Comparison of serum cytokine profiles of patients with pulmonary tuberculosis and aspergillus infections

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Abstract

Objectives: *aspergillus fumigatus* induced chronic pulmonary aspergillosis (CPA) is the most common sequelae of pulmonary tuberculosis (TB), which caused by intracellular infection pathogen *Mycobacterium tuberculosis* (*Mtb*). Timely and accurate detection of the potential infection of *A. fumigatus* in TB patients will undoubtedly greatly improve the prognosis of TB patients. At present, galactomannan (GM) antigen test is commonly used to detect *A. fumigatus*infection, but its poor sensitivity is not adequate for clinical practice.

Design or methods: given the different host immune responses induced by CPA and TB, we aimed to evaluate the serum cytokine profiles of TB, CPA-TB, and CPA patients without TB using multiple cytokine analyses.

Results: the results showed that the expression of a large number of proinflammatory cytokines such as IL-1β, IL-6, IL-8, IL-12p70, IFN-α, IFN-γ and TNF-α in peripheral blood of patients with CPA was significantly higher than that of patients with TB. Cytokine IL-8 alone has the best performance to diagnose TB patients from CPA-TB (AUC=0.949) or CPA (AUC=0.964) patients. Moreover, IL-8 and TNF-α combination (AUC=0.996) could distinguish patients with TB or CPA. Likewise, the combination of IL-8, TNF-α and IL-6 can help distinguish all CPA patients with TB or not from TB-alone patients.

Conclusions: our study provided multiple cytokines as potential markers to accurately diagnosis TB and CPA, and contribute to the prognosis of TB patients with CPA.Furthermore, the results help better understand the immune function disorder during *Mtb* and/or *A. fumigatus* infections.

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*) complex, affected an estimated 9.9 million people in 2020, with 1.5 million deaths from this disease worldwide ((WHO), 2021). Only 59% of pulmonary TB cases reported were bacteriologically confirmed, highlighting the urgent need to improve access to accurate TB diagnostics in areas of TB endemicity ((WHO), 2021; MacLean et al., 2020). China has the second largest burden of TB globally, only behind India, with approximately 0.85 million incident cases in 2020((WHO), 2021). Despite great achievements in TB control, a nationwide survey revealed that the prevalence of active TB was 442 cases per 100,000 population in China, posing a barrier to global end-TB strategy (Wang et al., 2014). More efforts are required to accelerate research on TB diagnostics, new drugs and vaccines.

Aspergillus fumigatus (*A. fumigatus*) is the major causative agent of aspergillosis, resulting in various manifestations of pulmonary infections (Kosmidis & Denning, 2015). Chronic pulmonary aspergillosis (CPA) is seen as a disease easily confused with pulmonary TB (PTB). In addition, CPA is the most frequent sequelae of PTB, and it is estimated that the global prevalence of CPA secondary to TB was approximate 1.2 million cases (Denning, Pleuvry, et al., 2011; Page et al., 2019). Although the occurrence and development of CPA may be the most subtle, CPA could result in an extremely higher 5-year mortality

of 80% by a retrospective study (Lowes et al., 2017; Ohba et al., 2012). Early initiation of oral azole drugs can effectively prevent CPA clinical progression and is associated with significantly decreased mortality (Al-Shair et al., 2013; Camuset et al., 2007). Timely and accurate identification of this causative agent is thus of great importance for proper management of the disease. Clinically, it is impossible to differentiate pulmonary aspergillosis from pulmonary TB based on clinical signs and symptoms. Although the galactomannan (GM) antigen testing has aided in the diagnosis of CPA (Zou et al., 2012), it yields poor sensitivities in clinical practice.

As a facultative intracellular organism, the major mechanism for elimination of *Mtb* is Th1 immune response via induction of Th1 cytokines such as IFN-γ; whereas *A. fumigatus* infection not only causes the host to produce Th1 cytokines as the dominant factor, but also induces cells to differentiate into Th2 cells (Allard et al., 2006; Bozza et al., 2009; Cramer et al., 2011), which antagonize Th1 cells. Although the interaction between TB and *A. fumigatus* infections is unclear and controversial, we hypothesized that the cytokine profiles in PTB patients with co-infection of *A. fumigatus* would differ from patients with PTB or *A. fumigatus* induced CPA. In this study, we characterized cytokine profiles in peripheral blood of PTB patients with concomitant *A. fumigatus* infection or not and identified potential diagnostic biomarkers for these patients.

Patients And Methods

Subjects and ethics statement

A total of 152 participants including 31 healthy controls (HC), 35 PTB, and 86 CPA were recruited at the Beijing Chest Hospital from December 2020 to November 2021. All the cases included in this study were hospitalized for the first time and were not treated with related drugs. PTB patients were diagnosed based on clinical symptoms suggestive of active TB plus positive evidence by sputum smear examinations for acid-fast bacilli (AFB) and/or GeneXpert MTB/RIF assay and/or mycobacterial culture. CPA patients were diagnosed by galactomannan (GM) antigen assay to detect *A. fumigatus* infection. All patients were primary treated as hospitalized individuals. Accordingly, 31 healthy volunteers were recruited after physical examinations without bacteriological and clinical evidence of TB or CPA and were confirmed to have negative results on the IFN-γ release assays (Table 1). All the blood samples we collected were fasting samples taken around 8 a.m. The residual clinical blood samples were stored in the refrigerator. Considering that this study presented no more than minimal risk of harm to patient subjects, the institutional review board approved a waiver of patient informed consent. This study was approved by the Ethic Committee of Beijing Chest Hospital, Capital Medical University.

Table 1
Demographic characteristics of participants included in the cohort.

Parameters	HC (31)	TB (35)	CPA-TB (40)	CPA+ (46)	*P* value*	OR(95%CL)
Age, y, median						
Age ≥ 45	51.5(4, 51–53)	57.5(18, 47–81)	66(30, 48–92)	69.5(42, 51–89)	0.062	2.490(0.954–6.501)
Age < 45	32(27, 24–42)	32(17, 14–43)	32(10, 17–43)	39(4, 25–43)		
Sex						
Male	11	22	30	32		
Female	20	13	10	14	0.258	0.564(0.209–1.520)
Blood cells, 10^6 /mL, median [IQR]						
Leukocytes		5.34(2.35–19.20)	6.96(2.79–25.40)	7.12(3.24–24.90)	0.048	0.050(0.045–0.054)
Neutrophils		3.20(1.14–17.82)	5.04(1.79–24.30)	4.99(1.75–17.11)	0.007	0.006(0.005–0.008)
Lymphocytes		1.28(0.37–3.30)	0.97(0.14–2.94)	1.11(0.10-14.67)	0.096	0.106(0.100-0.112)
Eosinophils		0.09(0.01–2.33)	0.09(0.00-1.31)	0.07(0.00-1.24)	0.372	0.378(0.369–0.388)
Monocytes		0.41(0.19–0.81)	0.45(0.02–5.60)	0.43(0.05–7.80)	0.228	0.230(0.221–0.238)
Platelets		247(116–598)	260(93–621)	187(22–442)	0.754	0.755(0.746–0.763)
Complication						
Hepatitis B		2(5.71)	1(2.50)	2(4.35)	0.272	0.274(0.027–2.758)
Diabetes mellitus		6(17.14)	11(27.50)	8(17.39)	0.289	1.833(0.598–5.619)
Cancer		1(2.86)	1(2.50)	0(0.00)	0.924	0.872(0.053–14.476)

OR, odds ratio; IQR, interquartile range; HC, healthy control; TB, tuberculosis infection; CPA-TB, tuberculosis infection and GM positive; CPA+, GM positive and Tumors/Pneumonia; CHD, coronary heart disease

***The** P **value represents the difference between TB and CPA-TB.**

Parameters	HC (31)	TB (35)	CPA-TB (40)	CPA+ (46)	*P* value*	OR(95%CL)
HIV/Rheumatism		3(8.57)	2(5.00)	1(2.17)	0.999	0.000
Hypertension/CHD		4(11.43)	6(15.00)	14(30.43)	0.651	1.368(0.353–5.305)
Cerebral infarction		1(2.86)	2(5.00)	2(4.35)	0.891	0.868(0.116–6.512)

OR, odds ratio; IQR, interquartile range; HC, healthy control; TB, tuberculosis infection; CPA-TB, tuberculosis infection and GM positive; CPA+, GM positive and Tumors/Pneumonia; CHD, coronary heart disease

***The P value represents the difference between TB and CPA-TB.**

Serum Gm Antigen Test

Peripheral blood samples were collected using heparin anticoagulant blood collection tube (Becton, Dickinson and Company, USA), and serum was isolated by centrifuge at 4000 rpm for 10 min. Serum GM antigen levels were measured with a commercial sandwich enzyme-linked immunosorbent assay (ELISA) kit in singlicate (Platelia™ Aspergillus; Bio-Rad, CA). Optical density (OD) index equal to or greater than 0.5 was identified as positive result.

Multiplex Cytokine Assay

Serum samples were stored frozen at − 80°C prior to being subjected to Luminex xMAP technology in singlicate (Luminex Austin, TX). Cytokine concentrations in serum were measured using twelve test kits for cytokines (AtomLife, Nanjing, China) according to the manufacturer's instruction. Briefly, the coupled beads, standards, and samples were added to 96-well plate, followed by detection antibodies and Streptavidin-PE. The mean fluorescence intensity was read for each cytokine using a Luminex xMAP technology (Luminex Austin, TX). The twelve cytokines and corresponding detection limits were as follows : IL-1β (3 ~ 7500 pg/mL), IL-2 (4 ~ 5000 pg/mL), IL-4(3 ~ 7500 pg/mL), IL-5 (3 ~ 7500 pg/mL), IL-6 (2 ~ 5000 pg/mL), IL-8 (3 ~ 7500 pg/mL),; IL-10 (3 ~ 5000 pg/mL), IL-12p70 (4 ~ 5000 pg/mL), IL-17 (5 ~ 5000 pg/mL), IFN-γ (5 ~ 5000 pg/mL), IFN-α (5 ~ 5000 pg/mL), and TNF-α (4 ~ 5000 pg/mL), respectively.

Statistical analysis

All statistical analyses were performed using the SPSS version 20.0 (IBM Corp., Armonk, NY). in combination with GraphPad Prism 5.0 (GraphPad software). The univariable logistic regression models were conducted to estimate risk factors for active PTB patients with CPA. Mann-Whitney U tests were used to compare the differences in cytokine responses between two groups. The diagnostic performance

of each cytokine or cytokine combination was evaluated by receiving operating characteristic (ROC) curve. Optimal sensitivity and specificity were estimated using Youden's index and the optimal cut-off values were estimated as the maximum of Youden's index. The proportion of patients correctly diagnosed is proportional to the area under the curve (AUC). The difference was declared significant if two-sided *P* values are less than 0.05.

Results

Characteristics of enrolled Patients

In this retrospective study, we included patients with pulmonary infection in Beijing Chest Hospital from December 2020 to November 2021. Laboratory examination revealed that 35 patients were afflicted with PTB, 40 patients with PTB and CPA, and 46 patients with CPA (Table 1). Statistical analysis of clinical information demonstrated the elevated levels of leukocytes (TB *vs*. CPA-TB: 5.34×10^6/mL *vs*. 6.96×10^6/mL, $P = 0.048$) and neutrophils (TB *vs*. CPA-TB: 3.20×10^6/mL *vs*. 5.04×10^6/mL, $P = 0.007$) were noted in CPA-TB group.

Difference In Cytokine Spectrum Between Ptb And Cpa

To diagnose PTB patients with concomitant CPA as early as possible and give correct treatment, we analyzed the serum cytokine profiles across the four groups included in this study. As shown in Figure. 1, there was no significant difference in serum cytokine profiles between the two groups with CPA. The levels of IL-4 and IL-5 were decreased after infection with either *A. fumigatus* or Mtb, but the contents of these two cytokines were not different among the three groups (IL-4: TB *vs*. CPA-TB, $P = 0.356$, TB *vs*. CPA+, $P = 0.325$, CPA-TB *vs*. CPA+, $P = 0.866$; IL-5: TB *vs*. CPA-TB, $P = 0.678$, TB *vs*. CPA+, $P = 0.961$, CPA-TB *vs*. CPA+, $P = 0.757$). In contrast, the serum levels of a variety of proinflammatory cytokines, including IL-1β, IL-6, IL-8, IL-12p70, IFN-α, IFN-γ and TNF-α, were significantly increased in patients infected with the pathogens, but there was no difference in IFN-α and IFN-γ levels between the two pathogen infected groups.

Roc Analyses

Since the levels of most cytokines in CPA were significantly different from those in PTB and HC, we propose a stepwise algorithm for the diagnosis of CPA-infected patients. We further compared the ROC analysis among the three groups except HC. As expected, there was no potential marker cytokine for differential diagnosis between CPA + and CPA-TB groups (Fig. 2). Furthermore, we analyzed the results of serum cytokines in TB group and CPA-TB group by ROC (Fig. 3). The results showed that IL-8, IL-1β and IL-6 had good accuracy in distinguishing the two groups, and the AUC was 0.949, 0.777 and 0.728 (Fig. 3), respectively. Because of the excellent accuracy of IL-8, IL-8 was used as the basis to combine IL-1β and IL-6 to analyze the two groups again (Fig. 3). The results showed that the best accuracy could be

obtained by combining IL-6 with IL-8, and the AUC was 0.958. In the same way, we also analyzed TB group and CPA + group (Fig. 4). The results showed that IL-8 was still the first choice among single cytokines, because its AUC was the highest (0.964), followed by IL-1β (AUC = 0.818) and TNF-α (AUC = 0.788) (Fig. 4A). Similarly, we analyze the combination of the three cytokines (Fig. 4B), and the results show that the AUC of the combination of the three cytokines is as high as 0.996 consistent with the result of IL-8 + TNF-α, and the AUC of the combination of IL-8 and IL-1β is slightly lower (0.974). Based on the above results, we are interested in whether this phenomenon is caused by the specificity of CPA. We combine the data of CPA-TB and TB and then carried out ROC analysis with TB group. The results of IL-8 are still the best (AUC = 0.957), followed by IL-1β (AUC = 0.798), IL-6 (AUC = 0.760) and TNF-α (AUC = 0.750). Once again, we take IL-8 as the basis, combined with the above factors to jointly analyze ROC. The results showed that the combination of IL-8 with TNF-α and IL-6 had the highest AUC (0.984). This combination has the potential to be used to identify the infection of *A. fumigatus* in pulmonary infections.

Discussion

A. fumigatus induced CPA are caused by inhalation of airborne conidia, which are common in indoor and outdoor environments(Wéry, 2014). *A. fumigatus* DNA was detected in 37% of healthy adult lung biopsies(Denning, Park, et al., 2011). The pathogenesis of CPA usually involves *A. fumigatus* colonization and proliferation in the lung cavity, most of which were caused by PTB. It was reported that 20% of cavernous TB patients develop the sequential CPA within 3 years after cure, and each year, and more than 350, 000 PTB patients after 12 months of anti-tuberculosis treatment progress to CPA(Denning et al., 2016; Patterson et al., 2016). At present, the diagnosis of CPA is based on clinical symptoms, imaging features and GM tests(Rhodes, 2006). However, due to the lack of gold standard for the diagnosis of CPA, it is difficult to evaluate the performance of GM Ag detection in different studies(Denning et al., 2016; Hayes & Novak-Frazer, 2016; Patterson et al., 2016).

In addition, the phenomenon of co-infection is common in clinic, but the clinical symptoms caused by different pathogens are similar, which makes the patients with co-infection cannot get accurate treatment at the first time, even misdiagnosis(Asner et al., 2014; Nongrum et al., 2019). This undoubtedly increases the burden of patients and has a great impact on the prognosis of patients. Therefore, targeted diagnosis of patients as soon as possible will greatly alleviate the unnecessary harm caused by missed diagnosis and misdiagnosis. In this study, we retrospectively studied 152 people and divided them into 4 groups according to AFB and/or GeneXpert MTB/RIF assay and/or mycobacterial culture and GM antigen test.

In addition, consistent with previous reports, *A. fumigatus* infection can lead to an increase in leukocytes and neutrophils in patients(Patel & Greenberger, 2019). *A. fumigatus* induce pulmonary epithelial cells to release inflammatory cytokines, which in turn promote lymphocyte recruitment and stimulate other inflammatory responses(Croft et al., 2016; Liu et al., 2021; Øya et al., 2019). The immune responses induced by CPA and PTB are different, which are Th1 and Th2 respectively(Abebe, 2019; Sales-Campos et al., 2013). In this study, we analyzed the differences in serum cytokine profiles among the four groups.

Interestingly, IL-4 and IL-5 showed a downward trend post *Mtb* and/or *A. fumigatus* infection, but there was no difference among TB, CPA-TB and CPA groups. On the contrary, a variety of pro-inflammatory cytokines increased significantly post *Mtb* and/or *A. fumigatus* infection, including IL-1β, IL-6, IL-8, IL-12p70, IFN-α, IFN-γ and TNF-α. Except IFN-α and IFN-γ, other cytokines in CPA-TB were significantly higher than those in TB, which is consistent with the results of a recent study on bronchoalveolar lavage fluid in patients with or without CPA(Salzer et al., 2018).

In view of the specificity of our results, we further compared the ROC analysis among the three groups of patients and found that IL-8 had best diagnostic performance among these cytokines, which were consistent with expectations. As the first characteristic cytokine possessing chemotactic and neutrophil-activation properties, the high expression of IL-8 in the host infected by *A. fumigatus* has been widely verified(Croft et al., 2016; Liu et al., 2021). On the other hand, IL-8 plays a central role in the host's effective defense against *Mtb*(Krupa et al., 2015). However, the level of IL-8 in patients with CPA was further increased, which may be a strategy for the immune system to respond to *A. fumigatus* infection. The AUC of IL-8 combined with TNF-α was the highest, suggesting that this combination had the potential to distinguish patients with TB or CPA. Correspondingly, the combination of IL-8, TNF-α and IL-6 could help diagnose all *A. fumigatus* infected CPA patients with TB or not from PTB-alone patients. Our results indicated that high expression of lots of cytokines in patients with CPA-TB or CPA cover the immune response caused by infection of *Mtb*. The experimental data of this study show that this combined diagnosis method of serum cytokines is superior to the sensitivity (56%-89%) and specificity (67%-99%) of GM shown by previous research data. And, Some Aspergillus are poor GM producer, cross reactivity with other fungal pathogens (Fusarium, Paecilomyces, Trichoderma, Histoserum and Penicillium species)(Lass-Flörl et al., 2021). In addition, we also acknowledge that the main advantage of cytokine-based diagnostic assay lays in the earliness of differentiation of TB and CPA patients in view of the delay of microbiological culture confirmation associated with their long doubling time, which would assist clinicians in decision making.

We also acknowledged several obvious limitations to the present study. First, we only included active patients having positive Interferon-γ release assays (IGRAs) rather than latent tuberculosis infection (LTBI) group, and GM antigen test is not sensitive to be used for immunocompetent patients, which given the lack of uniform diagnostic criteria. Hence, further study is needed to evaluate the accuracy of our diagnostic algorithm. Second, considering that there are large range of overlap of the levels of cytokines of interest between TB and CPA-TB/CPA groups, which is a major factor limiting the potential of these single cytokines as marker for presence of *A. fumigatus*. Therefore, the number of samples included in this study still needs to be further expanded to accurately clarify the results.

In conclusion, our data demonstrate that increased population of leukocytes and neutrophils are related factors for PTB patients with CPA. Cytokine IL-8 alone has best performance to diagnose TB patients from CPA-TB or CPA patients. Likewise, the combination of IL-8, TNF-α and IL-6 can help distinguish all CPA patients with TB or not from TB-alone patients. Our study provides multiple cytokines as potential

markers to accurately diagnosis TB and CPA and may help better understanding the immune function disorder during Mtb and/or *A. fumigatus* infections.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics committee of Beijing Chest Hospital, Capital Medical University (approval number: YJS-2019-016). The guidelines outlined in the Declaration of Helsinki were followed.

Consent for publication

Written informed consent was obtained from the patient.

Availability of data and material

All data contained in this study can be obtained from the corresponding author under reasonable request.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

YP, WR, and SL conceptualized the study. YP and WR designed the methodology. LH, YS, and XZ performed formal analysis. WR, CG, and HL conducted the investigation. HL, WR, and WW curated the data. HL, YP, and SL prepared the original draft. YP, SL, and HL reviewed and edited the manuscript. YP acquired funding. All authors contributed to the manuscript and approved the submitted version.

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Figures

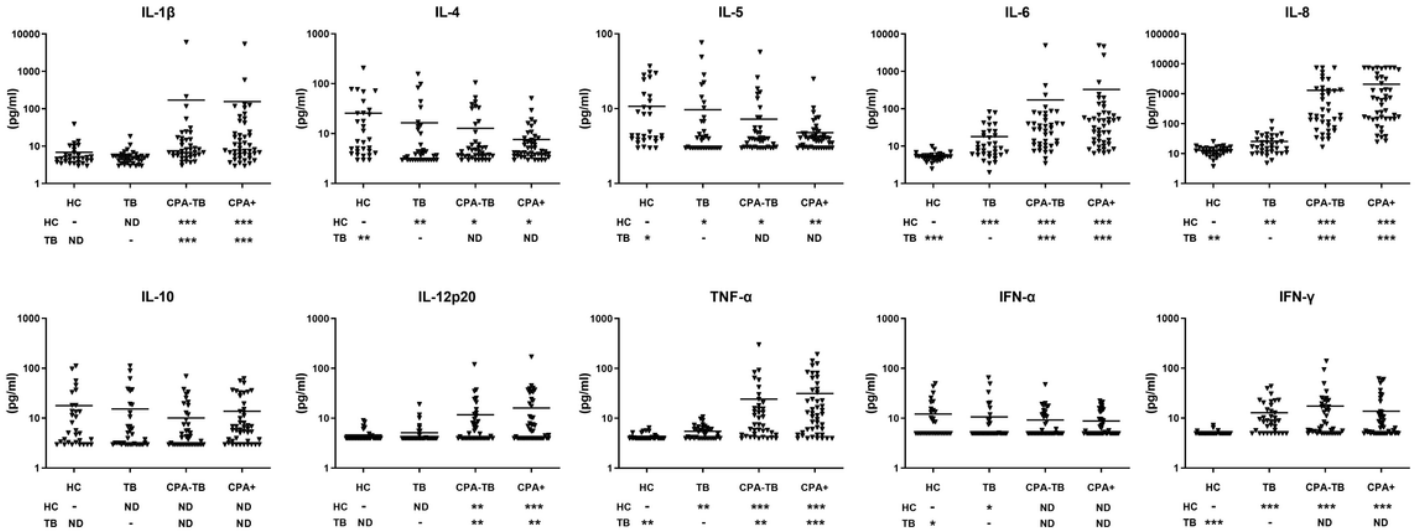

Figure 1

Difference in cytokine spectrum between tuberculosis (TB) and chronic pulmonary aspergillosis (CPA). HC, healthy control; CPA-TB, tuberculosis infection and GM positive; CPA+, GM positive and suffering from tumor and or pneumonia; Data denoting means ± SDs. ND, no difference. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

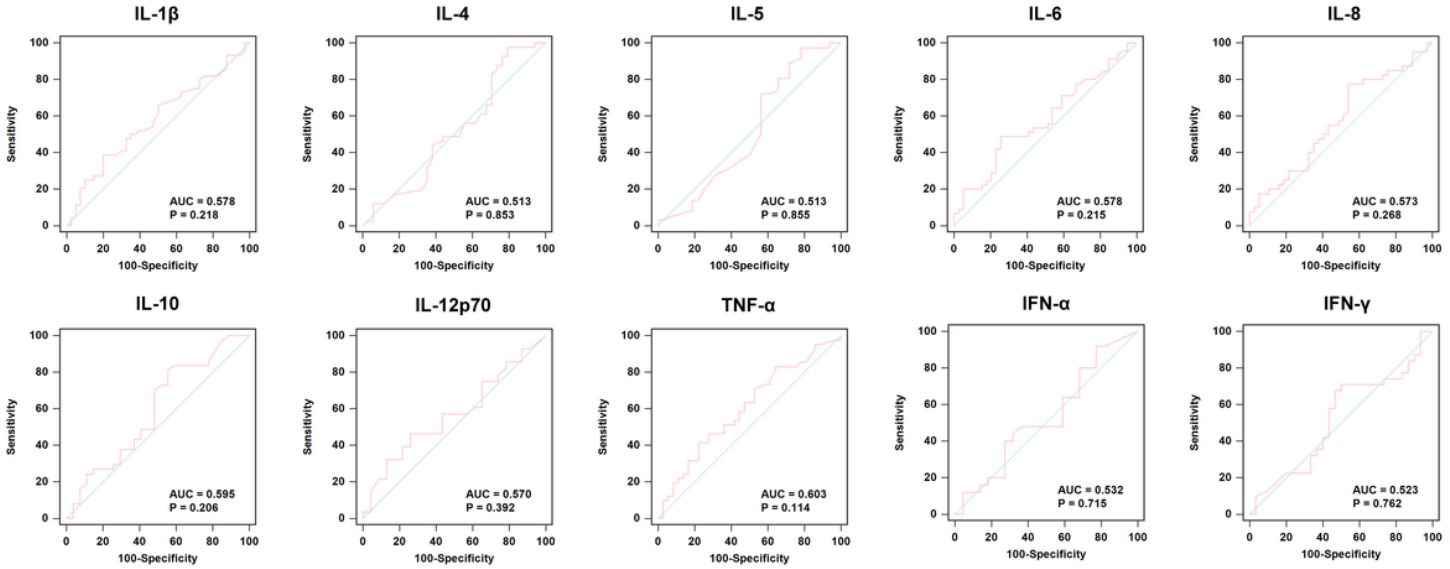

Figure 2

Receiving operating characteristic (ROC) curve analysis between CPA+ and CPA-TB groups. CPA-TB, tuberculosis infection and GM positive; CPA+, GM positive and Tumors/Pneumonia. AUC, area under the curve.

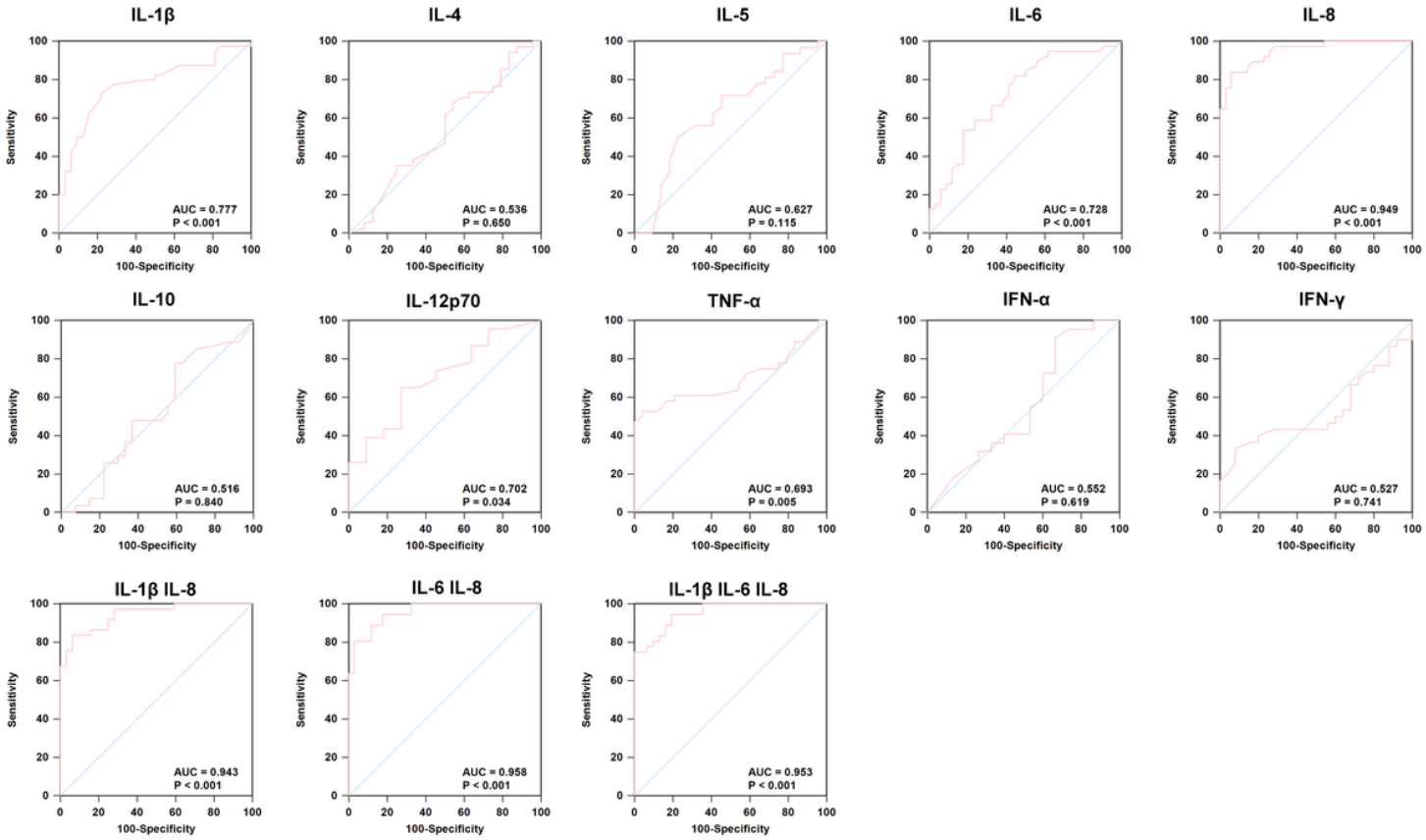

Figure 3

Receiving operating characteristic (ROC) curve analysis between tuberculosis (TB) and CPA-TB groups. CPA-TB, tuberculosis infection and GM positive. AUC, area under the curve.

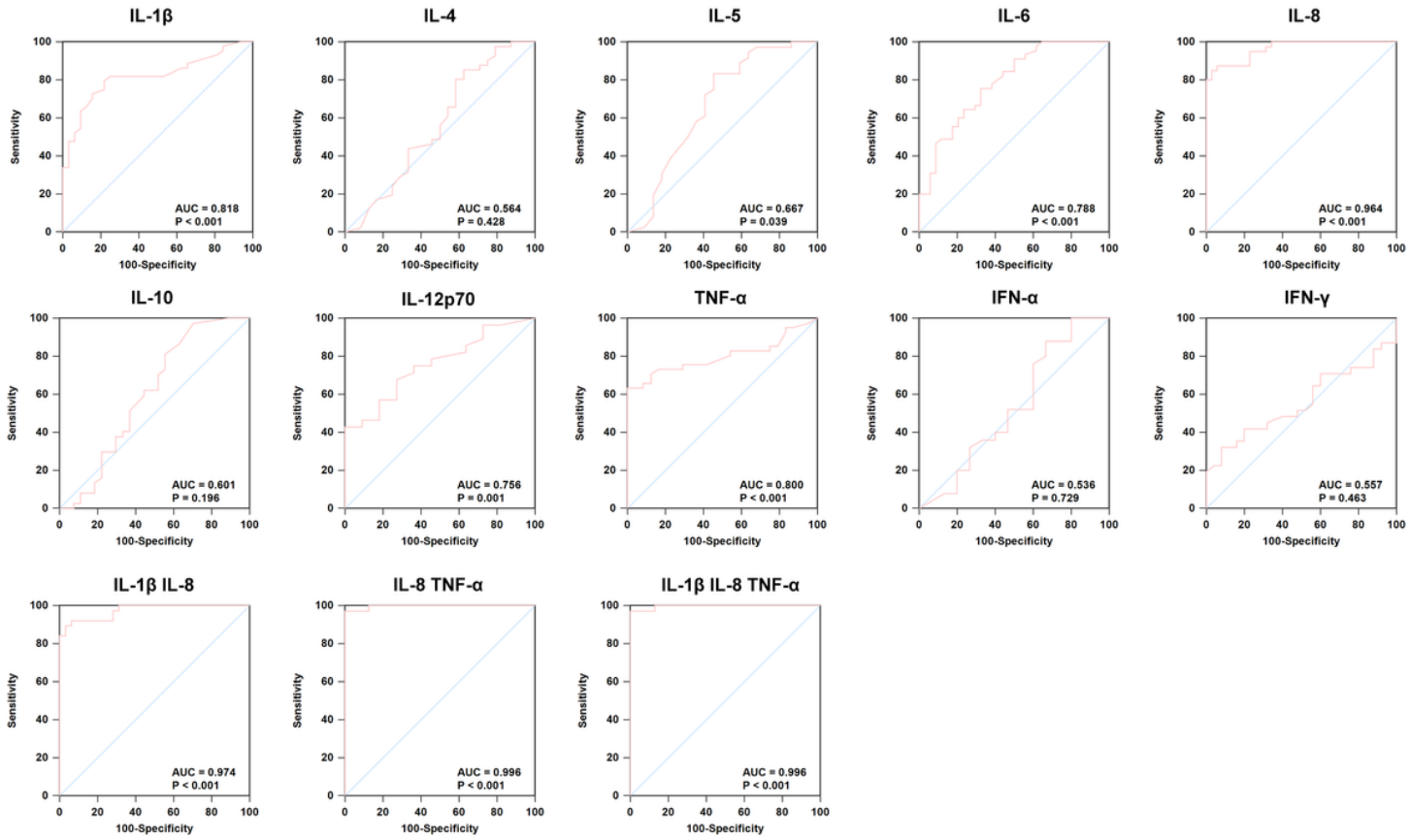

Figure 4

Receiving operating characteristic (ROC) curve analysis between tuberculosis (TB) and CPA+ groups. CPA+, GM positive and Tumors/Pneumonia. AUC, area under the curve.

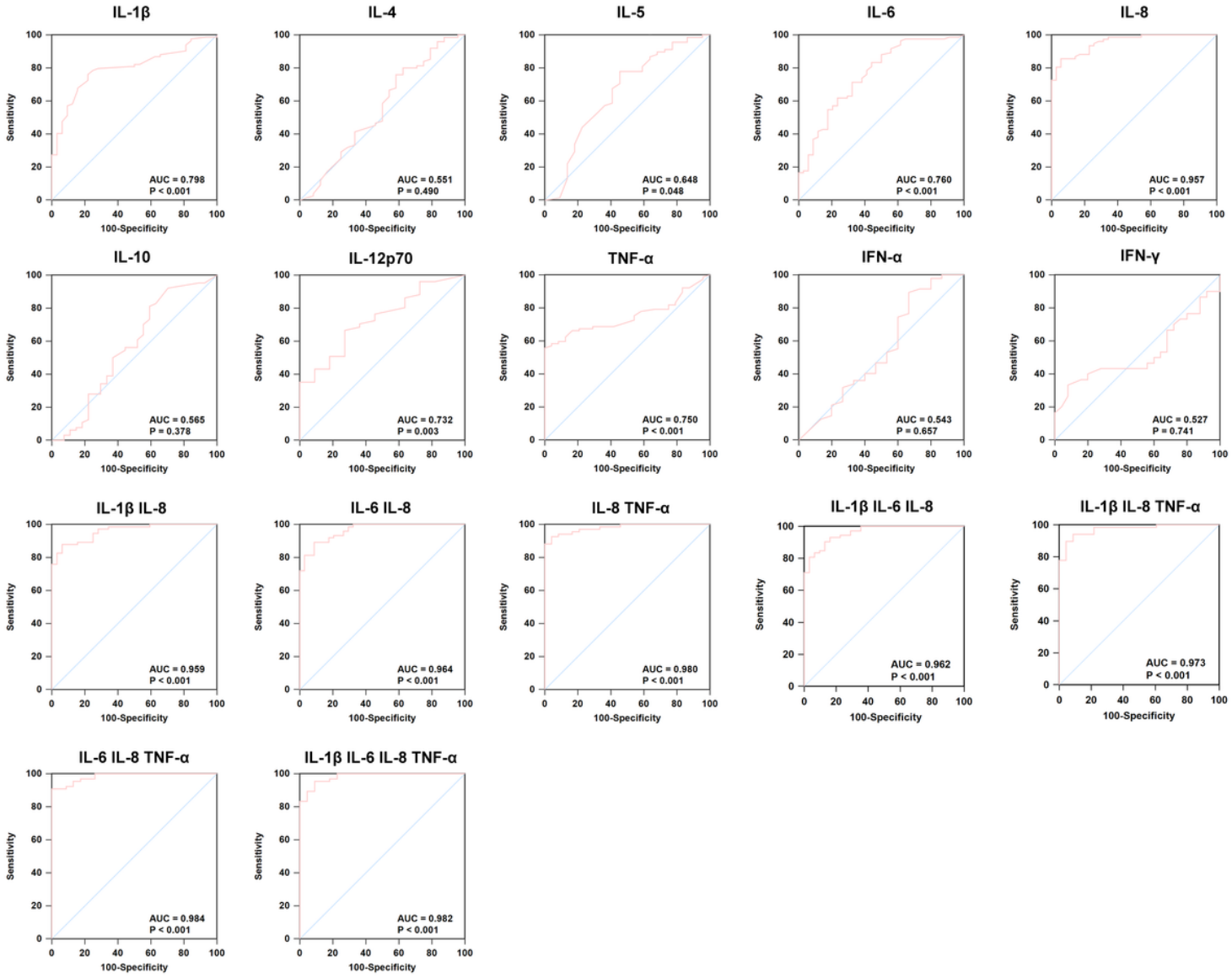

Figure 5

Receiving operating characteristic (ROC) curve analysis between tuberculosis (TB) and CPA+ plus CPA-TB groups. CPA-TB, tuberculosis infection and GM positive; CPA+, GM positive and Tumors/Pneumonia. AUC, area under the curve.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterial.xlsx