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Tumor Necrosis Factor-Alpha (TNF)-308G/A and Interleukin 8(IL-8)-251C/T Polymorphisms in Pulmonary Tuberculosis Patients from Congo

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Abstract

Background: Tuberculosis (TB) is one of the world's deadliest infectious diseases. Tumor necrosis factor-Alpha (TNF-α) and Interleukin 8 (IL-8) are involved in the pathogenesis of pulmonary TB (PTB). However, the contribution of polymorphisms of these cytokines to PTB susceptibility needed more investigation across geographic regions and ethnic groups. **Purpose:** The aim of this study was to investigate the association of the TNF-α-308 G/A and IL-8-251T/A polymorphisms with PTB risk in the Congolese population. **Methods:** This case-control study included 150 PTB patients and 160 control subjects. Blood samples were collected from all participants and were used for the TNF-α-308 G/A and IL-8-251T/A genotyping by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. Odds ratios (OR) were calculated to estimate the potential polymorphism associations. A P level of < 0.05 was considered significant. **Results:** A significant difference was found between PTB patients and controls regarding the TNF-α-308AA genotype ($P = 0.035$) distribution. Moreover, this genotype was associated with risk to TB (OR = 7.19, 95% CI = 0.85 - 60.65, $P = 0.035$). The A allele was significantly more frequent in PTB patients than in controls, and was associated with risk to PTB (OR = 1.68, 95% CI = 1.05 - 2.68, $P = 0.014$). Regarding the IL-8-251T/A gene, TA and AA genotypes were significantly more frequent in PTB patients compared to controls, and were associated with increased risk to PTB (OR = 2.64, 95% CI = 0.97 - 7.18, $P = 0.031$ and OR = 3.0, 95% CI = 1.13 - 7.98, $P = 0.014$, respectively). However, the

IL-8-251 A allele was not associated to PTB susceptibility (OR = 0.27, 95% CI = 0.15 - 0.44). **Conclusion:** TNF-α-308G/A and IL-8-251T/A polymorphisms may be associated to PTB susceptibility in the Congolese population, and the AA genotype of both cytokines could be a risk factor.

Keywords

Pulmonary Tuberculosis, Cytokine Polymorphism, Tumor Necrosis Factor-Alpha, Interleukin-8, PCR-RFLP

1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is one of the world's deadliest infectious diseases; approximately 10 million people are infected worldwide and about 2 million die every year [1]. Despite some major advances in prevention and treatment in recent years, TB remains a key infectious disease that causes many deaths every day, mostly in Asia and Africa. The Republic of Congo is among the countries with relatively high incidence of TB, with 382 new cases per 100,000 people each year [2]. The coronavirus (COVID-19) pandemic has caused dysfunction in the prevention and control measures of infectious diseases, including TB, which may have jeopardize efforts that have been made for years to reduce the incidence of this disease [3].

M. tuberculosis most often colonizes lungs but the infection can also be extra pulmonary, and the main clinical features of active TB include cough, chest pain, breathlessness, night sweats, and signs of pneumonia [4]. Human-to-human transmission of the bacterium occurs through inhalation of contaminated aerosols, making it difficult to control. However, a person's exposure *M. tuberculosis* does not necessary lead to TB disease. Only about 1/10 of the infected people fall ill with TB. Indeed, the pathogen can persist in many infected individuals in a latent state for many years. The risk of progression to TB disease is the highest for persons co-infected with HIV/AIDS or other immune-compromising conditions [5]. On the other hand, host genetic factors also strongly influence the individual susceptibility to TB. So, identifying these factors may lead towards better understanding the pathogenesis of TB and, thereby, to develop prophylactic and treatment strategies.

The sequencing of human genome has allowed the discovery of millions of DNA sequence variants, which are mainly present as single nucleotide polymorphisms (SNPs) [6]. An SNP can cause structural variations in the regulatory sites of the gene which can affect the production or function of the protein. This opened up the possibility of studying the influence of SNPs on the susceptibility or resistance of diseases in populations. Thus, for TB for example, SNPs in different gene families have been investigated, including human leukocyte antigen (HLA), Toll-like receptor (TLR), cytokines and chemokines, to name a few [7]. Cytokines are produced from various immune cells in response to external sti-

muli. They act as messengers enabling these cells to communicate with one another, and to generate a coordinated and robust immune response. Cytokines exert crucial roles in the development, homeostasis, activation, differentiation, regulation and functions of innate and adaptive immunity [8]. SNPs in promoter as well as coding region of cytokines genes may alter their transcriptional activation and production. Many studies have reported their association with susceptibility to infectious diseases and non-infectious diseases such as auto-immune diseases and cancer [9] [10].

A few studies have examined the association of cytokines gene polymorphisms and TB risk in various geographic populations, and these cytokines including tumor necrosis factor-Alpha (TNF-α) and interleukin 8 (IL-8) [11] [12]. One of the hallmarks of *M. tuberculosis* infection is the formation of granulomas within the lung. TNF-α is a Th1 cytokine that has pleiotropic effects on various cell types. It binds to its receptors, mainly TNFR1 and TNFR2, and then transmits molecular signals for biological functions such as inflammation and cell death [13]. TNF-α mediates resistance to mycobacteria by inhibiting bacterial growth and macrophage death [14]. It has been shown that the loss of TNF signaling increased mortality of *M. tuberculosis*-infected mice [15]. TNF blockage impairs the formation of granulomas [16]. Besides, IL-8 is a chemokine that attracts neutrophils, basophils, and T-cells towards the targeted site during the inflammatory process. It enhances the ability of neutrophils and macrophages to phagocyte and kills bacilli, and also promotes the formation of granulomas [17]. Granulomas consist of aggregates of macrophages, B and T lymphocytes, which are organized to control a pathogen or a foreign body that cannot be eliminated, thereby to restrict the spread of the pathogen [18].

Despite the wealth of association studies of the TNF-α-308 G/A (rs1800629) and IL-8-251T/A (rs4073) polymorphisms, a consensus has not yet emerged on which variants confer the susceptibility to TB. This requires studies to be repeated in different ethnic groups. To date, very few studies have been conducted in Africa but none in Central Africa. Therefore, the aim of the present study was to investigate the association of both cytokine polymorphisms with pulmonary TB risk in the Congolese population.

2. Materials and Methods

2.1. Study Design and Ethical Considerations

This case-control study was carried out from January to August 2021 in Brazzaville, the capital of the Republic of Congo. The study included 180 individuals with TB and 170 control subjects. TB patients were enrolled at the "Grandes Endémies of Brazzaville (GEB)", which is a national center dedicated to the diagnosis, treatment and follow-up of TB patients. The diagnosis of TB was performed at the GEB using the GeneXpert MPTB/RIF test or by chest-X-ray. TB patients followed at GEB, at least 18 years of age and who voluntarily agreed to take part in the study were included in this study. TB patients who were also

Human immunodeficiency virus (HIV)-positive were not included in the study. Those with extrapulmonary TB were excluded from the study. The control group consisted of blood donors who were enrolled at the National Blood Transfusion Centre (CNTS) of Brazzaville.

This study was approved by the Health Sciences Research Ethics Committee of the Ministry of Scientific Research of Congo (report number: 253/MIRSIT/IRSSA/CERSSA). A fact sheet describing the study was presented to participants. Before collecting data from participants, the purpose and expectations of the study were explained to them by the investigators. The participation in this study was voluntary, without any form of coercion or compensation. For confidentiality reasons, data were collected anonymously.

2.2. DNA Extraction and SNP Genotyping

Peripheral blood samples from TB patients and controls subjects were collected in tubes containing EDTA as anticoagulant, and stored at −20˚C until use. The whole genomic DNA was isolated and purified from whole-blood samples using the Zymo Research DNA extraction kit according to the manufacturer instructions (Zymo Research, Irvine, CA, USA). DNA was eluted in 100 µl of sterile water and stored at −20˚C. DNA quality was analyzed by agarose gel electrophoresis and by UV absorption at 260 and 280 nm. The TNF-*α*-308G/A (rs1800629) and IL-8-251T/A (rs4073) polymorphisms were genotyped using the PCR-RFLP method. The PCR reaction mixture of 30 µl contained about 50 ng genomic DNA, 15 µl of 2X Dream Taq Host Start Green (Thermo Fisher Scientific, Massachusetts, USA) and 10 picomole of each primer. For TNF-*α*-308G/A, the oligonucleotide primers were: forward: 5’ AGGCAATAGGTTTTGAGGGCCAT-3’ and reverse: 5’-TCCTCCCTGCTCCGATTCCG -3’ [19], and the thermal condition for PCR included 95˚C for 5 min; 35 cycles of 95˚C for 30 sec, 56˚C for 30 sec, and 72˚C for 30 sec; and final extension of 72˚C for 10 min. Approximately 20 µl of PCR products were digested with 5 units *Nco*I (Thermo Fisher Scientific, Massachusetts, USA) at 37˚C overnight. The undigested single fragment was of 107 bp and digested one give two 87 and 20 bp products. The oligonucleotide primers to amply the IL-8 251T/A gene, the sequence were: forward: 5’-ATC TTGTTCTAACACCTGCCACTC-3’, and reverse: 5’TAAAATACTGAAGCTCCACAATTTGG-3’ [20]. The thermal condition for PCR was as follows: 95˚C for 5 min; 35 cycles of 95˚C for 30 sec, 56˚C for 30 sec, and 72˚C for 30 sec; and final extension of 72˚C for 10 min. The PCR products were digested with 5 units *Mfe*I (Thermo Fisher Scientific, Massachusetts, USA) 37˚C overnight. The undigested single fragment was of 121 bp and digested one give two 82 and 39 bp products. PCR amplifications were carried out in the Bio-Rad T100™ thermal cycler. PCR and digested products were electrophoresed on 3% agarose gel and visualized under UV light using ethidium bromide stain.

2.3. Statistical Analysis

Data were analyzed using the statistical software program Epi-info version 7.2. Frequencies of allele and genotype were tested for Hardy-Weinberg equilibrium (HWE) using the χ^2 analysis, to determine the quality of the genotype data. Data are presented as means ± standard deviation (SD) or as percentages for categorical variables. Differences in genotypic and allelic frequencies between PTB patients and controls were assessed by means of Pearson x2 test and calculating the odds ratio (OR) with the 95% confidence intervals (CI). OR was defined with respect to the case groups. OR > 1 represents increased risk to PTB. A *P* level of < 0.05 was considered significant.

3. Results

3.1. Characteristics of Study Populations

Characteristics of study populations are shown in Table 1. Mean age of TB patients and control subjects were 42.8 ± 14.4 years (range 18 - 73) and 35.4 ± 8.7 years (range 18 - 52), respectively. Most of the TB patients were male (n = 114; 63.3%). Pulmonary localization of TB (PTB) accounted for 83.3% of all localizations. The PTB patients were included for genotyping of the cytokines studied.

3.2. Genotyping of TNF-*α*-308G/A (rs1800629)

Amplification of TNF-*α*-308G/A gene with specific primers yielded a 107 bp PCR product (Figure 1(a)). As expected, digestion of the PCR products with N*co*I generated two fragments of 87 and 20 bp representing homozygous *TNF-α*-308GG, three fragments of 107, 87 and 20 bp for heterozygous GA, and an uncut 107 bp fragment representing homozygous AA (Figure 1(b)).

Table 1. Characteristics of TB patients and controls.

Characteristics	TB patients (N = 180)	Control (N = 160)	*P* value
Male, n (%)	114 (63.3)	89 (55.6)	0.075
Female, n (%)	66 (36.7)	71 (44.4)	0.075
Mean age ± SD years (range)	42.8 ± 14.4 (18 - 73)	35.4 ± 8.7 (18 - 52)	<0.001***
Chest X radiology n (%)	31 (17.2)	NT	
GeneXpert MPTB/RIF, n (%)	149 (82.8)	NT	
PTB, n (%)	150 (83.3)	NT	
EPTB, n (%)	30 (16.4)	NT	

PTB: tuberculosis, PTB: pulmonary tuberculosis, EPTB: extra-pulmonary TB, NT: non tested. ***Significant difference ($P < 0.001$).

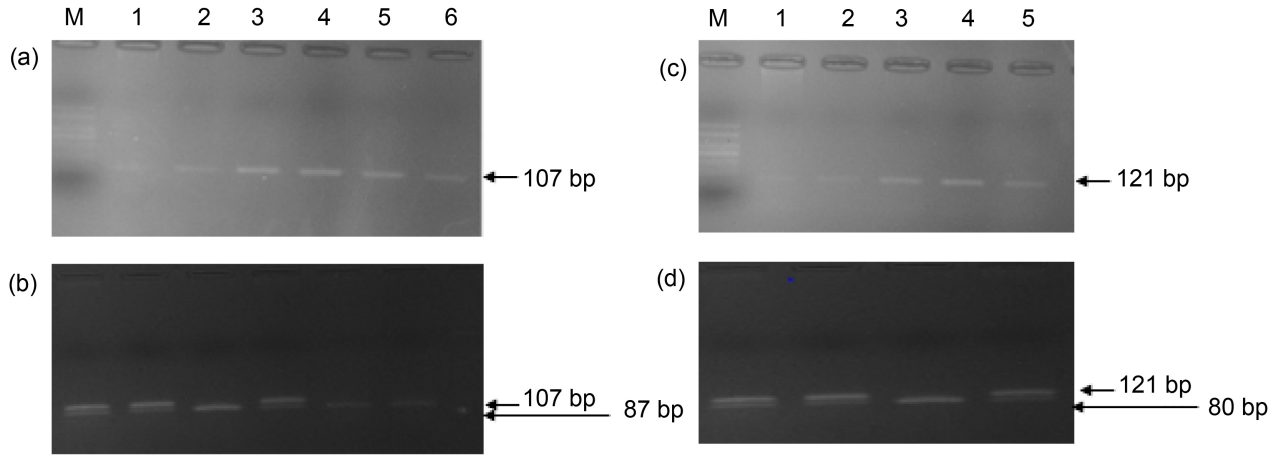

Figure 1. PCR amplification and RFLP of TNF-*α*-308G/A and IL-8-251T/A genes. Agarose gel electrophoresis 3% stained with ethidium bromide showing the profile of TNF-*α* -308 G/A gene PCR amplification (a) and after the digestion with *Nco*I (b), and of IL-8-251T/A gene PCR amplification (c) and following digestion with *Mfe*I (d). M: DNA marker; lane 1 to 6: samples.

The distribution of TNF-*α*-308G/A genotypes in both PTB patients and controls was consistent with the Hardy-Weinberg equilibrium (Table 2). Of the 150 PTB patients genotyped for TNF-*α*-308 G/A, the GG genotype was found in 70.7%, GA genotype in 25.3% and the AA genotype only in 4%. In the control group, the results were not much different since we observed GG genotype in 79.4%, GA in 20% and AA in 0.7%. There were significantly more variant of homozygote AA genotypes in PTB compared to the control group ($P = 0.035$). The Odd ratio value indicated a high association of this genotype with risk to PTB (OR = 7.19, 95% CI = 0.85 - 60.65). The carriers of the minor A allele (GA + AA) genotype were also in significantly higher frequency in PTB (29.3%) compared to the control group (20.6%), and were associated towards the PTB susceptibility (OR = 1.59, 95% CI = 0.95 - 2.68, $P = 0.038$). The A allele was in significantly higher frequency ($P = 0.014$) in PTB (16.7%) compared to the control group (10.6%), and the odd ratio indicated its association with risk to PTB (OR = 1.68, 95% CI = 1.05 - 2.68, $P = 0.014$). The frequencies of TNF-*α*-308GG and carrier of the major G allele (GG + GA) genotypes were not significantly different between PTB patients and controls. Taken together, these results suggest that TNF-*α*-308G/A polymorphism is associated with susceptibility to PTB in the Congolese population, and that the AA genotype and A allele may be involved.

3.3. Genotyping of IL-8-251T/A (rs4073)

The results of PCR-RFLP are shown in Figure 1(c) and Figure 1(d). The distribution of IL8-251A/T genotypes in both PTB patients and control subjects was consistent with the Hardy-Weinberg equilibrium (Table 3). Out of 180 PTB patients, 6 (4.0%) were homozygous TT, 56 (37.3%) heterozygous TA and the majority were homozygous AA 88 (58.7%). In the control group, 17 (10.6) had the TT genotype, 60 (37.5) the TA genotype and 83 (51.9) the AA genotype. Significant differences in the frequencies of TA and AA were observed between case

Table 2. Genotype and allele frequencies of the TNF-*α*-308G/A (rs1800629) gene in PTB patients and controls.

	PTB Patients (N = 150); n (%)	Control (N = 160); n (%)	OR [95% CI]	*P* value
Genotypes				
GG	106 (70.7)	127 (79.4)	1.00 [reference]	
GA	38 (25.3)	32 (20)	1.42 [0.83 - 2.43]	0.098
AA	6 (4)	1 (0.6)	7.19 [0.85 - 60.65	0.035*
AA *vs* GA	6 (4) *vs* 38 (25.3)	1 (0.6) *vs* 32 (20)	5.05 [0.58 - 44.19]	0.071
Dominant: AA *vs* GG + GA	144 (96)	159 (99.4)	1.08 [0.77 - 1.53]	0.32
Recessive: GG *vs* GA + AA	44 (29.3)	33 (20.6)	1.59 [0.95 - 2.68]	0.038*
Allele				
G	250 (83.3)	286 (89.4)	1.00 [reference]	
A	50 (16.7)	34 (10.6)	1.68 [1.05 - 2.68]	0.014*
HWE test (χ^2 (*P* value))	1.162 (0.281)	0.450 (0.502)		

TNF-*α*: tumor necrosis factor-alpha; PTB: pulmonary TB; OR: odds ratio; 95% CI: 95% confidence interval; HWE: Hardy-Weinberg equilibrium. *Significant difference ($P < 0.05$).

Table 3. Genotype and allele frequencies of the IL-8-251T/A (rs4073) gene in PTB patients and controls.

	PTB Patients (N = 150); n (%)	Control (N = 160); n (%)	OR [95% CI]	*P* value
Genotypes				
TT	6 (4.0)	17 (10.6)	1.00 [reference]	
TA	56 (37.3)	60 (37.5)	2.64 [0.97 - 7.18]	0.031*
AA	88 (58.7)	83 (51.9)	3.00 [1.13 - 7.98]	0.014*
AA *vs* TA	88 (58.7) *vs* 56 (37.3)	83 (51.9) *vs* 60 (37.5)	1.14 [0.71 - 1.82]	0.298
Dominant: AA *vs* TT + TA	62 (41.3)	77 (48.1)	0.3 [0.84 - 2.06]	0.114
Recessive: TT *vs* TA + AA	144 (96)	143 (89.4)	2.85 [1.09 - 7.44]	0.016*
Allele				
T	68 (22.7)	94 (29.4)	1.00 [reference]	
A	232 (77.3)	226 (70.6)	0.27 [0.15 - 0.44]	0.000001***
HWE test (χ^2 (*P* value))	0.632 (0.426)	1.481 (0.223)		

IL-8: interleukin 8; PTB: pulmonary TB; OR: odds ratio; 95% CI: 95% confidence interval; HWE: Hardy-Weinberg equilibrium. *Significant difference ($P < 0.05$), ***Significant difference ($P < 0.001$).

patients and controls ($P = 0$, Hardy-Weinberg equilibrium 038 and $P = 0.014$, respectively). Both genotypes were associated with an increased risk to PTB (OR = 2.64, 95% CI = 0.97 - 7.18 and OR = 3.0, 95% CI = 1.13 - 7.98). The recessive

model (TA + AA) was also associated with an increased risk to PTB (OR = 2.85, 95% CI = 1.09 - 7.44, $P = 0.016$). On the other hand, the IL8-251 A allele showed significantly higher frequencies in case groups than in control groups (77.3% vs. 70.6%, $P = 0.000001$) and was not associated to the PTB susceptibility (OR = 0.27, 95% CI = 0.15 - 0.44). These finding suggest that the IL8-251A/T polymorphism is associated with susceptibility to PTB in the Congolese population, and that the AA and TA genotypes, and the A allele may be involved.

4. Discussion

The pathogenesis of PTB is more complicated than that of many other common human diseases because there is potential confounding environmental, genetic and epigenetic factors that are involved in the outcome of the disease [21] [22]. Thus, the aim of this study was to investigate the association of TNF-α-308G/A and IL-8-25 A/T gene polymorphisms with risk to PTB in the Congolese population. We performed a case-control study using the PCR-RFLP method to determine genotypes of both cytokines among PTB patients and control subjects. The PCR-RFLP method is used in a wide range of screening applications to characterize single nucleotide polymorphisms (SNPs), since it offers a reliable and rapid way to genotype polymorphisms compared to the PCR-SSCP technique [23].

The TNF-α locus is located within the polymorphic human leukocyte antigen (HLA) complex III region. To date, in addition to TNF-α-308G/A, several other SNPs in the TNF-α gene have been identified including TNF-α-1031T/C, −863 C/A, −857 C/A, −851 C/A and −238 G/A [24]. For some of these genes, the association of genotypes and alleles with various diseases has been investigated in recent years. For example, it is reported that the TNF-α-863AA genotype and its allele A, and −1031 CT genotype and allele C were associated to the hepatitis B virus susceptibility among the Caucasoids [25]. TNF-α-308G/A but not −238 G/A may be a risk factor for type 2 diabetes mellitus in Caucasian and Asian populations [26]. On the other hand, a meta-analysis suggested that TNF-α-308G/A polymorphisms are correlated with an elevated risk to Hepatocellular carcinoma [27].

In Hardy-Weinberg equilibrium populations, TNF-α-308G homozygosity and allele G are the predominant genotypes [28]. In this study, the genotypic frequency distribution of TNF-α-308G/A showed that the homozygous GG genotype was largely in the majority in PTB patients (79.4%) and PTB-free control subjects (82.3%), while the homozygous AA genotype was very minority in both populations (2.8% and 1.8%). The G allele were also more predominant than the A allele. Our findings were consistent with other studies [29] [30]. Some reports document the significance of the TNF-α 308 G/A polymorphisms in the risk to PTB in different populations. A significant positive association with the TNF-308 GG genotype was found in the Shangai Chinese population [29]. A meta-analysis study reported the dominant GG + GA and homozygote AA models were not associated with significantly increased risk to PTB among African pop-

ulations [31]. However, when reviewed this meta-analyze study, it is noted that it included only two studies in African populations: one study carried out in the Tunisian population [32] and another one in Mozambique [30]. The linkage disequilibrium in each ethnic population may be a limited to generate a stable result. In our study, we found that the TNF-α-308G/A polymorphisms are associated with significantly increased risk to PTB and that TNF-α-308 AA genotype (OR = 7.19, 95% CI = 0.85 - 60.65, $P = 0.035$), A allele (OR = 1.68, 95% CI = 1.05 - 2.68, $P = 0.014$) and the carriers GA + AA model (OR = 1.59, 95% CI = 0.95 - 2.68, $P = 0.038$) may be involved. Similar findings have been reported elsewhere [30]. We found that the TNF-α -308 AA genotype would increase by 7-fold the risk to PTB. Whereas, Mabunda *et al.* reported that this genotype would increase by 14-fold the risk to PTB [30]. Their study included 102 patients PTB and 456 controls. So, differences in sizes of the study populations in their study with ours may explain this discrepancy.

IL-8 is part of chemokines and it is well known for its leukocyte chemotactic and inflammatory properties. During the immune response to M. tuberculosis infection, IL-8 attracts macrophages, neutrophils and lymphocytes to the site of infection leading to the formation of granulomas [17]. Several SNPs in the IL-8 gene have been identified including IL-8 −251T/A, +781 C/T and +2607 G/C, and can modulate the production of the cytokine [33]. The IL-8 gene polymorphisms have been associated with many various diseases. For example, it has been found that the IL-8 −251 AA genotype and A allele are associated to a higher risk to glioma, a central nervous system tumor [34]. The IL-8 +781 C/T polymorphism is associated with severe *Clostridium difficile* infection [35]. Regarding the relation of the IL-8 gene polymorphisms with TB, a previous study in the USA found that TA and AA genotypes of the IL-8 −251T/A gene was associated with TB susceptibility (OR > 2) in both African Americans and whites [33]. A recent meta-analysis study confirmed these findings by determining that the IL-8 −251 T/A polymorphism is associated with risk to TB in the general population under dominant, recessive and allelic models [36]. Our results are in agreement to these previous findings, suggesting that the IL-8 −251 T/A polymorphism also influences the risk to PTB in the Congolese population.

5. Conclusion

Our results indicate an association between TNF-α 308G/A and IL-8 −251T/A gene polymorphisms and susceptibility to TB in the Congolese population. However, a family-based association study will help to support these findings. Further studies evaluating the production of TNF and IL-8 cytokines by genotype of PTB patients are also needed and will help understand the pathogenesis of TB.

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Ethical Clearance

This study was approved by Health Sciences Research Ethics Committee of the Ministry of Scientific Research of Congo (CERSSA), reference number: 253/MIRSIT/IRSSA/CERSSA.

Authors' Contribution

All the authors have contributed to the achievement of this work and to the drafting of the manuscript.

Conflicts of Interest

The authors declare that they have no competing interests.

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