# Expression of lactoferrin in the peripheral blood of pulmonary tuberculosis patients and its clinical significance

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**Research Article**

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# Abstract

**Background:** Pulmonary tuberculosis (PTB) affects patients' lives and reduces their quality of life. Lactoferrin (LF) is a member of the transferrin family. We explore the expression level of LF in the peripheral blood of PTB patients and the clinical significance of LF in the diagnosis and course evaluation of PTB.

**Methods:** Plasma LF concentration and neutrophil LF concentration were measured, and their correlation was analyzed, as well as their diagnostic validity and specificity for PTB.

**Results:** Plasma LF concentration of active PTB patients was higher than that of stable PTB patients and patients in the control group. Neutrophil LF concentration of active PTB patients was higher than that of the control group, whereas it was reversed in stable PTB patients. However, there was no significant difference in the levels of neutrophil LF and plasma LF between primary and retreated PTB patients. There was a positive correlation between them, and the LF level was positively correlated with the TB replication load. Neutrophil LF had a higher specificity and sensitivity for diagnosing stable PTB, while plasma LF had higher specificity and sensitivity in the diagnosis of active PTB.

**Conclusions:** Plasma LF has a higher sensitivity and specificity for the diagnosis of active PTB, and LF might be a biological indicator of PTB.


# Highlights

The levels of plasma and neutrophil LF in active PTB patients were higher than those in stable PTB patients and control group; however, there was no significant difference in LF levels between initial treatment PTB patients and retreatment PTB patients.

There was a positive correlation between the levels of plasma LF and neutrophil LF in patients with active PTB.

LF level in active PTB patients is positively correlated with TB replication load.

Neutrophil LF has higher specificity and sensitivity for the diagnosis of stable PTB than plasma LF.

Plasma LF has higher specificity and sensitivity for the diagnosis of active PTB than neutrophil LF.

# Introduction

Pulmonary tuberculosis (PTB) is a chronic wasting disease of the respiratory system caused by *Mycobacterium tuberculosis* (MTB). Globally, PTB remains a major problem that needs to be tackled because of its long treatment cycle, easy recurrence, limited number of available therapeutic drugs, and the continuous emergence of multi-drug resistant *Mycobacterium tuberculosis* (MDR-TB)[1].

The diagnostic criteria for PTB, as defined by the World Health Organization, include clinical symptoms, sputum MTB isolation and culture, sputum smear microscopic examination, and chest radiograph examination[2]. However, only 5–10% of PTB patients have clinical symptoms and signs[3]. At present, laboratory diagnostic techniques for PTB still have some shortcomings, such as low specificity and sensitivity. There has been no breakthrough in early diagnosis. As the "gold standard," a bacterial culture is easy to cause false negatives and false positives, and the long culture cycle will delay the treatment of PTB. Culture conditions are strict, and laboratory safety risk is high[2, 3]. Sputum is an important specimen for the diagnosis of PTB. Acid-fast staining of sputum smears is widely used in the diagnosis of PTB. However, MTB cannot be distinguished from other acid-fast bacilli with low sensitivity and high bacterial content[4, 5]. Although chest radiography is helpful in the diagnosis of PTB, there are some concerns, such as radiation exposure and low specificity[6].

Lactoferrin (LF) is a member of the transferrin family. LF is an iron-binding glycoprotein. In most animals, especially mammals, their concentration is high. Additionally, the LF of peripheral blood mainly exists in neutrophils[7]. Besides, LF also exists in other body fluids, such as tears and saliva[8]. LF has a variety of biological functions, such as inhibiting and killing general bacteria, fungi, viruses, and even parasites, anti-inflammatory, regulating immunity, promoting cell growth, anti-tumor, and anti-oxidation, and participating in iron metabolism[9–12]. LF is a bridge between innate immune response and adaptive immune response[13]. LF can increase the number of NK cells, recruit blood polymorphonuclear cells, induce phagocytosis of phagocytes, and regulate the physiological function of the bone marrow[14]. Many studies have shown that LF is involved in the occurrence and development of lung diseases, such as coronavirus disease 2019 (COVID-19), tuberculosis, lung cancer, and others[15–17]. Therefore, the level of LF in the peripheral blood of patients with PTB and its clinical significance need to be further explored.

In this study, we collected samples from patients with PTB, estimated the concentration of LF in plasma and neutrophils, preliminarily explored the expression level of LF in the body, explored the clinical significance of LF level for PTB, and confirmed whether LF is of diagnostic value in PTB.

## Materials And Methods

# Patients

A total of 72 patients were recruited in this study, including 21 healthy volunteers (11 men and 10 women, aged 53–63 years), 21 stable PTB patients (13 men and 8 women, aged 52–64 years), and 30 active PTB patients (20 men and 10 women, aged 49–67 years). Active PTB patients included 16 primary treatment PTB patients and 14 retreatment PTB patients. Healthy volunteers had no history of tuberculosis. All patients were admitted to the Second People's Hospital of Weifang from 2019 to 2021 and signed an informed consent form.

The Ethics Committee of the Weifang Medical University approved this study.

Stable PTB patients include patients with stable PTB, tuberculosis eradication, loss of infectivity, focus improvement, and sputum negativity. Patients with active PTB include primary PTB treatment patients and retreatment PTB patients. Primary treatment PTB patients indicate patients who are found for the first time, have never received anti-tuberculosis treatment, or have irregular anti-tuberculosis treatment, but the course of treatment is not more than one month. Retreatment PTB patients include patients who had undergone initial treatment or have experienced anti-tuberculosis treatment ineffectiveness for more than one month.

# Materials and Reagents

The antibodies to mouse CD11b-Percp, CD15-PE,were supplied by BD

Biosciences (San Jose, CA, USA). Human neutrophil isolation kit was provided by Solarbio Science & Technology Co., Ltd (Beijing, China). PMSF lysate and Ripa lysate (strong) were obtained from Beyotime Biotechnology. Co., Ltd(Shanghai, China). The Human Lactoferrin Detection Kit (ELISA) was purchased from Abcam (Cambridge, UK), and the TB Nucleic Acid Assay Kit was purchased from the Da An Gene Company (Guangzhou, China).

# Isolation of neutrophils

Human peripheral blood neutrophils were isolated using a Human Neutrophil Isolation Kit. In a 15 ml centrifuge tube 3 ml reagent A was added, followed by addition of 2 ml reagent C to create a gradient interface. Next, 2 mL of human peripheral blood was added to the liquid level of the separation solution, and the mixture was centrifuged for 20–30 min at 500 g. The neutrophil layer was carefully removed and washed twice. The enriched cells were smeared, and the purity of neutrophils was examined using Wright's staining.

# Lysis of neutrophils

After the Ripa pyrolysis solution was dissolved, it was mixed properly, and PMSF was added to obtain a final concentration of 1 mM, and allowed to stand for 3–5 min. Neutrophils were collected after centrifugation, and 150 μL of lysate was added. After thorough lysis, the neutrophils were centrifuged at 13,000 rpm for 3 min, and the supernatant was extracted.

# Detection of lactoferrin

LF levels in plasma and neutrophil lysis supernatants were determined according to the instructions of the Human Lactoferrin Detection Kit.

# Detection of TB replication load

To achieve this, 4% sodium hydroxide was added to the sputum of the patient, and after shaking well, it was kept at room temperature for 3.5 h. Next, 0.5 ml of the mixture was taken in a centrifuge tube, to which 0.5 ml 4% NaOH was added, after which the resulting mixture was kept at room temperature for 10 min, then centrifuged at 15 000 rpm for 5 min. After the supernatant was discarded, physiological saline

was added to re-suspend the precipitate, centrifuged at 15,000 rpm for 5 min, and washed twice. The precipitate was mixed with 50 μL of DNA extract, bathed in water at 100°C for 10 min, and kept at 4°C for 8 h to ensure complete lysis. After centrifugation at 10,000 rpm for 5 min, 2 μL of the supernatant was collected for subsequent experiments. The replication load of TB was detected according to the operating instructions of the TB nucleic acid assay kit.

## Statistical analysis

The experimental data were statistically analyzed using GraphPad Prism 6 software, and the threshold for statistical significance was set at $P < 0.05$. All experimental continuous data are expressed as $X \pm SD$.

## Results

# 1. Determination of neutrophil LF concentration

LF, a glycoprotein with a molecular weight of 80 kDa, mostly exists in neutrophils[7]. The enriched cells suspension was stained with anti-CD11b and CD15 antibodies, and the purity of the isolated neutrophils was identified by flow cytometry. The results showed that the purity of neutrophils was 97% (Fig. 1A), indicating that most of the enriched cells were neutrophils, which meets the research requirements. The results also showed that the concentration of LF in neutrophils of the active PBT patients was higher than that in the control group and stable PTB patients (Fig. 1B). However, there was no significant difference between the LF levels of neutrophils in primary treatment PTB patients and those of retreated PTB patients (Fig. 1C).

# 2. Determination of plasma LF concentration

The results revealed that the concentration of plasma LF in the active PTB patients was higher than that in patients in the control group and stable PTB patients; however, there was no difference in the concentration between stable PTB patients and patients in the control group. (Fig. 2A). In addition, there was no significant difference in the level of plasma LF between primary PTB treatment patients and retreatment PTB patients (Fig. 2B).

# 3. Correlation analysis of plasma and neutrophils LF level

Correlation analysis of LF levels between plasma and neutrophils showed that the correlation coefficient of the LF concentration between plasma and neutrophils in the control group was 0.957 ($r = 0.957$), and that between plasma and neutrophils in the stable PTB patients was 0.947 ($r = 0.947$). The correlation coefficient of the LF concentration between plasma and neutrophils in active PTB patients was 0.580 ($r = 0.580$). These results suggest that there is a positive correlation between LF concentration in neutrophils and plasma.

# 4. Correlation between LF concentration and MTB load in patients with active PTB

The TB nucleic acid assay kit was used to measure the TB load of patients with active PTB, and the correlation between TB load and LF concentration in plasma and neutrophils was analyzed. The correlation coefficient between the plasma LF level and the MTB load was 0.740 (r = 0.740), and the correlation coefficient between neutrophil LF level and MTB load was 0.968 (r = 0.968). The above results suggest that the plasma and neutrophil LF concentrations of patients with active PTB were positively correlated with the MTB load.

# 5. Analysis of diagnostic validity of neutrophilic LF for PTB

In this study, the ROC curve was used to evaluate whether the neutrophil LF level is helpful in diagnosing PTB. The analysis showed that the AUC of the neutrophil LF ROC curve in stable PTB patients was 0.9044 (95% CI, 0.7953, 1.014, P < 0.0001), while that in active PTB patients was 0.7481 (95% CI, 0.5988, 0.8965, P = 0.0042) (Table 1, Fig. 3). Besides, the cut-off value of neutrophil LF level in stable PTB patients was 414.2 pg/ml, sensitivity was 76.19%, and specificity was 90.48%. The cut-off value of neutrophil LF in active PTB patients was 512.6 pg/ml, sensitivity was 86.67%, and specificity was 57.14%. Neutrophil LF level has a higher sensitivity and specificity in the diagnosis of stable PTB patients.

Table 1
Diagnosis of PTB patients using the neutrophil LF ROC curve

| Parameter | AUC | 95% CI | p-value |
|---|---|---|---|
| Stable PTB | 0.9044 | 0.7953, 1.014 | <0.0001 |
| Active PTB | 0.7481 | 0.5988, 0.8965 | 0.0042 |

# 6. Analysis of the diagnostic validity of plasma LF for PTB

In this study, the ROC curve was used to evaluate whether the plasma LF level is helpful in diagnosing PTB. The results showed that the AUC of the plasma LF ROC curve in stable PTB patients was 0.5388 (95% CI, 0.3440, 0.7336, P = 0.6827), and that in active PTB patients was 0.9286 (95% CI, 0.8431, 1.016, P < 0.0001) (Table 2, Fig. 4). The cut-off level of 571.9 pg/mL for plasma LF in stable PTB patients had a sensitivity of 38.10% and specificity of 90.48%. The cut-off level of 905.8 pg/mL for plasma LF in active PTB patients had a sensitivity of 83.33% and specificity of 100%. The plasma LF level has a higher sensitivity and specificity in the diagnosis of active PTB.

Table 2
Diagnosis of PTB using the plasma LF ROC curve

| Parameter | AUC | 95% CI | p-value |
|---|---|---|---|
| Stable PTB | 0.5388 | 0.3440, 0.7336 | 0.6827 |
| Active PTB | 0.9286 | 0.8431, 1.016 | <0.0001 |

# Discussion

LF has several biological functions such as anti-bacterial, anti-viral, and anti-tumor effects[9–12]. Besides, the immunomodulatory function of LF is extremely powerful, which is an immunomodulatory effect on innate and adaptive immunity[18]. LF can help maintain the dynamic balance of mucosal microorganisms, such as in the respiratory mucosa and digestive mucosa, and regulate the immune response caused by microorganisms in the mucosa[18–20]. LF has a direct bactericidal effect on common respiratory tract infection bacteria, such as *Actinobacillus pleuropneumoniae* and *Legionella pneumophila*[21, 22]. LF inhibits the growth and proliferation of iron-demanding bacteria by binding with iron[23]. The peptide fragment decomposed by LF has a higher antibacterial activity than natural LF, and its antibacterial mechanism is still not perfect[24]. In addition, LF has been shown to have bactericidal effects against many pathogen types by forming a complex with bacterial lipopolysaccharide, which perforates the outer membrane of Gram-negative bacteria[25]. However, recent studies have shown that the interaction between LF, iron, and bacteria might be much more complex than originally thought[25, 26]. Kerry J. Welsh et al. found that the bacterial content in the lungs of MTB-infected mice treated with LF was lower than that in the initial state, and lung inflammation in mice treated with LF was significantly reduced, suggesting that LF might have a certain therapeutic effect on PTB[27].

LF is involved in the occurrence and development of respiratory diseases. Studies have shown that compared with sedentary volunteers, LF levels in the saliva of street-running volunteers are higher in acute and chronic airway inflammation caused by air pollution[28]. Researchers found that knocking out the *IF* gene in mice increased the number of myeloid suppressor cells in the lungs, thereby promoting lung metastasis of melanoma[29]. In addition, the study also found that LF has clinical diagnosis and treatment effects on inflammatory bowel disease, spontaneous peritonitis of liver cirrhosis, viral hepatitis, nasopharyngeal carcinoma caused by EB virus infection, and dermatophytosis caused by fungal infection[30, 31].

The results showed that the plasma and neutrophil LF levels of active PTB patients were higher than those in the control group and stable PTB patients. However, there was no significant difference in plasma LF concentration between the stable PTB patient and the control group, and the neutrophil LF concentration of stable PTB patients was lower than that of patients in the control group. There was no significant difference in plasma and neutrophil LF concentrations between primary treatment patients

and retreatment PTB patients. The LF expression level was higher in patients with active PTB. Subsequently, the TB nucleic acid assay kit was used to detect the TB load in the samples of patients with active PTB, and the results suggested that the TB load was positively correlated with the expression level of LF in the body. Therefore, we speculate that the replication load of TB in active PTB patients is directly related to the expression level of LF. The expression level of LF in active PTB patients is generally higher than that in stable PTB patients and did not differ significantly from that of patients in the control group. This indicates that the expression level of LF might be associated with the severity of PTB.

In addition, ROC curves of plasma LF and neutrophil LF were drawn to analyze the sensitivity and specificity of LF in the diagnosis of stable and active PTB patients. The results revealed that the specificity and sensitivity of plasma LF in active PTB were 100% and 83.3%, respectively. Neutrophil LF has a higher specificity and sensitivity in the diagnosis of stable PTB than in the diagnosis of active PTB. LF levels of neutrophils in active PTB are not too high. The technique of neutrophil extraction is rigorous; thus, there are few potential sources of error. We speculate that this may be due to the uneven distribution of the peripheral blood neutrophil pool in patients, or the patient has low immunity and other factors. In the future, we will optimize the operation process, select more representative specimens, exclude the influencing factors, and further study the clinical diagnostic value of LF for active PTB.

This study not only detected the plasma LF concentration of patients with PTB at different infection stages but also measured the neutrophil LF level. At present, there are few studies on the expression level and clinical significance of LF in patients with PTB. In addition, the simultaneous determination of plasma LF and neutrophil LF is more comprehensive and convenient for statistical analysis and comparison of clinical data. PCR was used to detect the replication load of MTB *in vivo*. The correlation between copy number and LF expression level and the correlation between LF expression level and the severity of PTB infection were analyzed. Statistical analysis of the sensitivity and specificity of LF in the diagnosis of PTB in each group is helpful to further promote the clinical application of LF in the diagnosis of PTB.

The limitations of this study lie mainly in the low number of specimens selected. Owing to the limited study duration and the high standard of specimen selection, we selected the most representative specimens to facilitate statistical analysis and obtain valuable clinical results. For the follow-up experiments, we will conduct more in-depth research, assemble a large number of typical cases, and further explore the role of LF in the monitoring, diagnosis, and treatment of PTB.

In conclusion, the level of LF in active PTB patients increased, and it was associated with the peripheral blood neutrophil LF level. The practicability and clinical application value of LF biomarkers require further research.

# Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Weifang Medical University (Grant numbers: 2020YX066) and written informed consent was obtained from all participants.

In accordance statement

The research was conducted in accordance with relevant guidelines and regulations.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflict of interest.

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Author’s contributions

CM designed the experiments. FA, YT, XZ and YZ performed the experiments. FA ,QW, YZ, BD processed data. CM, YT, XZ and wrote the paper.

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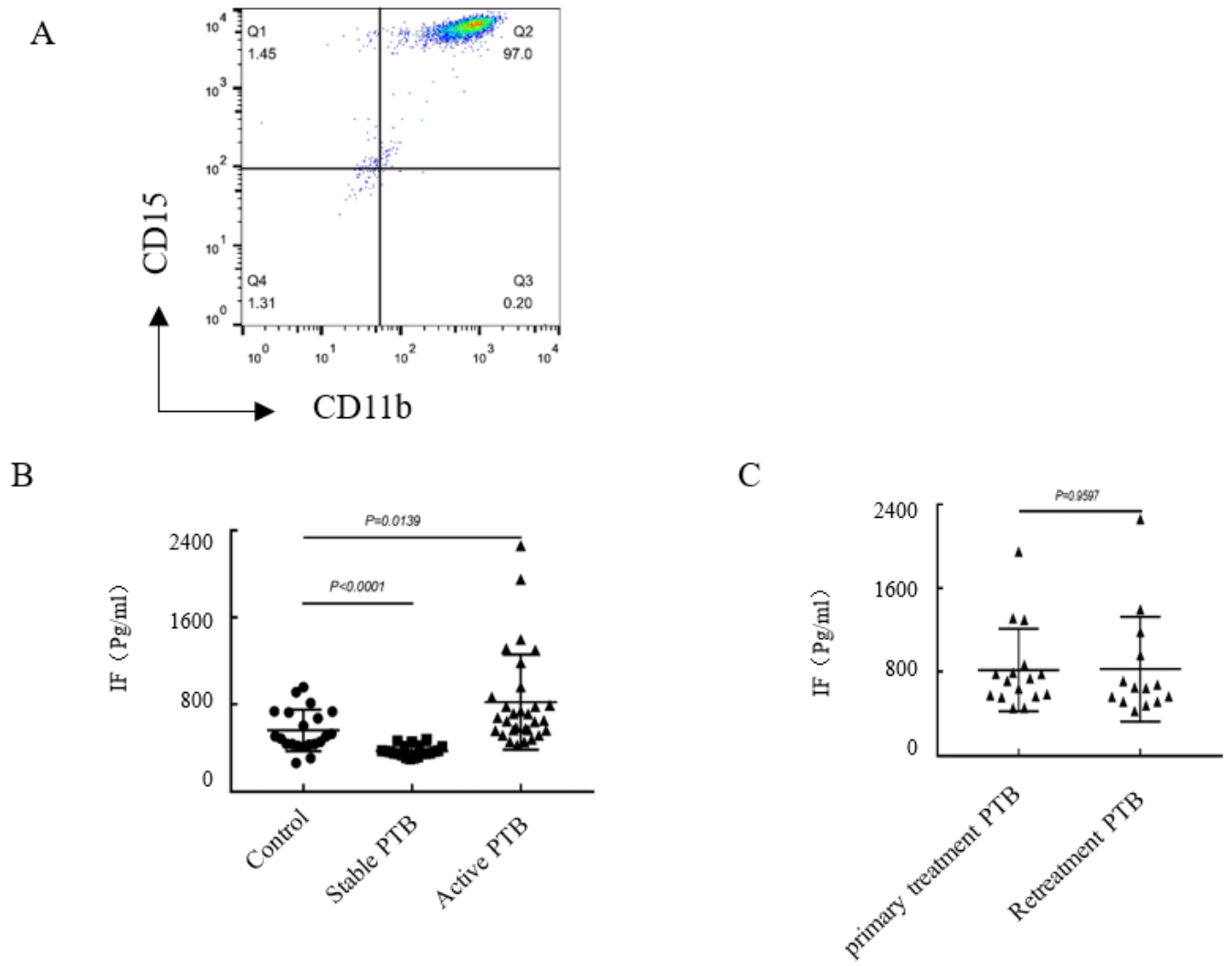
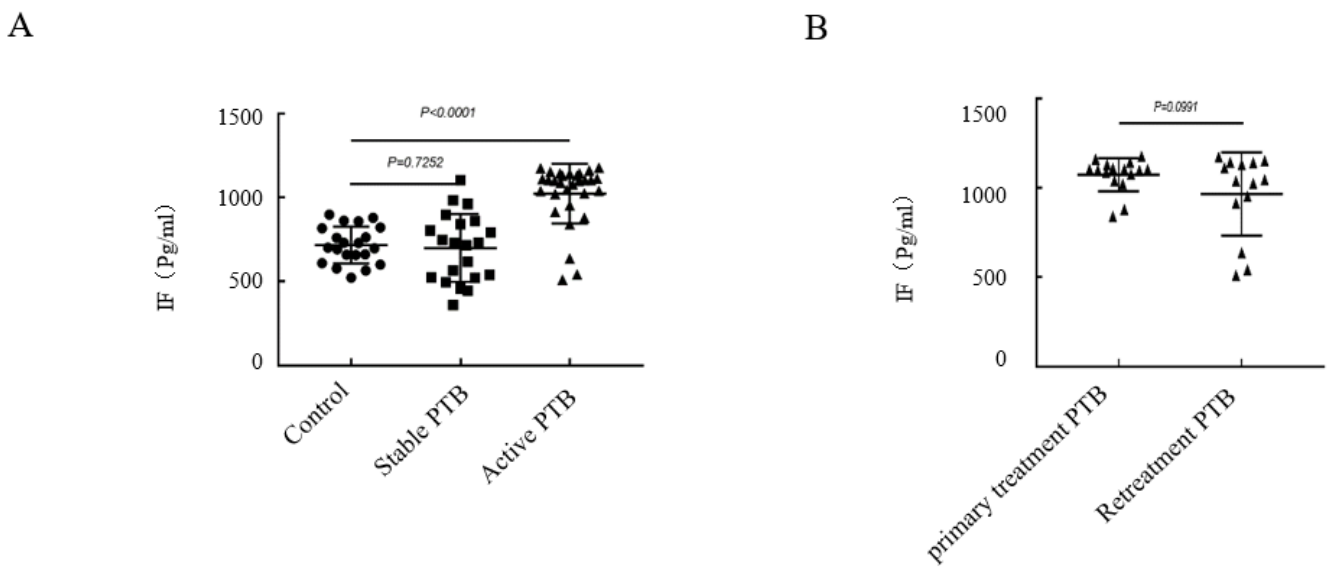
# Figures



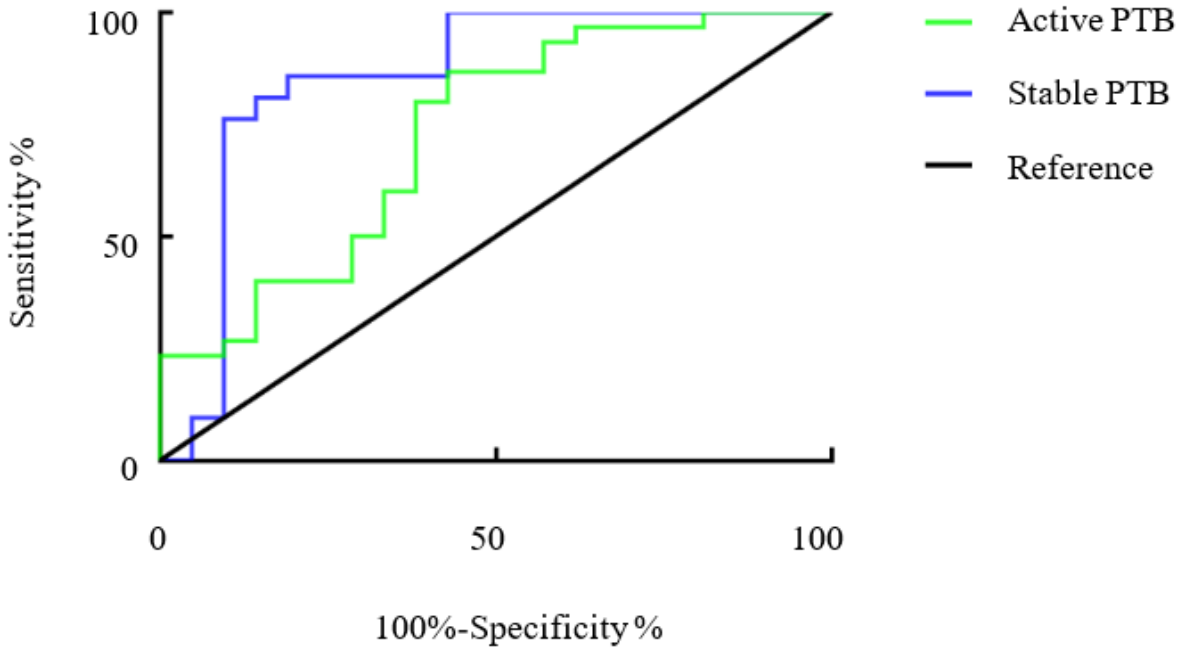

## Figure 1

Determination of neutrophil IF concentration. (A) Purity of enriched neutrophils was identified by flow cytometry. (B) The Human Lactoferrin Detection Kit was used to detect the LF level of neutrophils in the control group, stable PTB patients, and active PTB patients. (C) The Human Lactoferrin Detection Kit was used to detect the LF level of neutrophils in the primary treatment PTB patients and retreatment PTB patients.




## Figure 2

Determination of plasma LF concentration. (A) Plasma LF levels of the control group, stable PTB patients, and active PTB patients were detected using the Human Lactoferrin Detection Kit. (B) Plasma LF levels of the primary treatment PTB patients and retreatment PTB patients were detected using the Human Lactoferrin Detection Kit.




## Figure 3

Neutrophil LF diagnosis ROC curve. LF levels of neutrophils in stable PTB is represented by the blue line, active PTB by the green line, and reference value by the black line.

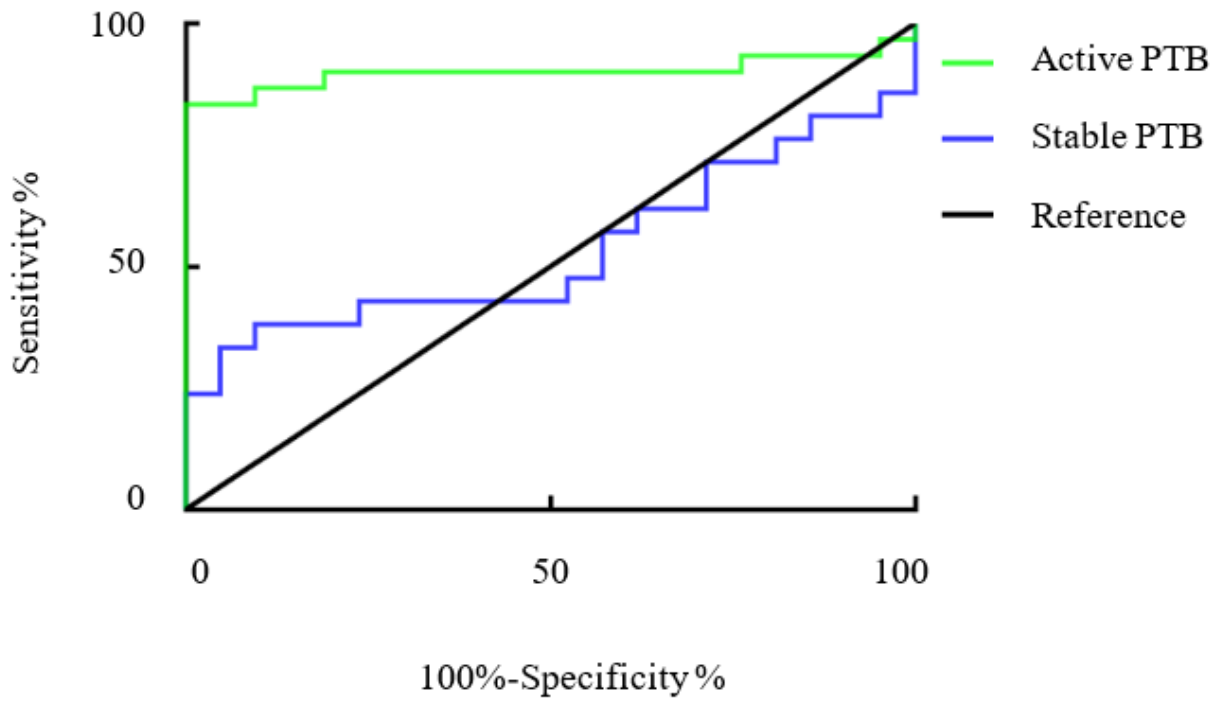



**Figure 4**

Plasma LF diagnosis ROC curve. Plasma LF level in stable PTB patients is presented as the blue line, active PTB patients as the green line, and reference value as the black line.

## Supplementary Files

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