/X is equivalent to “7-containing”. 5/5 + 5/6 + 6/6 is equivalent to all non-7 containing.

MIF –794	TB Disease, n (%) n = 98	No TB, n (%) n = 94	OR (95% CI)	p value
5/5 + 5/6 + 6/6	81 (82.7)	64 (68.1)	2.23 (1.08–4.71)	< 0.02
7/X	17 (17.3)	30 (31.9)	1	
5 + 6	178 (90.8)	156 (83.0)	2.03 (1.05–3.99)	< 0.02
7	18 (9.2)	32 (11.1)	1	

genotypes or alleles and TB disease was not observed. These patients comprised a more immunosuppressed population than the Durban patients (Table 2a) and included patients on ART therapy. Latent TB testing also was not conducted on subjects in this cohort, and this control group potentially included subjects with latent TB.

### 3.3. MIF serum levels and relationship with MIF genotype and disease and HIV immunosuppression

We observed significant differences in MIF serum values between the active TB group and control group in the Durban cohorts, with a median serum MIF concentration of 17.3 ng/ml (IQR 8.1–35.1) in the active TB group compared to a median concentration of 10.5 (IQR 6.7–19.7) in the control group ( $p < 0.015$ ,  $X^2$ ). We found increased circulating MIF concentrations in patients without TB disease who had a high expresser *MIF* haplotype (C7) in the Durban cohorts, with a median serum MIF concentration of 14.2 (IQR 9.3–22.2) in individuals with a C7 haplotype compared to a serum concentration of 9.4 (IQR 6.6–19.1) in individuals without the C7 haplotype ( $p < 0.05$ ,  $X^2$ ). However, *MIF* haplotype (or genotype) association with circulating MIF levels was not observed in subjects with TB disease (*data not shown*). A more detailed analysis of MIF concentrations in relationship to CD4 count levels, however, combining data from both Durban and Tugela Ferry and Greytown cohorts revealed a negative correlation between circulating MIF levels and CD4 count among HIV positive subjects, *i.e.*, significantly higher levels of MIF as CD4 cell levels decreased. (Fig. 2). This correlation between lower CD4 cell counts and higher levels of MIF was observed irrespective of TB status and independent of host *MIF* genotype.

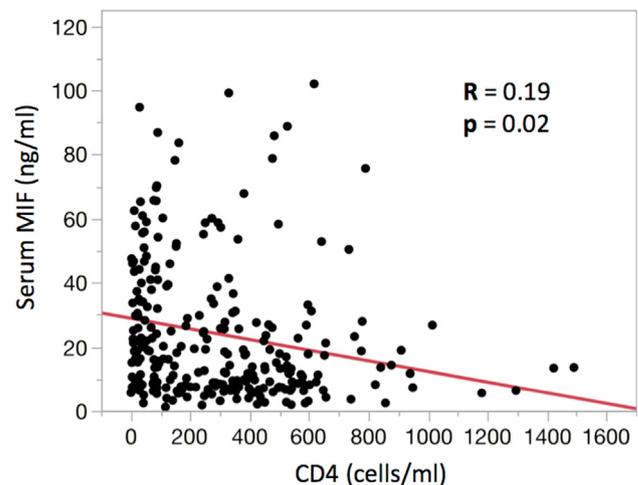
## 4. Discussion

To further define innate immunity to tuberculosis, particularly in the context of HIV, we explored the relationship between *MIF* gene polymorphisms and MIF levels in adults with and without TB disease with co-occurring HIV disease in KwaZuluNatal, South Africa. Accruing evidence supports a role for commonly occurring promoter polymorphisms in *MIF* in TB susceptibility or clinical severity across different populations [13,29–32]. Variant alleles at both the functional –794 CATT<sub>5,8</sub> and the closely associated –173 G/C *MIF* promoter sites occur commonly in the population (minor allele frequency > 5%) [16], and the observation that the highest global prevalence of low

**Table 2b**

Tugela Ferry and Greytown Cohorts. Genotypic and allelic frequencies of *MIF* CATT –794 in HIV positive patients with Active TB vs controls, with OR. “X” represents any allele, so 7/X is equivalent to “7-containing”. 5/5 + 5/6 + 6/6 is equivalent to all non-7 containing.

MIF –794	TB Disease, n (%) n = 100	Controls, n (%) n = 64	OR (95% CI)	p value
5/5 + 5/6 + 6/6	73 (73.0)	50 (78.1)	0.76 (0.36–1.59)	0.46
7/X	27 (27.0)	14 (21.9)		
5 + 6	172 (86.0)	110 (85.9)	1.01 (0.53–1.90)	0.99
7	28 (14.0)	18 (14.1)		



**Fig. 2.** Correlation of serum MIF levels with CD4 count in both active TB and control groups from the Durban and Tugela Ferry/Greytown populations (n = 266).

expression *MIF* alleles is in South Africa, where TB is highly endemic, prompted the present investigation. We were especially interested in studying subjects with HIV co-infection given the critical role of CD4 T cell immunity in controlling TB latency and influencing TB disease progression. Experimental studies support an important role for MIF in the innate control of experimental mycobacterial infection [13,33] and we hypothesized that a significant effect of variant *MIF* alleles would be uncovered by examining TB patients co-infected with HIV and in whom CD4 immunity may be impaired.

In the present case-control study, we investigated the association between *MIF* promoter polymorphisms and TB in HIV co-infected individuals in a Durban population and a Tugela Ferry/Greytown validation cohort, both from KwaZulu Natal province, South Africa. In the Durban patients, we observed that *MIF* promoter microsatellites associated with lower expression (*i.e.*, –794 CATT<sub>5,6</sub>) were significantly overrepresented in the TB disease group compared to the control group. Conversely, the high expression C7 *MIF* promoter haplotype was observed to be significantly overrepresented in the control group, which is consistent with MIF being protective of TB disease.

The functional *MIF* promoter microsatellite was not found to be associated with active TB in the Tugela Ferry/Greytown cohort, however this cohort included cases that were more immunosuppressed than in the Durban cohort, and immunosuppression may have dominated over the protective effect of the high expression *MIF* polymorphisms. Additionally, less rigorous criteria were employed for defining the control group in this cohort. A negative symptom screen and patient-provided history were the basis for inclusion of controls; chest x-ray radiographs were obtained for all subjects only in the Durban population leading to the possibility of including subjects with TB disease in the control group.

Elevated levels of MIF in serum were found in the TB disease group when compared to controls in both the Durban and Tugela Ferry/Greytown populations, which is likely due to an inflammatory response

to TB disease [13]. However, an association between circulating MIF and genotype was not observed in subjects with active TB. Conceivably, active pulmonary disease stimulates tissue inflammatory responses that supervene the influence of *MIF* genotype on MIF levels as measured in the serum compartment. Yende *et al.* [34] for instance, observed protection of high *MIF* expresser genotypes in community-acquired pneumonia but no relationship with circulating MIF levels.

The current and previously published findings collectively suggest that low genotypic *MIF* expression is a risk factor for the development of TB disease in HIV infected hosts, presumably due an inadequate level of constitutive or induced MIF production. MIF facilitates macrophage clearance of *Mycobacterium* by enhancing downstream innate cytokine and reactive oxygen production, but it also promotes neutrophil survival and metalloproteinase production, which may enhance pulmonary tissue damage [13]. Indeed, these later mechanisms have been invoked to explain the contribution of high genotypic *MIF* expression to invasive pneumococcal infection [35,36], increased mortality from meningococcal disease [37], and cystic fibrosis severity [38].

Acute HIV infection elevates circulating MIF levels, which decrease after initiation of anti-retroviral therapy [39,40]. This response is in accord with the down-regulation of systemic inflammation and promotion of immune recovery that occurs with effective anti-retroviral treatment. Among all subjects, and irrespective of TB status, we observed a previously undescribed negative correlation between circulating MIF levels and CD4 lymphocyte counts. MIF is produced by several cell types [41] and progressive CD4 cell decline and immunosuppression may remove immunologic restraints on MIF production or prompt increased MIF production from diverse sites affected by advancing disease and different opportunistic infections. Serial measurement of circulating MIF levels may thereby offer prognostic information in HIV infection and consideration may be given for its potential value as a clinical biomarker in high risk and TB co-infected cohorts. Additional parasitic or other infectious diseases as well as significant malnutrition were not evident in this studied cohort, however follow-up investigations of the relationship between MIF expression and CD4 cell counts will need to control for these potential confounders. At the clinical level, high levels of MIF production and pro-inflammatory cytokines may contribute to organ failure and the high mortality rate associated with active TB in patients with very low CD4 cells [5–7,42].

This first examination of *MIF* promoter variants in TB cases in South Africa supports the hypothesis that the high frequency of low expression *MIF* alleles in South Africans: > 70% for the –794 CATT<sub>5</sub> allele in this study and higher than that reported in any other region globally [16], is an important contributor to the prevalence of active TB in this population. Closer study of *MIF* promoter variants and their interaction with HIV infection and HIV induced immunosuppression, may contribute to better understanding of severe TB disease pathogenesis in HIV co infected patients and offer prognostic biomarker information for TB progression and measures of therapeutic response and support more aggressive or targeted therapies in those carrying *MIF* low expression risk alleles.

## Conflicts of interest

R Bucala is listed as a co-inventor on patents describing the clinical utility of MIF genotype determination.

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