Concordance between IFNγ gene +874 A/T polymorphism and interferon-γ expression in a TB-endemic indigenous setting

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Abstract

Introduction: Interferon-γ (IFN-γ) plays a crucial role in resistance to mycobacterial diseases; accordingly, variants of the gene encoding this cytokine may be associated with elevated risk of contracting pulmonary tuberculosis (TB). **Methods:** Blood samples were collected from 135 Warao indigenous individuals with newly diagnosed sputum culture-positive TB. Of these, 24 were diagnosed with active tuberculosis (ATB). The study comprised 111 participants, who were grouped as follows: 1) 14 tuberculin skin test (TST)-positive Warao indigenous individuals and 4 that were QuantiFERON-TB®Gold In-Tube (QFT-IT) test-positive, collectively comprising the latent TB infection group (LTBI, n = 18), and 2) healthy controls who were QFT-IT- and TST-negative, comprising the control group (CTRL, n = 93). Detection of the IFN γ gene (IFNG) +874A/T polymorphism was performed via PCR and quantification of IFNG expression via qPCR. **Results:** Relative to indigenous and white Americans, ATB and CTRL groups had a higher frequency of the IFNG SNP (+874A): 23 (95.8%) and 108 (97.3%), respectively. Indigenous Warao individuals homozygous for the IFNG (+874) A allele exhibited 3.59-fold increased risk of developing TB (95% confidence interval, 2.60-4.96, p =0.0001). A decreased frequency of the AT genotype was observed in individuals with pulmonary TB (4.16%) and controls (0.90%). The frequency of the TT genotype was decreased among controls (1.80%); none of the patients with TB were found to have this genotype. The differences in IFNG expression between the groups, under unstimulated and stimulated conditions, were not statistically significant. **Conclusions:** Preliminary results demonstrate concordance between IFNG +874 A/A genotype and low expression of IFNG.

Keywords: Warao. TB. IFNG +874 A/T polymorphism. Genotype. Allele.

INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis,* is one of the most common infectious diseases with high morbidity and mortality worldwide. According to a report of the World Health Organization (WHO), it causes > 2 billion infections and about 2 million deaths annually, mainly in the developing world[1]. The Venezuelan Health Services reported a rate of TB infection between 24.8 and 26.1 per 100,000 among the non-indigenous population, and a rate of between 129.4 and 155.6 per 100,000 inhabitants among the indigenous population between 1997 and 2001[2,3,4].

One-third of the population is infected with *M. tuberculosis*; however, it is estimated that only 5-10% will develop clinical disease[5]. Interferon-γ (IFN-γ) is a regulatory cytokine that participates in the immune response by acting as a pro-inflammatory mediator; it has been shown that IFN-γ levels influence susceptibility towards, and the outcome of, infectious such as TB[5]. IFN-γ is the most important cytokine in resistance to mycobacterial diseases. Macrophage activation by IFN-γ, which secreted by CD40 ligand-expressing Th1 cells, is central to the host response to pathogens, such as *M. tuberculosis*, that proliferate in macrophage vesicles[5,6]. Several polymorphisms in the promoter region of the IFN-γ gene (IFNG), in particular those at position +874, are considered to influence the levels of expression of this cytokine. Several studies have demonstrated the association of this single nucleotide polymorphism (SNP) with susceptibility towards, and severity of, this disease[7,8]. Local and systemic IFN-γ levels correlate with TB severity.

Active tuberculosis (ATB) is characterized by lower levels of IFN-γ production by peripheral blood mononuclear cells (PBMCs) relative to latent infection. In this context, an earlier study carried out in patients with pulmonary TB revealed that the combination of the IFNG +874 polymorphism and allele A homozygosity was associated with significantly lower levels of IFNG compared to those in individuals carrying one or two copies of the T allele[7, 8].

In Venezuela, there are 28 different ethnic groups; among these, the Warao peoples are of low socio-economic status and lack access to healthcare compared with the Creole peoples from urban areas[9]. We previously investigated cytokine secretion by PBMCs and purified protein derivative (PPD)-induced responses in Warao and Creole individuals with TB. Our findings revealed that PBMCs from Creole patients exhibited a tendency for secretion of TNF-α, IL-12, and IFN-γ, while those of Warao individuals exhibited a higher tendency for IL-5 and IL-4 secretion. Therefore, among Warao patients, a tendency for production of lower IFN-γ and higher IL-4 levels, relative to Creole patients, was observed[10]. The aim of the present work was to examine the incidence of the IFNG (+874 A/T) polymorphism in the Warao indigenous population and elucidate its association with *M. tuberculosis* active infection relative to white American individuals. We also aimed to identify concordance between the IFNG +874A/T polymorphism present and IFNG expression in this population.

METHODS

Study design and inclusion and non-inclusion criteria

A transversal study was carried out among individuals of both sexes from the Warao indigenous population living in the Orinoco Delta area. A total of 135 Warao indigenous patients and controls were enrolled and classified according a previously reported scheme, which took into account clinical, nutritional, and epidemiological factors such as tuberculin skin test (TST)-positive and -negative status (TST+ and TST-). Participants were grouped as follows: A) indigenous individuals with active TB (ATB, n = 24), according to clinical evaluation of smear and sputum culture-positive tuberculosis, and B) healthy volunteers (n = 111). Whole peripheral blood samples from all

healthy indigenous individuals without TB symptoms or disease activity were drawn for QuantiFERON®–TB Gold In-Tube (QFT-IT) testing (Commercial test QuantiFERON®–TB Gold In-Tube, Cellestis, Victoria, Australia). These individuals were additionally subjected to TST; results were used as the basis for classification as the TB latent infection group (LTBI, n = 18), which consisted of Warao indigenous TST+ individuals, (n = 14) and QFT-IT-positive (QFT-IT+), (n = 4) individuals. The healthy control group, (CTRL, n = 93) was composed of Warao indigenous QFT-IT- and TST- individuals (**Table 1**).

Inclusion and non-inclusion criteria for individuals with or without evidence of clinical symptoms of pulmonary TB. Individuals with evidence of clinical symptoms suggesting pulmonary TB infection were diagnosed as having pulmonary TB, using at least one of the following previously applied criteria: X-ray suggestive of TB and positive sputum smear or positive sputum culture[11]. For confirmatory diagnosis of TB, sputum was collected for investigation of alcohol/acid-fast bacilli among all individuals presenting respiratory symptoms. Smears were stained using the Ziehl-Neelsen direct method. For each sputum sample, 2 tubes of modified Ogawa egg medium and Lowénstein-Jensen were inoculated using the swab method of Kudoh and Kudoh[12]. Patients with coinfection with the human immunodeficiency virus were excluded from the study. Tuberculosis medication was initiated in all cases of TB identified via X-rays, bacilloscopy, or culture as recommended by the Venezuelan National TB Control Program.

Inclusion criteria for healthy controls comprised lack of evidence of clinical symptoms suggesting pulmonary TB infection, in which HIV and active TB were ruled out by blood tests, microbiological assays, and X-rays, respectively. Individual prescribed immunosuppressive drugs (i.e., corticosteroids, azathioprine, and cyclophosphamide) were excluded. Participants who did not sign an informed consent agreement were additionally excluded.

Ethical considerations

The present study complied with the Helsinki Declaration. This study was approved by the Ethical Committee of the Biomedicine Institute. (Protocol number CDCH-

TABLE 1

Age and gender characteristics and immunological and bacteriological markers of the study population.

Characteristics	ATB	LTBI	CTRL
Ages in years (Mean±SD)	32.0±17.5	42.3±19.8	36.6±14.3
Number of Male/Female	10/14	10/08	28/65
Smear+ (%)	64.0[a]	0[b]	ND
Culture+ (%)	100.0[c]	0[d]	ND
QFT-IT + (%)	25.0[e]	22.0[f]	0.0[g]
TST+ (%)	ND	77.7[h]	0.0[i]

The ATB group: Patients with active TB. The LTBI group: Individuals with TB latent infection (QFT-IT or tuberculin skin test-positive (TST+)), and the CTRL group: Healthy individuals (QFT-IT negative). Statistically significant differences were found between: [a] & [b], [c] & [d] p < 0.0001, [e] & [g], p<0.0005, and [f] & [g], [h] & [i] p < 0.0001. Not done (ND). **ATB:** active tuberculosis; **LTBI:** latent tuberculosis infection; **CTRL:** healthy control; **ND:** not done.

UCV-8007/2011-FONACIT-2013002319/2013-UNU-BIOLAC/2016). Voluntary informed consent forms were signed by individuals.

Quantification of IFN-γ

Blood samples were transported and processed soon after collection. Briefly, tubes were incubated for 24h at 37°C; then, plasma was separated from blood by centrifugation. Measurement of IFN-γ levels in plasma was performed by enzyme-linked immunosorbent assay (ELISA) using the QFT-IT test kit, following the manufacturer's instructions. The cut-off value for a positive result was ≥ 0.35 International Units (IU)/mL of IFN-γ in the sample after stimulation with a mixture of the TB7.7 antigen and two synthetic peptides of; the early secreted antigen-6 (ESAT-6), the culture filtrate protein-10 (CFP10), and TB7.7, regardless of the result obtained for the positive control. In accordance with the manufacturer's instructions, the test result was considered indeterminate if the value of the antigen-stimulated sample was negative or if the value of the positive control was < 0.5IU/mL after subtraction of the value of the nil control, and/or if the negative control was > 8.0IU/mL.

Tuberculin skin test

Tuberculin skin test (TST) was carried out after performing the QuantiFERON®–TB test on Warao indigenous individuals without TB symptoms or disease activity. The TST was administered according to the Mantoux method, by injecting intradermally 2 tuberculin units; 0.1 mL of purified protein derivate (RT23 PPD; Statens Serum Institute, Copenhagen, Denmark) as previously described[9, 10]. Reading was performed by trained professionals between 48 and 72 hours after administration. The criterion for positive test reactivity was based on measurements of the transversal diameter of the indurations on the volar surface of the forearm; (value ≥ 10mm), according to national guidelines[2]. Due to ethical considerations and norms of the Venezuelan National TB Control Program, the TST test was not performed on Warao indigenous diagnosed with active TB.

Genotyping of subjects

The two alleles of the polymorphic region of the first intron of IFNG (+874 A/T), corresponding to each individual were analyzed in two different reaction samples using amplification refractory mutation system polymerase chain reaction (PCR). Briefly, a blood sample of 3-5 mL was collected in a Vacutainer tube with EDTA as anticoagulant. Total DNA was extracted from peripheral blood cells using the Total DNA Isolation System kit (Promega Corporation, WI, US), following the instructions of the supplier. Two sense primers; 5'-TTCTTACAACACAAAATCAAATCA-3' (primer A) and 5'-TTCTTACAACACAAAATCAAATCT-3' (primer T), and one antisense primer 5'-TCAACAAAGCTGATACTCCA-3' were used to amplify the IFNG +874 A/T polymorphism. Each reaction employed a generic antisense primer and one of the two allele-specific sense primers. In every reaction sample, a pair of primers based on the nucleotide sequence of the human growth hormone (Gh1f); 5′-GCCTTCCAACCATTCCCTTA-3′ (sense) and 5′-TCACGGATTTCTGTTGTGTTTC-3′ (antisense) was included as internal control. The PCR protocol comprised 10 cycles:

95°C for 1 min, 95°C for 15 s, 62°C for 50 s, and 72°C for 40 s, followed by 20 cycles; 95°C for 20 s, 56°C for 50 s, and 72°C for 50 s. The PCR assay was performed using 2μl of DNA (30 ng/μl), 5μl of the master-mix SYBER Green (Promega Corporation, WI, US), 1.2μl of sterile and nuclease-free water and 0.6 μl of each primer (5 mM) added in a final volume of 10 μl. The amplified products were separated by electrophoresis on a 2% agarose gel stained with SYBR Green (Sigma-Aldrich Co, St. Louis MO, US) and visualized by a Benchtop UV transilluminator, MultiDoc-It Digital Imaging System camera combination (**Figure 1**).

IFNG amplification

Quantitative PCR (qPCR) was performed on 50 blood samples under non-stimulation and stimulation; under stimulation, 3-5ml of peripheral blood sample was collected in a Vacutainer tube with EDTA as anticoagulant and stimulated with antigens (ESAT-6, CFP10, and TB7.7) for 24h at 37°C, after incubation, both non-stimulated and stimulated blood cells were obtained by centrifugation. Total RNA was extracted from blood cells using the Total RNA Isolation System kit (Promega Corporation, WI, US) following the instructions of the supplier, and the RNA content was measured using a spectrophotometer at 260nm. cDNA was generated from 5μg of RNA using the Reverse Transcription System kit (Promega Corporation, WI US), following the manufacturer's protocol. Briefly, RNA was incubated with a 1μl of oligo dT primer (50μM); the reaction was made up to 12μl with sterile and RNaseOut-free water, and incubated at 70ºC for 10 min, after which it was quickly cooled on ice. A total of 2μl of 10 × first-strand buffer (100mM Tris-HCl, pH 8.8 at 25ºC; 500 mM KCl; 1% Triton X-100), 2μl MgCl$_2$ (25mM), 2μl deoxynucleoside trisphosphate mix (10mM of each dATP, dGTP, dCTP, and dTTP) and 1 μl of RNaseOut RNase inhibitor (40U/μl) were added. The mix was incubated at 42ºC for 2 min after which it was further incubated at 42ºC for 50 min and 70ºC for 10 min with 1μl of AMV Reverse Transcriptase. The cDNA content was measured in a spectrophotometer at 280/260nm, and cDNA samples were stored at -80°C until use.
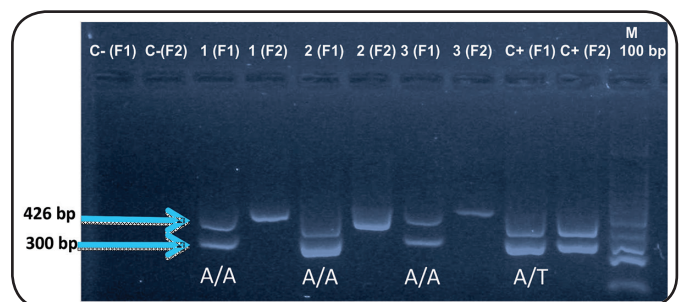

FIGURE 1 - **Genotypes of Warao indigenous individuals according to IFNG polymorphism status.** The genotype of each individual (+874A/T) was analyzed using the amplification-refractory mutation system-PCR technique. A pair of primers based on the nucleotide sequence of the human growth hormone (GH1f) was added to amplify a 426-bp product (Control +) to assess the success of PCR; negative control (C-), A allele (F1), T allele (F2), Base pair marker (100bp) (M).

Primers for IFNG selected gene were was designed using the GENBANK database; GTTTTGGGTTCTCTTGGCTGTTA (sense), and AAAAGAGTTCCATTATCCGCTACATC (antisense), (IDT®, USA, NM-000619.2 amplicon size: 112 (bp)) were used to amplify IFNG, data were normalized to those for the reference gene[5]. We analyzed expression levels of the frequently used reference genes; ACTβ, HPRT, and GAPDH to identify the most suitable ones for our study. ACTβ and HPRT were excluded as their amplification rates were too low. GAPDH was used as the reference gene. AGCCACATCGCTCAGACAC (sense), and GCCCAATACGACCAAATCC (antisense), (IDT®, USA, NM-002046.3 amplicon size: 66 (bp) were used as primers for GAPDH. All primers were designed to anneal at 60°C, and primer specificities and assay efficiencies were tested on control cDNA.

Quantitative PCR was performed as follows: 2μl of cDNA (50ng) was incubated with 8μl of reaction mix, which was composed of 5μl of SsoFast™ EvaGreen® 2x (BioRad, USA), 2.5μl of sterile and nuclease-free water, and 0.25 μl of IFNG or GAPDH forward and reverse primers (20 μM) each. The reaction mix was placed in a Light Cycler 480® (Roche Life Sciences, USA). Cycling parameters were 1 cycle for enzyme activation at 95°C for 30 s, 45 cycles of amplification at 95°C for 5 s and 60°C for 10 s, 1 cycle for melting curve analysis at 60°C and 90C for 15 s, and 1 cycle for cooling at 40°C for 30 s. Assay efficiencies were tested on positive and negative cDNA control samples prepared by pooling cDNA from several individuals, as well as on blank control without cDNA; these controls were included in every assay.

Quantification of gene expression

Acceptance criteria for specific amplification of each targeted cDNA were set as follows: 1) Crossing point (Cp) value for specific amplification of control cDNA to be below 40; 2) Single dominant peak in the derivative of the melting curve; 3) No amplification of non-template controls.

The mRNA relative expression of each gene was calculated based on the values of Cp. Normalization of Cp was performed against corresponding values for the constitutive gene GAPDH, using the following equation: $2^{-\Delta\Delta ct}$ [13].

$$ER=2^{-((Cp\ problem-Cp\ GAPDH)-(Cp\ control-\ Cp\ GAPDH\ control))}$$

Data analysis

Genotype frequencies were compared by the Fisher's exact test, and the relative risk and odds ratio for disease susceptibility or clinical course were calculated. Genotype frequencies in patients and control subjects were not significantly different from those predicted under Hardy-Weinberg equilibrium. Results were analyzed by the Mann-Whitney rank-sum test. A multiple logistic regression model employing the likelihood ratio was used to examine the influence of different genotypes and *ex vivo* IFNG expression on susceptibility to tuberculosis. For comparison of IFNG expression between groups, Shapiro-Wilks for normality, Kruskal-Wallis, and Dunn's multiple comparison tests were performed using GraphPadPrism, 6.0-Windows (San Diego, Ca. USA).

RESULTS

Characteristics of study participants

A total of 135 participants were enrolled into this study, 24 (17.8%) of who were found to have culture-positive pulmonary TB or ATB. Of the 135 study participants, 111 (82.2%) were classified as follows: 18 (16.2%) with LTBI (QFT-IT+ or/and TST+) and 93 (83.8%) were healthy controls (CTRL) with no reactivity to QFT-IT (QFT-IT-) or TST (TST-). The age results are shown as value X ± SD years. The average age was 32.0 ± 17.5 years, 42.3 ± 19.8 years, and 36.6 ± 14.3 years for ATB, LTBI, and CTRL groups (Table 1). There were no statistically significance differences between males and females in the ATB group (10/14), the LTBI group (10/8), and the CTRL group (28/65), respectively (**Table 1**). Demographic and clinical data for the participants are shown in **Table 1**.

Bacteriological, QFT-IT, and TST

Table 1 shows results for the bacteriological, QFT-IT, and TST. Smears or smear plus culture were performed for all indigenous individuals with TB symptoms or disease activity (**Table 1**). Bacteriological assays revealed a statistically significant difference between ATB and LTBI groups, both for smears as well as cultures; p < 0.0001 (**Table 1**). Chest radiograph studies confirmed radiographic characteristics with lesions suggestive of active TB (data not shown). QFT-IT results showed a statistically significant difference between indigenous QFT-IT+ and QFT-IT- in ATB (6/24) and LTBI (4/18) versus CTRL (0/93) groups; p < 0.0001and p < 0 .0005, respectively (**Table 1**). The skin reactivity test was carried out in all indigenous individuals without TB symptoms or disease activity in order to study delayed-type hypersensitivity (DTH); reactions for which the transversal diameter of indurations ≥ 10mm were considered positive. There was a statistically significant difference between indigenous TST+ and TST- in LTBI (14/18) and CTRL (0/93) groups, p < 0.0001 (**Table 1**).

Cytokine genotype and allele frequencies of Warao individuals

Analysis of genotype and allele frequencies for IFNG +874 A/T in indigenous patients with TB as well as controls revealed that the AA genotype for IFNG was the most common (**Table 2**). The AA genotype was present in 108 (0.973) controls and 23 (0.958) patients, respectively, p < 0.0001 (**Table 2**); the AT genotype in 1 (0.009) control and 1 (0.041) patient, respectively, and the TT genotype in 2 controls (0.018); this genotype was not found in the patient group (data not shown). Using the approximation of Katz in indigenous groups, a significant difference in genotype frequencies was observed when comparing the A allele between controls and patients with TB: AA homozygous indigenous individuals showed a relative risk (RR) of [0.98 (95% CI; 0.9011-1.077, p < 0.0001)]. However, there was no significant difference between indigenous individuals with the AA genotype and those with other genotypes such as TA, RR of [4.62 (95% CI; 0.2995-71.423, p < 0.32)] .

Analysis of allele frequency for the IFNG +874 A/T genotype showed that the A allele was markedly overrepresented among Warao indigenous individuals compared with the T allele. A significant difference in allele frequencies was observed when comparing A allele between controls [217 (0.977)] and patients with TB [47 (0.979)]. Using the approximation of Katz, a RR of [0.21 (95% CI; 0.1457-0.3113, $p < 0.0001$) was found (**Table 2**); however, there was no significant difference between indigenous controls [5 (0.022)] and patients with TB [1 (0.020)] with respect to the T allele, RR of [0.20 (95% CI; 0.032-1.241, $p < 0.080$)], (**Table 2**).

Cytokine phenotype frequencies for Warao indigenous and white American individuals

Genotype and allele frequencies for IFNG +874 A/T among the Warao indigenous and white American individuals are presented in **Table 3.** Compared with the white American population, Warao indigenous individuals exhibited a higher frequency of the IFNG +874 allele SNP, whose phenotypic expression is associated with decreased production of IFN-γ. Results showed that the AA genotype for IFNG +874 A/T differed significantly between Warao indigenous and white Americans: the frequency of this genotype was higher in Warao indigenous individuals [131(0.97)] compared with white American individuals 27 (0.27) (**Table 3**). However, AT and TT genotypes were less common among Warao indigenous individuals [2 (0.014) for both genotypes] compared with white American individuals [53 (0.53) and 20 (0.20)], respectively, (**Table 3**). Using the approximation of Katz, a significant difference in genotype frequencies was observed when comparing AA genotype between indigenous and white Americans; AA-homozygous individuals had a RR of [3.59 (95% CI; 2.600-4.968, $p < 0.0001$)], (**Table 3**). Additionally, a significant difference was found between indigenous with the AT and TT genotypes and white Americans RR of [0.02 (95% CI; 0.0069-0.1120, $p < 0.0001$)] and RR of [0.07 (95% CI; 0.0177-0.3098, $p < 0.0001$)], respectively, **Table 3**. In relation to allele frequency for IFNG +874A/T, results showed that the A allele was markedly overrepresented among Warao indigenous individuals compared with the T allele. Using the approximation of Katz, a significant difference for allele frequencies was obtained when comparing A allele between Warao indigenous [264 (0.98)] and white American individuals [107 (0.54)] RR of [1.82 (95% CI; 1.604-2.082, $p < 0.0001$)], (**Table 3**). Additionally, there was a significant difference between indigenous [6 (0.02)] individuals and white Americans [93(0.47)] with respect to the T allele RR of [0.04 (95% CI; 0.0213-0.1069, $p < 0.0001$)], (**Table 3**).

Analysis of IFNG expression by quantitative assay

Figure 2 shows the medians of mRNA relative expression between groups studied. A total of 50 individuals composing the quantitative PCR set were studied; these were grouped into three diagnostic categories: 4 indigenous patients with ATB, 18 indigenous patients with LTBI, and 28 healthy indigenous controls (CTRL). The findings are shown as medians and inter-quartile range (IQR) of expression relative of the ATB, LTBI,

TABLE 2

Genotype distribution and allele ratio for the Warao indigenous population.

Genotypes/Alleles	Healthy Controls (n = 111)	Patients with TB (n = 24)	RR	CI (95%)
A:A	108(0.973)[a]	23(0.958)[b]	0.98	0.9011-1.0077
T:A	1(0.009)	1(0.041)	4.62	0.2995-71.423
T:T	2(0.018)		-	-
Fischer's exact Test (p value)		$p < 0.0001$*		
A Allele/T Allele	217(0.977)[c]	47(0.979)[d]	0.21	0.1457-0.3113
	5(0.022)	1(0.020)	0.20	0.0320-1.2410
Fischer's exact Test (p value)		$p < 0.0001$**		

There were statistically significant differences between controls and patients with TB with AA genotype, [a] & [b] $p < 0.0001$; and controls and patients with TB cases with the A allele, [c] & [d] $p < 0.0001$. **TB:** tuberculosis; **RR:** relative risk; **CI:** confidence interval.

and CTRL for non-stimulated and stimulated conditions. There were no statistically significant differences between groups (**Figure 2**).

Eighteen blood samples from indigenous patients in the LTBI group, under non-stimulated conditions, showed a decreased IFNG level (4.83 ± 14.51 of mRNA relative expression under non-stimulated conditions, while under stimulated conditions was 6.94 ± 23.53 of mRNA relative expression); however, there was no statistically significant difference between the two conditions, $p < 0.15$ (**Figure 3**). Analysis of the 28 blood samples from CTRL indigenous individuals under non-stimulated conditions showed that the median IFNG levels were higher in this group: 17.50 ±51.15 of mRNA relative expression compared with that under stimulated conditions; 14.45 ± 41.31 of mRNA relative expression; there were no statistically significant differences between groups under each condition, $p < 0.96$ (**Figure 3**). Analysis of 4 blood samples from indigenous individuals with ATB without stimulation revealed that the median IFNG levels in response to *M. tuberculosis*-specific antigens at baseline evaluation was 0.56 ± 0.59 of mRNA relative expression. In comparison, the median IFNG levels under stimulated conditions were 1.54 ± 2.62 of mRNA relative expression; there were no statistically significant differences under the two conditions ($p < 0.49$) (data not shown).

DISCUSSION

The Venezuelan Warao indigenous group consists of 25,125 individuals, comprising 17% of the total American Indian population of Venezuela[2]. Although the Warao have remained largely isolated for millennia due to their inaccessible swampland habitat, this group has had increased contact with the Mestizo population ("Creoles") of urban areas in recent years, consequently contracting infectious diseases such as TB from the latter.

TABLE 3

IFN-γ genotype frequencies in Warao indigenous and white American individuals.

Genotypes/Alleles	Warao Indigenous (n = 135)	White American (n = 100)	RR	CI (95%)
A:A	131(0.970)[a]	27(0.270)[b]	3.59	2.600-4.968
T:A	2 (0.014)[c]	53(0.530)[d]	0.02	0.006-0.112
T:T	2 (0.014)[e]	20(0.200)[f]	0.07	0.017-0.309
Fischer' s exact Test (p value)	p < 0.0001 p < 0.0001 p < 0.0001			
A Allele/ T Allele	264(0.977)[g] 6(0.022)[i]	107(0.535)[h] 93(0.465)[j]	1.82 0.04	1.604-2.082 0.021-0.106
Fischer' s exact Test (p value)	p < 0.0001 p < 0.0001			

AA genotype and AT + TT genotype frequencies were significantly different between Warao indigenous and white American individuals, [a] & [b], [c] & [d] and [e] & [f] p < 0.0001, Additionally, statistically significant differences in A and T allele frequencies were observed between Warao indigenous and white American individuals, [g] & [h], [i] & [j] p < 0.0001; Relative risk (RR) and Confidence Interval (CI).

The genetic component of TB susceptibility in this population likely involves interaction between alleles located on different genes, as well as chromosomes; this higher susceptibility is considered to arise as a result of the different genetic backgrounds of Warao indigenous individuals and white Americans. Immunological studies performed in the Warao population confirmed inheritance and segregation of DW, an antigen of class II Human Leukocyte Antigen (HLA), HLA haplotypes, linkage disequilibrium defined only in homozygous typing cells of Warao origin, and DR/DQ associations not seen previously in other human populations[14,15]. The role of immunogenicity factors in influencing the incidence of pulmonary TB in a population is well known[14, 15]. In the present work, we aimed to examine a polymorphism in IFNG, which is the most important cytokine in resistance to mycobacterial diseases, and its association with elevated susceptibility to TB in the Warao indigenous population. Present findings showed the highest frequency of the AA genotype of IFNG among Warao CTRL or indigenous controls and Warao patients with ATB. Further, a low frequency of heterozygous AT (AT genotype) among these indigenous groups: Warao CTRL and ATB, and a low frequency of T homozygous (TT genotype) within the Warao CTRL group, were observed. A significant difference in genotype frequencies was obtained when comparing the AA genotype between CTRL and ATB groups; AA homozygous indigenous individuals showed a relative risk of 0.98. The IFN-γ cytokine is the archetypical readout for cell-mediated immune response (CMI) assays[16], and has been recognized as the defining cytokine of Th1 cells. IFN-γ–mediated immune activation appears to play an important role in immunity to intracellular pathogens, both in mice and humans[17-19]. Early studies demonstrated that mice with disruption of IFNG are more susceptible to infection by *M. tuberculosis* than wild-type strains[20]. Zembruzuski et al. have investigated the Xavante
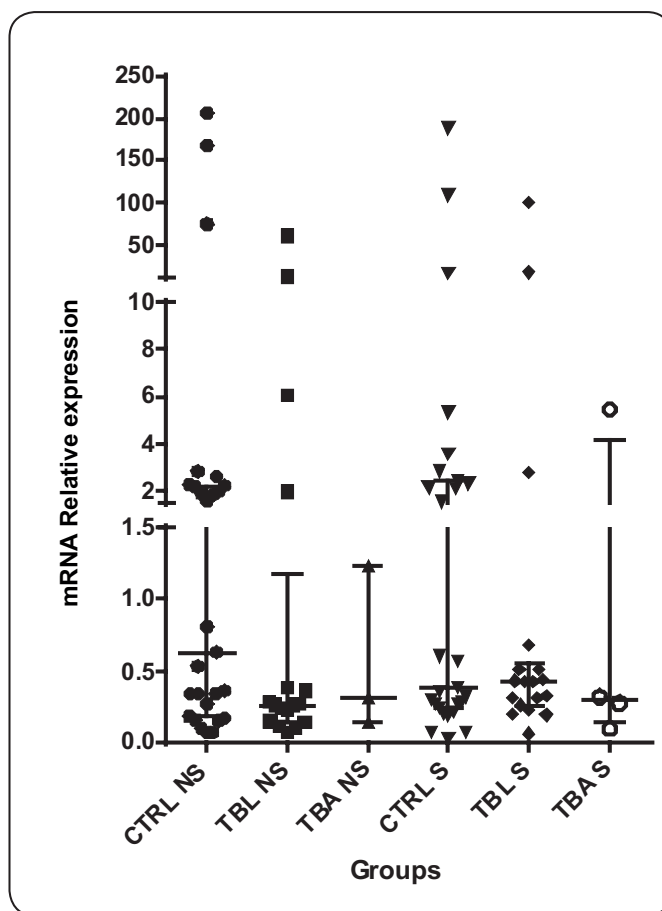

FIGURE 2 - Differences in IFNG expression levels between the groups. Horizontal bars represent medians and inter quartile range (IQR) of IFN-γ expression under non-stimulated (NS) condition; CTRL NS (●), LTBI NS (■) and ATB NS (▲) and for stimulated condition (S); CTRL S (▼), LTBI S (♦) and ATB S (○). Kruskal-Wallis test and Dunn's multiple comparison were used to compare groups. No statistically significant differences were found. :
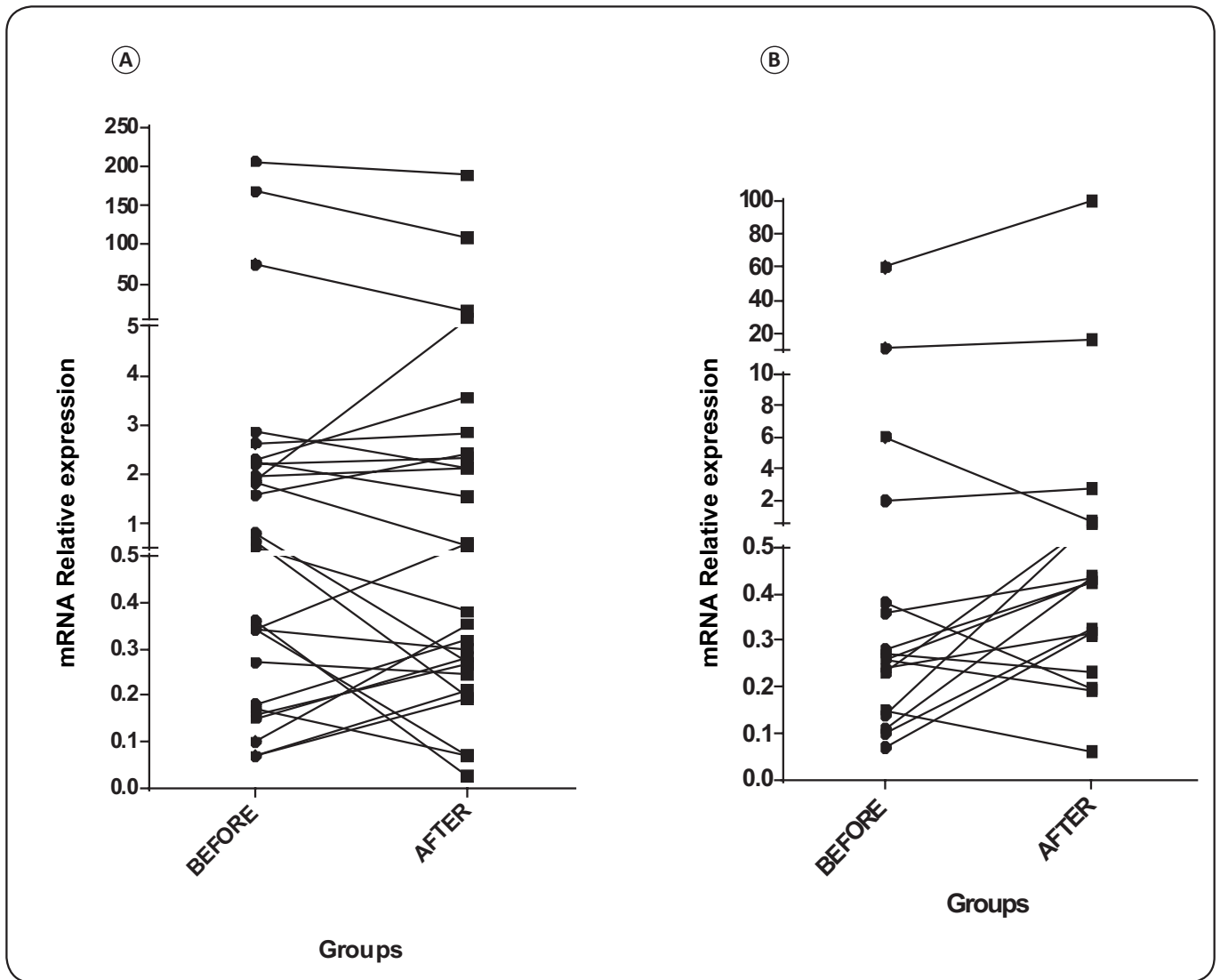
FIGURE 3 - Relative expression of IFNG measured by quantitative PCR before and after the stimulated condition for CTRL and LTBI groups; (A) and (B), respectively. The differences between the relative expression values in each group before and after stimulation were not significant by Mann Whitney test.

population, who live in Brazil and exhibit high TB prevalence, and revealed that the absence of TST response (anergy) may be associated with a predominantly Th2 cytokine pattern, which may increase susceptibility to TB[21].

The present work describes the relative expression of IFNG in Warao individuals with TB. The expression of this cytokine reflects the antigen-specific response of cells stimulated *ex vivo* in whole blood samples from ATB, LTBI, and CTRL indigenous groups by specific-*M. tuberculosis* T-cell antigens for 24h. There were no statistically significant differences between non-stimulated and stimulated conditions between groups; IFNG was found to be expressed at low levels, especially in the ATB and LTBI groups (0.56 ± 0.59 and 4.83 ± 14.51 of mRNA relative expression, respectively under non-stimulation, and 1.54 ± 2.62 and 6.94 ± 23.53 of mRNA relative expression, respectively, under stimulation). There were no statistically significant differences between either condition for the CTRL group,

demonstrating the existence of concordance between the highest frequency of the AA genotype among Warao healthy controls and patients with TB, and IFNG expression. IFN-γ cytokine exhibits poor potential as a biomarker in the Warao indigenous population owing to its failure to discriminate between ATB and LTBI indigenous individuals. The low IFNG expression levels detected may be correlated with the present findings in relation to QFT-IT assays; among the LTBI indigenous individuals tested, only 4 of were QFT-IT+. In this context, the value of the QFT-IT as an adjunct in diagnosing latent infection in Warao individuals may be limited: in Warao children for whom both TST and QFT-IT results are available, the proportion of children with a positive TST was high compared with those with a positive QFT-IT[22]. The latter correlates with the present results; therefore, replacement of the TST by QFT-IT for detection of *M. tuberculosis* infection is not recommended in this indigenous population. However further are required to fully evaluate the utility of QFT-IT test in this population.

It has been reported that the clinical symptoms in patients with TB correlate with *M. tuberculosis*–stimulated IFN-γ production by PBMCs, which is lower in patients with TB than in healthy tuberculin reactors, and even lower in patients with extensive disease[10,23]. Differences in the distribution of the IFNG genotype may explain the lower production of this cytokine in patients with TB; these patients have a higher frequency of the homozygous A (AA genotype) associated with lower PPD-stimulated IFN-γ production[23]. The latter findings correlate with those of our previous study examining the capacity of antigen-induced proliferation by PBMCs and IFN-γ production in Warao indigenous individuals with pulmonary TB and Warao healthy controls. The results revealed that IFN-γ production in Warao patients and controls was significantly lower after stimulation for 24h and 48h compared with that in the Creole groups[10].

As IFN-γ plays a crucial role in facilitating macrophage containment of *M. tuberculosis*, we aimed to determine whether the observed allelic variation associated with low expression of IFNG may in part contribute to the high rates of TB among Warao indigenous individuals relative to the white American population. The latter population was used for comparison because of the historical context in which the two populations evolved in relationship to their microbial environments, particularly in the context of their response to the selective pressures of their respective microbial environments, as found by Larcombe et al.[18].

Results showed that AA genotype frequency for IFNG +874 A/T differed significantly between Warao indigenous and white American individuals. Higher frequencies of these genotypes were observed in the Warao indigenous population (0.970 compared with 0.270 in white Americans, while lower frequencies of the AT and TT genotypes were observed in the Warao individuals: 0.014 for each genotype compared with 0.530 and 0.200, respectively, in white Americans). This comparison additionally reveals that the IFNG +874 A/T polymorphism is significantly associated with pulmonary TB, the findings showed that indigenous homozygous for the +874 A allele had a 3.59-fold increased risk of contracting TB. Our results correlate with previous findings showing that individuals homozygous for the +874 A allele had a 3.75-fold increased risk of developing TB and the lowest IFNG expression level in the groups studied, therefore supporting that the IFNG +874 A/T polymorphism is significantly associated with pulmonary TB[18,19].

Ethnic-specific genetic variations may greatly influence host immunity to TB, causing variations in TB susceptibility in the ethnic population studied[24]. Accordingly, several studies of genetic diversity in ethnic populations have reported an association between the IFNG +874 polymorphism and TB[18,25]. Studies in relation to genotype profile in Canadian aboriginal and Filipino populations showed that both populations have a higher frequency of cytokine SNPs associated with low production of IFNG (AA genotype) compared with white Americans; these aboriginal populations exhibited a significantly higher frequency of the A allele at the IFNG loci[18]; in contrast, white Americans exhibit a relatively higher frequency of cytokine SNPs associated with delayed-type hypersensitivity

IFN-γ response, the latter suggest that white Americans tend to mount a Th1 immune response, while Canadian aboriginal and Filipino populations show a tendency towards a Th2 immune response[18]. In this context, intestinal parasites are endemic among the Warao population, which is consistent with the considerably higher levels of total serum IgE found in this population[26].

Recent data suggest that, similar to Canadian aboriginal and Filipino populations[18], in other populations, such as Spanish (RR = 3.75, CI 2.26-6.63, p = 0.0017)[17], Hong Kong Chinese (RR = 3.79, CI 1.93-7.45, p = 0.001)[27], South African colored (RR = 1.46, CI 1.12-1.91, p = 0.0062)[28], and Warao indigenous populations (RR = 3.59, CI 2.600-4.968, p = 0.0001), common polymorphisms in the IFNG +874 AA genotype are associated with the risk of TB disease. However, the +874AA genotype was not significantly more frequent in cases than in control subjects (OR, 1.16; 95%CI, 0.89–1.51; p = 0.25) in individuals from West African populations[28], and in Brazilian patients with TB[29]. However, the influence of gene polymorphisms on protein production may differ as a result of linkage with functional variants in other loci in the regulatory regions of the genes, or with other cytokine polymorphisms necessary for adequate immune response to *M. tuberculosis* infection. Additional studies are needed to confirm the influence of the IFNG +874 A/T alleles and other cytokine genes on TB susceptibility in the Warao population.

Conflict of interest

The authors declare that there is no conflict of interest.

REFERENCES

1. World Health Organization. World Health Statistics 2012. WHO, Geneva, 2012.

2. Ministerio de Sanidad y Asistencia Social (MSAS). Programa Integrado de Control de la Tuberculosis. Seminario Técnico-Administrativo. Caracas: 1999. 150pp.

3. Ministerio de Salud y Desarrollo Social (MSDS). Evaluación del Programa Nacional de Control de la Tuberculosis. Año Evaluado 2001. Caracas: 2002. 201pp.

4. Ministerio de Salud y Desarrollo Social (MSDS) 2006. Evaluación del Programa Nacional de Control de la Tuberculosis. Año Evaluado 2006. Caracas: 2006. 200 pp.

5. Bibova I, Linhartova I, Stanek O, Rusnakova V, Kubista M, Suchanek M, et al. Detection of immune cell response to *M. tuberculosis*-specific antigens by quantitative polymerase chain reaction. Diagn Microbiol Infect Dis. 2012;72(1):68-78.

6. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. J Exp Med. 1993;178(6):2249-54.

7. Trajkov D, Trajchevska M, Arsov T, Petlichkovski A, Strezova A, Efinska-Mladenovska O, et al. Association of 22 cytokine gene polymorphisms with tuberculosis in Macedonians. Indian J Tuberc. 2009;56(3):117-31.

8. Khalilullah SA, Harapan H, Hasan NA, Winardi W, Ichsan A, Mulyadi M. Host genome polymorphisms and tuberculosis infection: What we have to say? Egypt J Chest Dis Tuberc. 2014;63(1):173-85.

9. Araujo Z, de Waard JH, de Larrea CF, Borges R, Convit J. The effect of Bacille Calmette-Guérin vaccine on tuberculin reactivity in indigenous children from communities with high prevalence of tuberculosis. Vaccine. 2008;26(44):5575-81.

10. Giampietro F, de Waard JH, Rivas-Santiago B, Enciso-Moreno JA, Salgado A, Araujo Z. *In vitro* levels of cytokines in response to purified protein derivative (PPD) antigen in a population with high prevalence of pulmonary tuberculosis. Hum Immunol. 2010;71(11):1099-104.

11. Larrea CF, Fandiño C, López D, del Nogal B, Rodríguez N, Convit J, et al. Childhood tuberculosis in the Warao population in Venezuela. Invest Clin. 2002;43:35-48.

12. Kudoh S, Kudoh T. A simple technique for culturing tubercle bacilli. WHO/TB/51. Geneva Bull World Health Organ. 1974;51(1):71-82.

13. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real time quantitative PCR and the 22DDCT method. Methods. 2001;25(4):402-8.

14. Layrisse Z, Heinen HD, Balbas O, García E, Stoikow Z. Unique HLA-DR/DQ associations revealed by family studies in Warao Amerindians. Haplotype and homozygosity frequencies. Hum Immunol. 1988;23(1):45-57.

15. Makhatadze NJ, Franco MT, Layrisse Z. HLA class I and class II allele and haplotype distribution in the Venezuelan population. Hum Immunol. 1997;55(1):53-8.

16. Chegou NN, Heyckendorf J, Walzl G, Lange Ch, Ruhwald M. Beyond the IFN-γ horizon: biomarkers for immunodiagnosis of infection with *Mycobacterium tuberculosis*. Eur Respir J. 2014;43:1472-1486.

17. López-Maderuelo D, Arnalich F, Serantes R, González A, Codoceo R, Madero R, et al. Interferon-γ and Interleukin-10 Gene Polymorphisms in Pulmonary Tuberculosis. Am J Respir Crit Care Med. 2003;167(7)970-5.

18. Larcombe L, Rempel JD, Dembinski IJ, Tinckam K, Rigatto C, Nickerson P. Differential cytokine genotype frequencies among Canadian Aboriginal and Caucasian populations. Genes Immun. 2005;6(2):140-4.

19. Larcombe L, PH Lodge AM, Brown JS, Dembinski IJ, Milligan LC, Larcombe EA, et al. Functional gene polymorphisms in Canadian Aboriginal populations with high rates of tuberculosis. J Infect Dis. 2008;198(8):1175-9.

20. Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russell DG, Orme IM. Disseminated tuberculosis in interferon gamma gene-disrupted mice. J Exp Med. 1993;178(6):2243-7.

21. Zembruzuski VM, Basta PC, Callegari-Jacques SM. Cytokine genes are associated with tuberculin skin test response in a native Brazilian population. Tuberculosis. 2010;90(1):44-9.

22. Verhagen LM, Maes M, Villalba JA, d´Alessandro A, Perez-Rodriguez L, España MF, et al. Agreement between QuantiFERON®-TB Gold In-Tube and the tuberculin skin test and predictors of positive test results in Warao Amerindian pediatric tuberculosis contacts. BMC Infect Dis. 2014;14:383.

23. Prabhu Anand S, Harishankar M, Selvaraj P. Interferon gamma gene +874A/T polymorphism and intracellular interferon gamma expression in pulmonary tuberculosis. Cytokine. 2010;49(2):130-3.

24. Maronna R, Longhi P, Marques Zembrzuski V, Basta PC, Croda J. Genetic Polymorphism and immune response to tuberculosis in indigenous populations: a brief review. Braz J Infect Dis 2013;17:363-368.

25. Cox AJ, Moscovis SM, Blackwell CC, Scott RJ. Cytokine gene polymorphism among Indigenous Australians. Innate Immun. 2014;20(4):431-439.

26. Araujo Z, Giampietro F, Rivas-Santiago B, Luna-Herrera J, Wide A, Clark W, et al. Patients exposed to *Mycobacterium tuberculosis* infection with a prominent IgE response. Arch Med Res. 2012;43(3):225-32.

27. Tso HW, Ip WK, Chong WP, Tam CM, Chiang AKS, Lau YL. Association of interferon gamma and interleukin 10 genes with tuberculosis in Hong Kong Chinese. Genes Immun. 2005;6(4):358-63.

28. Bennett S, Lienhardt C, Bah-Sow O, Gustafson P, Manneh K, Del Prete G, et al. Investigation of environmental and host-related risk factors for tuberculosis in Africa: II. Investigation of host genetic factors. Am J Epidemiol. 2002;155(11):1074-9.

29. Peresi E, Cardoso Oliveira LR, da Silva WL, Pellison Nunes da Costa EA, Pessoa Araujo Jr J, Ayres JA, et al. Cytokine polymorphisms, their influence and levels in Brazilian patients with pulmonary tuberculosis during antituberculosis treatment. Tuberc Res Treat. 2013; 2013:Article ID 285094.