

## Single Nucleotide Polymorphisms (SNPs) of the TNF- $\alpha$ (-238/-308) gene among TB and non TB patients: Susceptibility markers of TB occurrence?\*

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**Background:** Host genetic factors may play a role in the susceptibility to active tuberculosis (TB), and several polymorphisms in different cytokine coding genes have been described and associated with diseases to date.

**Objectives:** To investigate whether polymorphisms within the promoter region of the TNF- $\alpha$  (-238/-308) coding genes are associated to the occurrence of active TB.

**Methods:** SNPs within the TNF- $\alpha$  gene were analyzed by PCR-RFLP among two groups of individuals: patients with TB (n = 234, and patients non TB (n = 113).

**Results:** In this study, the presence of the -238A allele was associated with susceptibility to TB disease occurrence and severity ( $p = 0,00002$ ; OR = 0,15; IC = 0,06-0,36. On the contrary, the -308A allele was associated with protection to the occurrence of another pulmonary diseases.

**Conclusions:** These results suggest the importance of genetics studies on TB occurrence. Further studies are needed pursuing a better understanding of the human pathogenesis of *M. tb*.

**Key words:** uberculosis/genetics. Polymorphism, single nucleotide/genetics. Tumor necrosis factor/genetics. Alleles. Genotype. Lung diseases.

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## INTRODUCTION

The World Health Organization has estimated that more than 1.7 billion people are infected with *Mycobacterium tuberculosis* (Mtb), the causative agent of tuberculosis (TB)<sup>(1)</sup>. For individuals who are infected but asymptomatic (latent TB), the risk for developing the active form of the disease ranges from 5% to 10%. In such individuals, the degree of disease risk depends on the ability of their immune system to prevent the dormant Mtb from multiplying<sup>(2,3)</sup>.

The immune response to Mtb depends on a response mediated by cells, whose interaction with lymphocytes and monocytes culminates in the production of pro- and anti-inflammatory mediators. Notable among the cytokines involved, the tumor necrosis factor-alpha (TNF- $\alpha$ ) has been characterized as one of the most important molecules in inflammatory response promotion. Smith et al.<sup>(4)</sup> demonstrated that the local role played by TNF- $\alpha$  in the pulmonary response to Mtb infection, especially in the production of granulomas, is complex. In addition to aiding bacterial clearance, TNF- $\alpha$  participates actively in the modulation of pulmonary inflammation. Inhibiting the activity of TNF- $\alpha$  in the lungs in the initial phase of infection causes persistent inflammation. Variations in cytokine production among individuals may be related to various genetic polymorphisms, thereby determining the immune response to the disease. Different allelic forms of various cytokine genes have been identified, including single nucleotide polymorphisms (SNPs) at positions -238<sup>(5)</sup> and -308<sup>(6)</sup> in the TNF promoter region. A GA substitution occurs in these polymorphisms, resulting in mutations that seem to be related to differences in gene expression and protein secretion<sup>(7,8)</sup>, although this is still controversial<sup>(9,10,11)</sup>. The presence of -308 SNP has been related to an increase in TNF- $\alpha$  gene transcription<sup>(12,13)</sup>. The presence of the -308 A allele TNF2 has been related to clinical susceptibility to various diseases, such as cerebral malaria<sup>(14)</sup>, mucocutaneous leishmaniasis<sup>(15)</sup>, and lepromatous leprosy<sup>(16)</sup>. All of these diseases have in common high levels of TNF- $\alpha$  circulating in the peripheral blood. On the other hand, the presence of this mutation has also been related to protection against some diseases, such as severe forms of leprosy<sup>(17,18,19)</sup>. The SNP at position -238

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### Síglas e abreviaturas utilizadas neste trabalho:

COPD – Chronic obstructive pulmonary disease  
Mtb – *Mycobacterium tuberculosis*  
SNP – Single nucleotide polymorphism  
TB – Tuberculosis  
TNF – Tumor necrosis factor  
VDR – Vitamin-D receptor

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has been associated with reduced TNF- $\alpha$  transcription<sup>(17,19)</sup>. The presence of the mutant -238A allele in patients with rheumatoid arthritis<sup>(13)</sup>, dengue with HIV<sup>(20)</sup>, or cancer<sup>(21)</sup> has been related to protection against and susceptibility to some diseases, such as chronic hepatitis B and C<sup>(22)</sup>, multiple sclerosis<sup>(23)</sup> and psoriasis in males<sup>(24)</sup>.

The objective of the present study was to determine whether promoter region polymorphisms of the gene encoding TNF- $\alpha$  (-238 and -308) are related to the incidence of TB in patients treated in hospitals in the state of Rio de Janeiro. Moreover, we evaluated whether these polymorphisms correlate with the various clinical forms of TB and with HIV co-infection.

### Patients, Materials and Method

**Population studied.** The study comprised 205 patients diagnosed with TB, admitted to the Hospital Tuberculosis Control Program and treated at two locations within the *Universidade Federal de Rio de Janeiro* (UFRJ, Rio de Janeiro Federal University) medical complex: the *Instituto de Doenças do Tórax* (Institute for Thoracic Disease) of the *Hospital Universitário Clementino Fraga Filho* (HUCFF, Clementino Fraga Filho University Hospital) and at the *Hospital Estadual Santa Maria* (Santa Maria State Hospital). A diagnosis of TB was considered confirmed if the culture tested positive for mycobacteria and subsequent biochemical tests of a clinical sample identified the species as Mtb. Mean age was  $48.5 \pm 7.3$  (range, 15 to 82), and the group comprised 136 males and 69 females. In addition, 113 patients diagnosed with lung diseases other than TB (45 with pneumonia, 5 with malignant carcinoma, 13 with COPD, 15 with meningitis, 12 with asthma, 3 with pulmonary fibrosis and 20 with sepsis) were also included. In this group, ages ranged from 18 to 81, and there were 56 males and 57 females. The Research Ethics Committee of the HUCFF-UFRJ approved this project. All participants gave informed written consent and completed a standardized

questionnaire on demographic data and history of diseases that could be related to the occurrence of the polymorphisms analyzed in the present study. Clinical samples were collected and stored at  $-20^{\circ}\text{C}$  for later use.

**DNA extraction.** We used a protocol based on a commercial extraction kit (Invitrogen, Carlsbad, CA, USA) and a DNA isolation reagent (DNAzol, Gibco BRL/Life Technologies, Gaithersburg, MD, USA), adapted for use on a small scale directly from total blood (fresh or frozen) in our laboratory. In summation, after thawing the samples,  $300\ \mu\text{L}$  of blood were transferred to a fresh test tube, and  $1\ \text{mL}$  of  $0.9\%$  NaCl solution were added. After centrifugation, the resulting sediment was resuspended in a hypotonic TE solution ( $20\ \text{mM}$  Tris-HCl;  $10\ \text{mM}$  EDTA) at  $4^{\circ}\text{C}$ . After another centrifugation, DNAzol was added in order to break down the sediment and liberate the DNA, which was then precipitated with the addition of absolute ethanol. The precipitate was dried at room temperature and redissolved in  $50\ \text{mL}$  of alkaline solution ( $8\ \text{mM}$  NaOH). After having been redissolved, the DNA sample was electrophoresed on a  $1\%$  agarose gel, stained with ethidium bromide to determine integrity and concentration, and then stored at  $-20^{\circ}\text{C}$ .

**DNA amplification using polymerase chain reaction and genotyping.** The genotyping of the polymorphism within the TNF- $\alpha$  promoter gene was carried out as follows: for the -308 position, after amplification of the region of interest (a  $107\text{-bp}$  fragment) as described by Wilson et al.<sup>(6)</sup>, *NcoI* enzyme was used for digestion; for the -238 position, after amplification of a  $165\text{-bp}$  fragment, *BamHI* enzyme was used for digestion. In short, approximately  $100\ \text{ng}$  of the DNA extracted, consisting of  $50\ \text{mM}$  of KCl,  $10\ \text{mM}$  of Tris-HCl pH 8.3,  $1.5\ \text{mM}$  of  $\text{MgCl}_2$ ,  $200\ \mu\text{M}$  of dNTP and  $1.25\ \text{U}$  of AmpliTaq Gold DNA Polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA), was added to each polymerase chain reaction. Mutation-specific oligonucleotides ( $1\ \mu\text{M}$  for -308 and  $12.5\ \text{pmol}$  for TNF -238) were also added, resulting in final volumes of  $50\ \mu\text{l}$  (-308) and  $40\ \mu\text{l}$  (TNF -238). All mixtures were incubated at  $95^{\circ}\text{C}$  for 10 minutes and subsequently submitted to 38-cycle amplifications: (for TNF -308) at  $94^{\circ}\text{C}$  for 1 minute, at  $57^{\circ}\text{C}$  for 1 minute and at  $72^{\circ}\text{C}$  for 1 minute; (for TNF -238) at  $60^{\circ}\text{C}$  for 30 seconds,  $72^{\circ}\text{C}$  for 30 seconds,  $94^{\circ}\text{C}$  for 30 seconds and  $72^{\circ}\text{C}$  for 7 minutes.

**Statistical analysis:** Statistical significance between proportions of sociodemographic data and the differences between genotypic and allelic frequencies was determined using the chi-square test or, when appropriate, Fisher's exact test (Epi Info, version 6, Centers for Disease Control and Prevention). The magnitude of each correlation was estimated as an odds ratio (OR), with its respective confidence interval (CI). The level of significance adopted was  $5\%$ .

## RESULTS

### Genotypic and allelic distribution of -238G/A and -308G/A polymorphisms in the population studied

**Patients with TB.** The presence or absence of SNPs in the gene encoding TNF- $\alpha$  was determined. This determination was made for the -238 position in 200 TB patients and for the -308 position in 205 TB patients. In the analysis of mutation at positions -238 and -308, no significant differences related to gender or HIV co-infection were detected in the incidence of the mutant allele or in any of the possible genotypes (data not shown).

Patients were stratified by clinical presentation of the disease (pulmonary versus extrapulmonary or disseminated). For the mutation at the -238 position, a significantly higher incidence of the wild-type GG homozygote genotype was seen in the group of patients with pulmonary forms of TB than in those with extrapulmonary or disseminated forms ( $p = 0.003$ ; OR = 5.54; CI = 2.01-15.00). In contrast, incidence of the mutant GA and AA genotypes was significantly lower in the group with pulmonary forms than in those with extrapulmonary or disseminated forms ( $p = 0.04$ ; OR = 0.33; CI = 0.11-1.07 and  $p = 0.001$ ; OR = 0.05; CI = 0-0.44, respectively). A significant increase in the incidence of the -238A allele was also observed in the group of patients with the more severe (extrapulmonary or disseminated) forms ( $p = 0.00002$ ; OR = 0.15; CI = 0.06-0.36). Regarding the -308 position, no significant differences in genotypic and allelic frequencies were seen among the clinical presentation forms of TB (data not shown).

**Patients with other lung diseases.** Genetic variability at positions -238 and -308 of the gene encoding TNF- $\alpha$  was evaluated in, respectively, 100 and 113 patients diagnosed with lung diseases other than TB. After gender stratification, only 3 males were found to carry the -238A allele with a heterozygote genotype. No female patients carried

the -238A mutant allele. No HIV-negative patients carried the -238A allele, and only 3 HIV positive patients (6.7%) carried the mutant allele, all of the GA genotype. Among the 113 patients in whom the mutation at the -308 position of the TNF- $\alpha$  gene was analyzed, no significant differences were found in relation to gender or co-infection with HIV (data not shown).

#### Distribution of -238 and -308 G/A polymorphisms in TB and Non-TB patients

The distribution of genotypic and allelic frequencies of polymorphisms at the -238 and -308 positions in the group of patients diagnosed with TB and in the group diagnosed with lung diseases other than TB is shown in Table 1. In the group of patients with other diseases, a significant increase in the incidence of the wild-type -238 GG genotype was observed. Inversely, the frequency of the heterozygote GA genotype was significantly higher in the group of patients with TB, and no patients from the non-TB group presented the mutant homozygote AA genotype. The analysis of allelic frequency showed that the incidence of the -238A allele was significantly higher in the TB group than in those in the non-TB group ( $p < 0.01$ ).

Comparison of the genotypic distribution of SNP in the position -308A between the groups showed that, in the group of patients with TB, there was significantly higher incidence of both the mutant

homozygote (AA) genotype and the -308A allele ( $p = 0.04$ ; OR = 2.48; CI = 0.93-0.97 and  $p = 0.02$ ; OR = 1.94; CI = 1.07-3.58, respectively).

#### DISCUSSION

The participation of genetic background in resistance and susceptibility to initial infection, and in the progression from latent to active TB has been elegantly demonstrated in various animal models<sup>(25,26,27)</sup>. Various studies with humans have shown a genetic component to host susceptibility, resistance to TB infection and conversion to active TB<sup>(28)</sup>. Concordance between monozygotic twins in the development of active TB ranges from 65% to 85%, compared with 20% to 35% for dizygotic twins. Stead et al., in an innovative tuberculin survey conducted in a home for the elderly in Arkansas, reported that Afro-Americans became infected with Mtb more often than Caucasians<sup>(29)</sup>. Other studies have shown that patients carrying mutations in IFN- $\gamma$  and IL-12 receptors present infection with BCG and atypical mycobacteria more frequently and develop more severe clinical manifestations of the disease<sup>(30,31)</sup>. More recently, correlation studies have been carried out involving the genes encoding various factors, such as NRAMP1, vitamin-D receptor (VDR), IL-10, IL-1 and IFN- $\gamma$ , that are important in the pathogenesis of TB<sup>(32,33,34)</sup>. Four polymorphisms, deletions or mutations in the gene encoding NRAMP1 have been correlated with susceptibility to TB in populations in Gambia, Japan, Korea and

TABLE 1  
Genotypic distribution of SNPs at the -238 and -308 positions in the TNF- $\alpha$  promoter region in tuberculosis and non-tuberculosis patients

	TB patients <i>n</i> = 200	NTB patients <i>n</i> = 100	<i>p</i> value	OR	CI
-238					
GG	173 (86.5%)	97 (97%)	0.004	0.20	0.05-0.71
GA	20 (10%)	3 (3%)	0.031	3.59	0.98-15
AA	7 (3.5 %)	0 (0%)			
f (A)	0.085	0.015	0.0007	6.10	1.77-25
-308	<i>n</i> = 205	<i>n</i> = 113			
	<i>n</i> (%)	<i>n</i> (%)			
GG	174 (84.8%)	102 (90.3%)	0.17	0.61	0.27-1.32
GA	06 (2.9%)	05(4.4%)	0.34	0.65	0.93
AA	25 (12.1%)	06 (5.3%)	0.04	2.48	0.93
f (A)	0.13	0.084	0.02	1.94	1.07-3.58

TB: tuberculosis; NTB: non-tuberculosis; *n*: number of patients studied; OR: odds ratio; CI: confidence interval; GG: wild-type homozygote genotype; GA: heterozygote genotype; AA: mutant homozygote genotype; f: frequency

Guinea<sup>(32,33)</sup>. The SNPs found in the IL-1 gene cluster and in the VDR gene have been related to susceptibility to TB in patients in India<sup>(34,35)</sup>. Selvaraj et al.<sup>(39)</sup>, in a recent case-control study, reported no correlation between the -238 and -308 polymorphic sites of TNF- $\alpha$  promoter gene and the development of pulmonary TB. However, the authors did not compare the incidence of these polymorphisms with the various clinical forms of TB. In the present study, we analyzed the possible correlations between these polymorphisms and the severity of the various forms of TB. In addition, we investigated potential correlations between these polymorphisms and various clinical and demographic characteristics, such as severity of clinical manifestation, gender and HIV co-infection.

#### Polymorphisms in the TNF- $\alpha$ promoter region and their correlation with TB: severity and infection

In the present study, only 8% of patients in the non-TB group carried the -238A allele, whereas 15% of patients in the TB group presented this mutation ( $p < 0.01$ ). These results confirm data found in the literature, when comparing patients with TB to those who have been in contact with TB patients and test positive on tuberculin tests, since the presence of this allele (-238A) can be considered a marker of susceptibility to TB<sup>(40)</sup>. The correlation of the -238A allele incidence with extrapulmonary and disseminated TB, as evidenced in the present study, has never before been reported in the literature. Future studies evaluating this correlation and Mtb virulence may confirm its usefulness as a marker of clinical severity, especially in recently infected patients who are at risk for developing the forms of TB that present high rates of morbidity and mortality.

One of the principal limitations of our study was that patients diagnosed with lung diseases other than TB were not submitted to tuberculin tests. Therefore, in our analysis of mutant allele correlations, we could not compare non-TB to TB patients.

However, when the distribution of mutation at the -308 position was studied in combination with increased gene transcription and the consequent higher protection against the incidence of a given disease, we observed significantly higher incidence of this allele and of AA genotype in TB patients than in non-TB patients. A possible explanation for this discrepancy is that the non-TB group comprised

some patients diagnosed with diseases related to high TNF- $\alpha$  production, which is characteristic of carriers of such alleles. Therefore, this group cannot be considered a "clean" control group. It is also known that the TNF- $\alpha$  gene is regulated at various transcriptional and post-transcriptional levels<sup>(38)</sup>. Therefore, another possibility is that some patients diagnosed with TB and carrying the -308 allele (and therefore functionally more able to produce TNF- $\alpha$ ) also carry mutations in other anti-TB immunoregulatory genes, such as that encoding IFN- $\gamma$ , or even in the TNF receptor itself. Therefore, although these patients would present a mutation that induced greater TNF- $\alpha$  production, the absence of receptor malfunction would prevent this cytokine from exerting its inflammatory action, thereby favoring the development of TB.

The immune response, whether protective against TB development (or even TB severity) or not, is related to a network of cytokines produced over the course of the infection. Therefore, identifying the molecular mechanisms that precede the production of these cytokines may represent a powerful tool for researching new vaccines and therapeutic drugs.

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