의학박사 학위논문

혈청학적 결핵 진단법의 유용성에 관한 연구

A study on the usefulness of serological tuberculosis diagnosis

2021년 2월

서울대학교 대학원

의학과 미생물학전공

이 지 연

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지도교수 국 윤 호

이 논문을 의학박사 학위논문으로 제출함
2021년 2월

서울대학교 대학원
의학과 미생물학전공
이 지 연

이지연의 의학박사 학위논문을 인준함
2021년 2월

위 원 장 김범준 (인)

부위원장 국 윤 호 (인)

위 원 임재준 (인)

위 원 김동현 (인)

위 원 장원중 (인)

Abstract

Background: Tuberculosis remains a major public health problem. Conventional tests are inadequate to distinguish between active tuberculosis (ATB) and latent tuberculosis infection (LTBI). Serological tests offer the potential to improve diagnosis of tuberculosis (TB). This study aimed to investigate new biomarkers in the diagnosis of TB.

Methods: Antibody responses to *Mycobacterium tuberculosis* antigens (*Mycobacterium tuberculosis* chorismate mutase (TBCM), antigen 85B (Ag85B), early secreted antigen-6 (ESAT-6), and culture filtrate protein-10 (CFP-10)) and macrophage migration inhibitory factor (MIF) were measured in groups of 65 ATB, 53 LTBI, and 62 non-infected (NI) individuals. Enzyme-linked immunosorbent assay was used to measure levels of IgG and IgA from sera of study participants. The QuantiFERON-TB Gold In-Tube assay was used to diagnose LTBI.

Results: IgG levels against TBCM were significantly higher in LTBI than NI subjects. IgG and IgA levels against Ag85B and IgG levels against CFP-10 were significantly higher in ATB, followed by LTBI, and then NI. When the ATB group was subdivided, IgG levels against Ag85B and CFP-10 were significantly higher in each

subgroup compared with those in LTBI and NI groups. Positive correlation trends between interferon-gamma (IFN-γ) and IgG levels against Ag85B, TBCM, and CFP-10 and IgA levels against Ag85B in LTBI and NI subjects were observed. Age- and sex-adjusted models showed that IgG against TBCM and CFP-10 was independently related to LTBI diagnosis, and IgG against Ag85B was independently related to the diagnosis of ATB and could distinguish between LTBI and ATB.

The level of IgA against MIF was significantly lower in LTBI and ATB patients than in NI individuals and was significantly related to LTBI diagnosis, ATB, and the discrimination between LTBI and ATB. The level of IgG against MIF was significantly lower in LTBI patients than in NI individuals and was significantly related to LTBI diagnosis. Levels of IgA against MIF were significantly lower in AFB-negative TB, minimal TB, and new patients of the ATB group than in the NI group. Both IgA and IgG levels against MIF showed significant negative correlations with IFN-γ levels induced in QFT-GIT test.

Conclusion: Overall, IgG antibody responses to TBCM, Ag85B, and CFP-10 can discriminate among ATB, LTBI, and NI groups. Also, results suggest the possibility of using IgA antibody responses to MIF in the diagnosis of LTBI and ATB.

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Keyword : tuberculosis, serology, diagnosis, biomarkers, macrophage migration inhibitory factor, cytokines

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List of Abbreviations

Abbreviations

TB	Tuberculosis
Mtb	*Mycobacterium tuberculosis*
NTM	Nontuberculous mycobacteria
ESAT-6	Early secreted antigen-6
CFP-10	Culture filtrate protein-10
TBCM	*Mycobacterium tuberculosis* chorismate mutase
IFN-γ	Interferon-gamma
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LTBI	Latent tuberculosis infection
ATB	Active tuberculosis
IGRA	Interferon-gamma release assay
MIF	Macrophage migration inhibitory factor
NI	Non-infected
OD	Optical density

ANOVA	One-way analysis of variance
ROC	Receiver operating characteristics
AUC	Area under the curve
IPTG	Isopropyl β-D-thiogalactoside
DAT	2,3-diacyltrehalose
TAT	2,3,6-triacyltrehalose
LAM	Lipoarabinomannan
TNF-α	Tumor necrosis factor alpha
IL	Interleukin
AFB	Acid-fast bacilli
HIV	Human immunodeficiency virus
PBS	Phosphate-buffered saline
BSA	Bovine serum albumin
RT	Room temperature
ELISA	Enzyme-linked immunosorbent assays
EIA/RIA	Enzyme immunoassay/radio immunoassay
QFT-GIT	QuantiFERON-TB Gold In-Tube
TST	Tuberculin skin test
BCG	Bacillus Calmette-Guérin

Chapter 1. General Introduction

1.1. Study Background

1.1.1. Epidemiology of Tuberculosis

Tuberculosis (TB) remains a major global public health issue. In 2018, 7.0 million new cases of TB were reported worldwide, with an estimated 1.2 million TB deaths among human immunodeficiency virus (HIV)−negative people and an additional 251 000 deaths among HIV−positive people [1]. More specifically, 26,433 new TB cases (51.5 cases per 100,000 people) were reported in Republic of Korea in 2018 [2]. However, since the establishment of the national TB control program in 1962 [3], the number of reported pulmonary TB patients in Republic of Korea has rapidly declined, from 101 per 100,000 people in 1995 to 79 in 2002. Meanwhile, more recently the decline has reportedly slowed due to an increase in the elderly population and the number of immunocompromised patients, as well as frequent outbreaks of TB in group facilities [4].

1.1.2. The importance of early diagnosis of active and latent tuberculosis infection in tuberculosis management

Early diagnosis and treatment of TB is essential to prevent the spread of TB and reduce its prevalence. Moreover, diagnosis and treatment of latent tuberculosis infection (LTBI) as well as active tuberculosis (ATB) is necessary for effective TB management. In Korea, when the prevalence of ATB was quite high, treatment of infectious TB was considered to be more urgent. However, as the rate of TB began to decrease, the importance assigned to detecting and treating LTBI increased in an effort to further lower the incidence of TB. Since 2016, the World Health Organization has launched the End TB Strategy, which strives to reduce the mortality rate associated with TB by 90% and the incidence rate of new cases by 80% by 2030 compared to 2015. It also seeks to eliminate the burden placed on households due to TB. In accordance with this strategy, the Korean government also prepared a TB-safe national plan and implemented a nationwide LTBI infection screening project.

1.1.3. Limitations of current tests for tuberculosis diagnosis

Rapid diagnosis and treatment of TB is essential to contain the disease at an early stage and to lower the prevalence. However, diagnosis and treatment of LTBI infection (LTBI) as well as ATB

are required for effective TB control. Latency and active disease are components of the dynamic TB spectrum [5]. As latent TB, bacilli can become reactivated later to cause active TB. Hence, effective diagnosis and treatment of LTBI is also critical.

However, the currently used tests for TB diagnosis have many limitations. For instance, although initial diagnosis of TB is confirmed by *Mycobacterium tuberculosis* (*Mtb*) culture, the proper responsive measures are often delayed due to the 2–8 week required period for the results [6]. Moreover, approximately 50% of the mycobacterial smears can be negative, and the sensitivity is further reduced in extrapulmonary TB [6]. However, molecular analysis is cost-intensive and requires the latest technology and high-end laboratory instruments, which is not always available to the medical professionals [7].

Moreover, no diagnostic gold standard has been described for LTBI, with all existing tests comprising indirect approaches that provide immunological evidence of host sensitization to TB antigens [8]. Two tests currently used to diagnose LTBI are the tuberculin skin test (TST) and the blood interferon-gamma release assay (IGRA), both of which have many limitations. Specifically, they cannot distinguish between ATB and LTBI [8-10]. In particular, IGRA is not only technically complex and expensive, but also poorly

reproducible [8, 11]. Considering that these tests detect the presence of an immune response to *Mtb*, rather than a directly detecting a small number of *Mtb* organisms in the body, the test can yield positive results even for patients with resolved TB. In fact, Lee et al. reported that 66.1% of patients tested positive for IGRA after six months of treatment in a study in which the IGRA test was repeated during anti-tuberculosis treatment in patients with ATB[12].

In the case of TST, false-positives may occur due to cross-reaction with Bacillus Calmette-Guérin (BCG) strains, and the possibility of false-negatives should be considered in immunocompromised conditions, such as HIV infection.

Unlike TST, the IGRA test has the advantage of having a high specificity for diagnosis of TB infection as it is less affected by BCG vaccination or most nontuberculous mycobacteria (NTM) infections. However, there remains a possibility of cross-reaction with certain NTM species. Moreover, challenges have arisen with being able to effectively reproduce IGRA results as it is affected by several variables during the testing process [8].

1.1.4. Advantages of serological diagnostics

Serological testing, which is performed using blood samples, has become an attractive alternative for the diagnosis of various diseases. This approach is rapid and does not require a lesion site specimen, making point-of-care testing possible. In addition, it is inexpensive and requires little laboratory infrastructure; therefore, it can be conducted even in resource scarce conditions.

1.2. Purpose of Research

The purpose of this study was to develop new biomarkers for the diagnosis of TB. Hence, differentially abundant immunological markers between normal healthy individuals, patients with LTBI, and patients with ATB were investigated.

First, IgA and IgG responses against TBCM, an enzyme essential for the survival of *Mtb*, as well as Ag85B, ESAT-6, and CFP-10 (reported promising antigens) were analyzed. TBCM is a new TB antigen that has not yet had its serologic usefulness described, and is of fundamental value for this study. The serological diagnostic value of this new antigen was compared with existing antigens and evaluated through a combination of results.

Second, the IgA and IgG responses to MIF, a cytokine recently reported to play an important role in controlling inflammation and infection, were analyzed. This is the first study to examine the

antibody responses to MIF in TB patients, and thus, serves as a basis for subsequent studies.

Additionally, since the current standard tests used LTBI diagnosis are not able to effectively distinguish between ATB and LTBI, this study also sought to determine whether new immunological indicators could distinguish these two groups.

Previous studies have reported that the more severe a TB case, the higher the probability of detecting mycobacterium in the sputum mycobacterial smear, and the higher the sensitivity for detecting antibodies via serological testing. However, since not all ATB patients encountered in the clinic have advanced infection, a tool that can more effectively differentiate patients according to the TB spectrum, including those with mild or smear-negative pulmonary TB and LTBI, is needed. However, as described above, data for serological tests in sputum smear-negative TB are currently insufficient. Therefore, this study sought to verify the diagnostic capacity for each group, by subdividing ATB according to imaging severity and sputum smear results.

Chapter 2. Diagnostic Potential of IgG and IgA Responses to *Mycobacterium tuberculosis* Antigens for Discrimination among Active Tuberculosis, Latent Tuberculosis Infection, and Non-Infected Individuals

2.1. Introduction

To develop a diagnostic method that can be more readily distributed in place of microscopic examination, serological assays based on antibody response have been investigated in many studies. Recent studies have suggested the utility of antibody responses to TB antigens for the diagnosis of TB. Legesse et al. reported that IgA levels for early secreted antigen-6 (ESAT-6), culture filtrate protein-10 (CFP-10), and Rv2031 can be used to distinguish patients with pulmonary TB, from those with LTBI, or non-infected (NI) individuals [13]. Other studies have also reported that antibody levels against *Mtb* components are markers for bacterial load and are associated with disease risk [14]. Sireci et al. compared the antibody responses before and after TB treatment, and showed that IgM and IgG to HSP16 in childhood TB significantly decreased after TB treatment [15]. Meanwhile, another study reported that the antibody level against the *Mtb* component serves as an effective marker for bacterial load and is related to disease risk [14]. These results demonstrate that antibody responses to *Mtb*-specific antigens have promising potential for diagnosis of TB, suggesting that TB antigen-specific IgA and IgG can be used to develop accurate and simple ELISA tests for TB diagnosis.

However, the accuracy of serological tests in the diagnosis of pulmonary TB and extrapulmonary TB varies greatly from study to study. In the case of Anti-TB IgG, the most commonly evaluated test, the integrated sensitivity is 76% for smear-positive TB and 59% for smear-negative TB, and the sensitivity of the individual tests is 54-85% for smear-positive TB, and 35-73% for smear-negative TB. In addition, a meta-analysis of 25 extrapulmonary TB studies reported the sensitivity to be 0-100% and the specificity as 59%-100% for each individual study [16]. Similarly, a meta-analysis of studies involving at least 25 pulmonary TB patients and using purified antigens, 38 kDa, MPT51, malate synthase, CFP-10, TbF6, Ag85B, α-crystallin, 2,3-diacyltrehalose, DAT, TAT, cord factor, and TbF6 plus DPEP (multi-antigen) antigens, did not detect sufficient sensitivity to replace sputum smear microscopy. Although protein antigens had higher specificity, and multiple antigens were determined to be more sensitive than single antigens, data from sputum-negative TB and children were insufficient [17].

As various TB antigens have been isolated and purified, ELISA methods have been designed using multiple antigens, which have reported clinical value. However, due to the variability in TB antigen components, antigen production methods, and diversity of

study subjects, large variation was observed in the results between studies.

Nevertheless, antigens play a significant role in serological diagnosis. TB-specific antigens must induce a significant immune response, and the sensitivity and specificity for their detection is increased in the absence of cross-reactions. However, due to the complexity of *Mtb* antigen components, cross-reaction can occur with other strains. Therefore, efforts must be made to purely isolate and assay antigenic components specific to *Mtb*. Accordingly, new TB antigens are constantly being developed, and various studies for diagnosis of TB, as well as the development of therapeutic drugs and vaccines, are being undertaken.

Among the previously studied antigens, ESAT-6 and CFP-10 are TB-specific secreted proteins encoded by the RD1 gene of *Mtb* which together form a 1:1 complex structure [18]. Importantly, these antigens are not present in Bacillus Calmette-Guérin strains or in most NTM species [19]. Moreover, ESAT-6 has been identified as a promising component for vaccine development with regard to human T cell recognition and protective efficacy [20, 21].

Similarly, Ag85B has been investigated as a major antigen in candidate vaccines due to its adaptability and ability to induce CD4 and CD8 T lymphocyte responses in a wide range of vertebrate

hosts [21]. Ag85B is a secreted protein of TB and has been considered a potential drug target for TB treatment due to its enzymatic activity as a mycolyl transferase, as well as its importance in the construction of the mycobacterial envelope [22]. Ag85B is highly immunogenic, resulting in specific humoral and cell-mediated immune responses in both LTBI and ATB patients, and has been shown to induce partial protection in murine models of infection [23, 24].

In recent studies exploring potential targets for new anti-tubercular agents, chorismate mutase, found in *Mtb*, has been described as a key regulator of amino acid biosynthesis [25]. *Mycobacterium tuberculosis* chorismate mutase (TBCM) converts chorismate to prephenate to form the essential amino acids phenylalanine and tyrosine in the shikimate biosynthetic pathway, which plays an important role in the survival and pathophysiology of *Mtb*. Therefore, TBCM is considered a promising target for potential anti-tubercular agents that inhibit this pathway [25]. However, to date, no studies have investigated antibody responses to TBCM in TB patients.

Ag85B, ESAT-6, and CFP-10 have been evaluated for serological usefulness in several studies, however, have elicited variable results for diagnostic accuracy. These inconsistent results

may be due to the purity of the antigen, optimization of the ELISA, and differences in characteristics between study groups. In previous studies, chromatographic methods were primarily used for antigen purification. Among immunoglobulins, the results for IgA were relatively scarce, whereas those for IgG were mainly used. In addition, there were many cases where the sample size was small, or a separate latent TB group was not included. In particular, results confirming the usefulness of the differential diagnosis between LTBI and ATB were rare. In a previous study, the sensitivity and specificity of IgG responses to various antigens, including ESAT-6 and CFP-10, were investigated among three groups of ATB patients, LTBI patients, and healthy individuals. However, the sensitivity of differential diagnosis between LTBI and ATB was not as high as 50-60% for all antigens [26].

This study compared the serological diagnostic value of a new antigen (TBCM) with existing antigens (Ag85B, ESAT-6, and CFP-10) and evaluated them through a combination of results. Because no test method exists to distinguish between LTBI and ATB so far, the study subjects were evaluated by dividing them into ATB, LTBI, and normal groups. In addition, this study attempted to evaluate the IgA response, which is relatively insufficient in the existing research results, as well as the IgG response.

2.2. Materials and Methods

2.2.1. Participants and Clinical Samples

The inclusion criteria allowed for men and women over 19 years of age, diagnosed with ATB or who were selected for LTBI examination (direct contact with ATB persons, health-care workers, nursery workers, or welfare facility workers) at the National Medical Center, Seoul, Republic of Korea. Individuals under the age of 19 were excluded. Five milliliters of peripheral blood was collected in plain tubes and centrifuged to separate the serum within 24 h of collection. Subsequently, the tubes were stored at −70 ° C until further analyses.

ATB was confirmed in patients with sputum or bronchoscopy specimens that were positive for either mycobacterial culture or the TB nucleic acid amplification test. The subjects selected for LTBI examination were tested with the QuantiFERON-TB Gold In-Tube (QFT-GIT) assay. Patients with a positive test result in the absence of clinical symptoms and chest radiographic abnormalities were classified as LTBI, and those with a negative test result were classified as non-infected (NI). Clinical and laboratory data were

collected during baseline examination and at the time of usual hospital visits.

2.2.2. Preparation of *Mtb* Antigens

Recombinant Ag85B, CFP10, ESAT-6, and TBCM proteins were purified from *Escherichia coli* as previously described [27, 28]. Briefly, *E. coli* BL21 strains (RBC Bioscience, Taipei City, Taiwan) were transformed with pET28a-Ag85B, pET28a-CFP10, pET28a-ESAT-6, or pET28a-TBCM for the expression and purification of each fusion protein. Protein expression was induced by adding 0.4 mM isopropyl β-D-thiogalactoside (IPTG, Duchefa Biochemie, Haarlem, Netherlands). Cultured bacterial cells were disrupted by sonication (10 min, 4 ° C), and the resultant lysates were centrifuged (1,600 × *g*, 20 min, 4 ° C). The pellets containing each protein (Ag85B, CFP-10, ESAT-6, or TBCM) were resuspended in binding buffer containing 4 M urea (Sigma Aldrich, St. Louis, MO, USA). Each protein was purified with Ni-NTA His binding resin (Merck, Darmstadt, Germany) and eluted with elution buffer (300 mM NaCl, 50 mM sodium phosphate buffer, and 250 mM imidazole) containing 4 M urea. Purified proteins were dialyzed serially against the elution buffer to remove imidazole, urea, and residual salts.

2.2.3. Antibody Enzyme-Linked Immunosorbent Assays (ELISA)

Serum levels of antibody isotypes IgA and IgG against TBCM, ESAT-6, Ag85B, and CFP-10 were measured using enzyme-linked immunosorbent assays (ELISA). Corning 96-well Enzyme Immunoassay/Radio Immunoassay (EIA/RIA) plates (Corning Inc., Kennebunk, ME, USA) were coated with TBCM (5 μg/mL), ESAT-6 (5 μg/mL), Ag85B (5 μg/mL), or CFP-10 (5 μg/mL), diluted in 0.05 M carbonate-bicarbonate coating buffer and incubated overnight at 4 °C. Plates were washed three times with PBST (phosphate-buffered saline (PBS) containing 0.05% tween 20) and blocked with PBS containing 5% bovine serum albumin (BSA) for 1 h at room temperature (RT). After washing, 100 μL of sample diluted 1:10 in PBS was added to each well, and plates were incubated at RT for 2 h. After washing, 100 μL of anti-human IgG or IgA HRP-conjugated secondary antibody (IgG: Promega, W4038, Madison, WI, USA; IgA: Invitrogen, PA1-74395, Rockford, IL, USA) diluted at 1:500 in 5% BSA was added to each well of the respective plates. Plates were incubated at RT for 1 h, and after washing, 100 μL of 3, 3′5, 5′-tetramethylbenzidine substrate reagent (BD OptEIA substrate; BD Biosciences, San Diego, CA, USA) was added to each well. After 10

min, the reaction was stopped with 1 N sulfuric acid (50 μL) and analyzed at 450 nm. Optical density (OD) values were used for analysis.

2.2.4. Data Analysis

Antibody levels were presented as OD values, and one-way ANOVA with Bonferroni's multiple comparisons were used to compare antibody responses among ATB, LTBI, and NI groups. Correlations between the OD values of IgG or IgA and the level of interferon-gamma (IFN-γ) were assessed using Pearson's correlation coefficient. P values less than 0.05 were considered statistically significant. SPSS version 17.0 (SPSS Institute, Inc., Chicago, IL, USA) and GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla California, USA, http://www.graphpad.com) were used for data analyses. For multivariate analysis, logistic regression with the backward elimination method was performed to predict LTBI or ATB. The area under the curve (AUC) of the receiver operating characteristics (ROC) curve was calculated to compare the predictive power of each model using the ROCR package in R (version 3.6.0). To generate the decision tree for the prediction of diseases, the tree package was used. Tree is a nonparametric statistical procedure containing

classification using a set of if-then-else logical conditions to assign unknown features to predefined categories. Algorithms to construct trees work from the top down, by choosing a variable at each step that best separates the set of items. Training and test sets were divided at a 7:3 ratio for cross validation, and the number of pruning nodes was selected by K-fold cross validation.

2.2.5. Ethics Statement

All subjects provided written, informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the institutional review board of National Medical Center (IRB no. H-1811-096-002) and Seoul National University Hospital (H-2006-090-1132).

2.3. Results

2.3.1. Characteristics of the Study Participants

Between May 11, 2017 and Jan 13, 2019, a total of 180 patients were enrolled at the National Medical Center, of which 65 were assigned to the ATB group, 53 to the LTBI group, and 62 to the NI group. The baseline characteristics of the participants are summarized in Table 2-1. Age and sex differed significantly between the three groups. Age was highest in the ATB group and lowest in the NI group ($p < 0.0001$). The proportion of males in the ATB group was highest ($p < 0.0001$). None of the subjects in the NI and LTBI groups had a previous history of TB.

Table 2-1. Demographic and clinical characteristics of the study population

	NI (n = 62)	LTBI (n = 53)	ATB (n = 65)
Demographics			
Age (years), median (IQR)	33 (29-33)	49 (33-57)	60 (51-67)
Sex			
Male	21 (33.9%)	21 (39.6%)	56 (86.2%)
Female	41 (66.1%)	32 (60.4%)	9 (13.8%)
Body-mass index (kg/m^2), median (IQR)	22.4 (20.2-24.9)	23.7 (21.4-26.7)	20 (17.0-21.7)
Comorbidities			
Diabetes	3 (4.8%)	6 (11.3%)	14 (21.5%)
Chronic alcoholics	0 (0%)	1 (1.9%)	9 (13.8%)
Cancer	0 (0%)	5 (9.4%)	4 (6.2%)
Liver disease	0 (0%)	4 (7.5%)	7 (10.8%)
Chronic kidney disease	0 (0%)	2 (3.8%)	2 (3.1%)
Heart disease	0 (0%)	3 (5.7%)	3 (4.6%)
Previous history of TB treatment			
New patients	n/a	n/a	34 (52.3%)
Retreatment	n/a	n/a	27 (41.5%)
Unknown previous TB treatment history	n/a	n/a	4 (6.2%)
Bacteriological examinations			

Acid-fast staining of sputum	n/a	n/a	
Negative			31 (47.7%)
1+			16 (24.6%)
2+			7 (10.8%)
3+			5 (7.7%)
4+			6 (9.2%)
Positive *Mtb* culture of sputum			
Liquid media	n/a	n/a	46 (70.8%)
Solid media	n/a	n/a	39 (60.0%)
Positive Xpert MTB/RIF assay	n/a	n/a	59 (90.8%)
Drug resistance	n/a	n/a	14 (21.5%)
Radiographical examinations			
Normal	61 (98.4%)	51 (96.2%)	n/a
Previously healed TB*	1 (1.6%)	2 (3.8%)	n/a
Radiologic severity of ATB			
Minimal	n/a	n/a	25 (38.5%)
Advanced	n/a	n/a	40 (61.5%)
Presence of cavity	n/a	n/a	31 (47.7%)

IQR: interquartile range; NI, non-infected; LTBI, latent tuberculosis infection; ATB, active tuberculosis; TB, tuberculosis; *Mtb*, *Mycobacterium tuberculosis*; n/a, not applicable. *Radiographic lesions suggesting TB sequelae without clinical or microbiological evidence of active pulmonary TB

2.3.2. Serum Levels of IgG and IgA Against TBCM, Ag85B, ESAT-6, and CFP-10

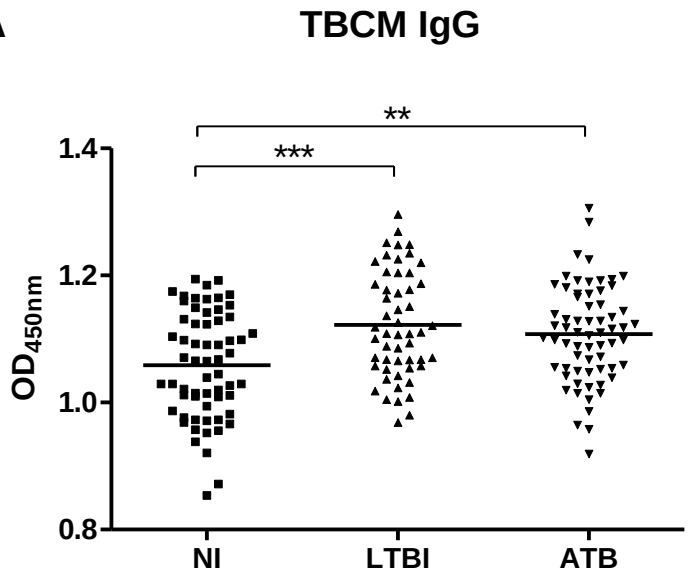
Figure 2-1 shows the IgG and IgA responses to TBCM, Ag85B, ESAT-6, and CFP-10 in each participant group. IgG levels against TBCM were significantly higher in LTBI ($p < 0.0001$) and ATB groups ($p = 0.004$) than in the NI group. No significant difference was observed between LTBI and ATB individuals (Figure 2-1A). IgA levels against TBCM were significantly higher in the ATB group than in the NI ($p < 0.0001$) and LTBI groups ($p = 0.047$). No significant difference was observed between the NI and LTBI groups (Figure 2-1B).

The levels of both IgG and IgA against Ag85B were significantly higher in the LTBI ($p = 0.005$, $p = 0.022$, respectively) and ATB ($p < 0.0001$ in both cases) groups than the NI group, as well as in the ATB group compared to those in the LTBI group ($p < 0.0001$, $p = 0.002$, respectively; Figure 2-1C, 2-1D). The levels of IgG against ESAT-6 were not significantly different among NI, LTBI, and ATB groups (Figure 2-1E). The levels of IgA against ESAT-6 were significantly higher in the ATB group than in the NI group ($p = 0.001$). There was no significant difference in levels between the

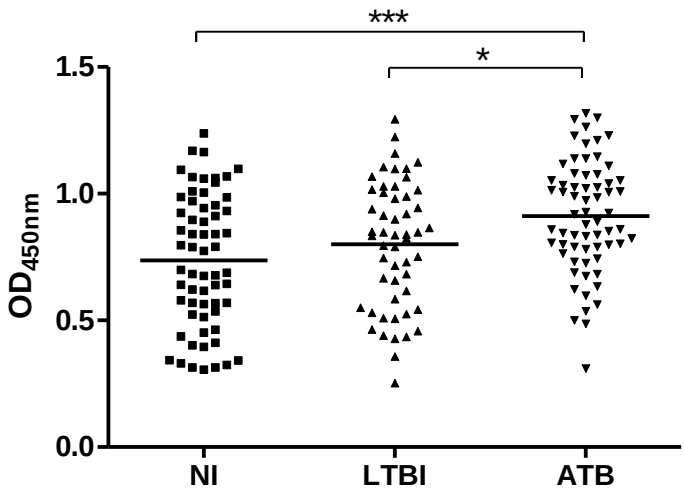
NI and LTBI groups, or between the LTBI and ATB groups (Figure 2-1F).

The IgG levels against CFP-10 were significantly higher in LTBI (p = 0.010) and ATB (p < 0.001) groups than in the NI group, as well as in the ATB group (p < 0.0001) compared to the LTBI group (Figure 2-1G). The IgA levels against CFP-10 were significantly higher in the ATB group than in the NI group (p = 0.001). There were no significant differences in CFP-10 IgA levels between the NI and LTBI groups, or between the LTBI and ATB groups (Figure 2-1H).

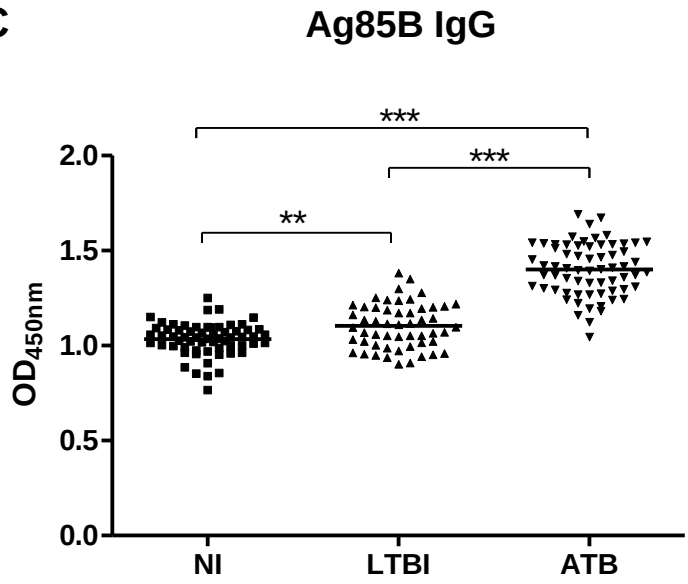
A


TBCM IgG
**
***
1.4
1.2
1.0
0.8
OD450nm
NI
LTBI
ATB

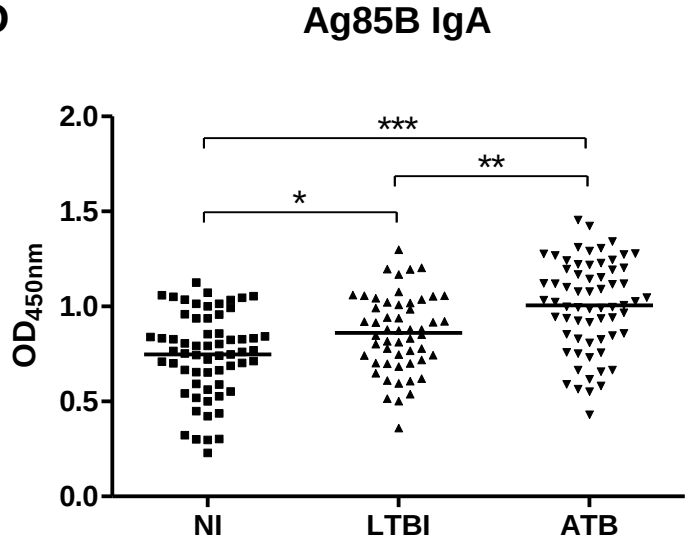
B


TBCM IgA
***
*
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
ATB

C


Ag85B IgG
***
***
**
2.0
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
ATB

D


Ag85B IgA
***
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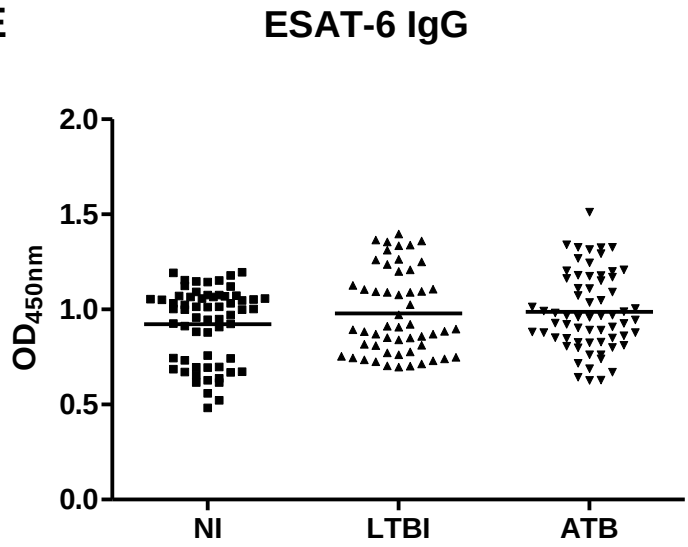
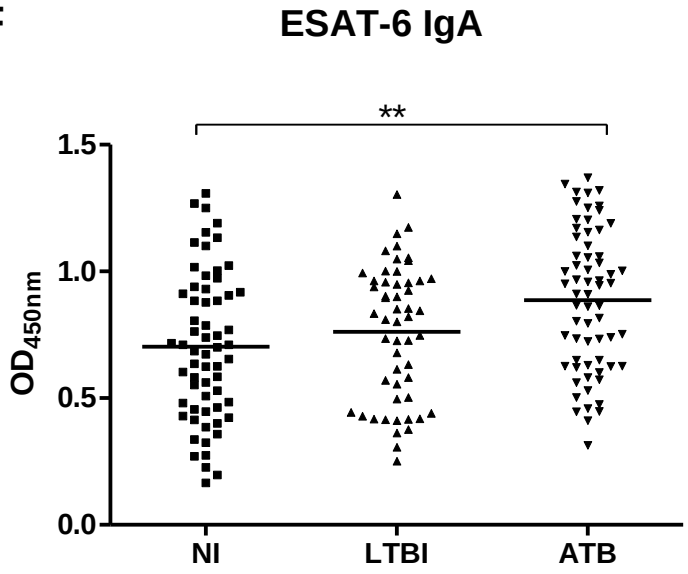
*
2.0
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
ATB

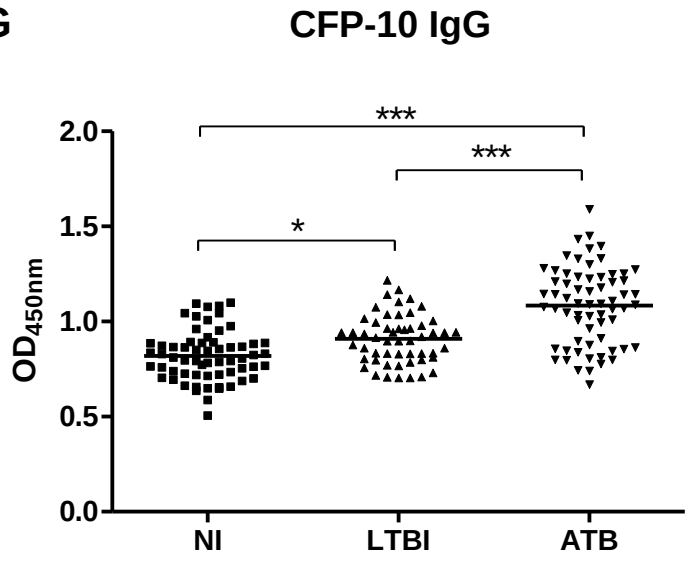
E


ESAT-6 IgG
2.0
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
ATB

F


ESAT-6 IgA
**
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
ATB

G


CFP-10 IgG
***
***
*
2.0
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
ATB

H

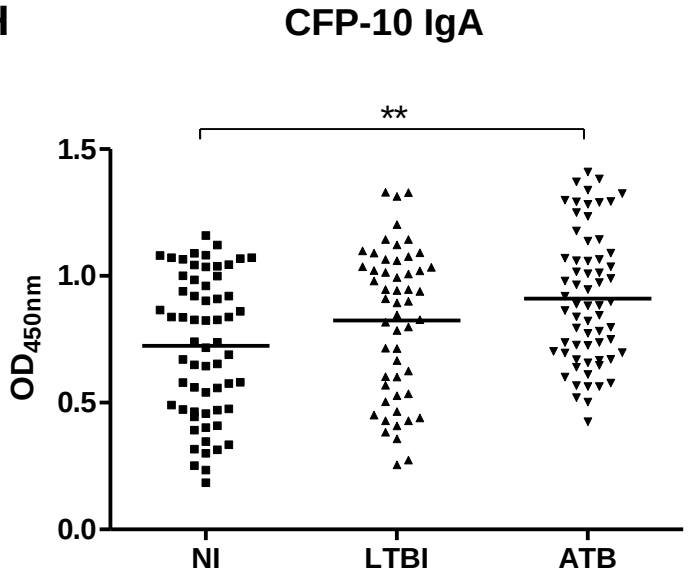

CFP-10 IgA
**
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
ATB

Figure 2-1. IgG and IgA responses to TB antigens.

The optical density (OD) values of the anti-TBCM (*Mycobacterium tuberculosis* chorismate mutase) immunoglobulin G (IgG) (**A**), anti-TBCM immunoglobulin A (IgA) (**B**), anti-antigen 85B (Ag85B) IgG (**C**), anti-Ag85B IgA (**D**), anti-ESAT-6 (early secreted antigen-6) IgG (**E**), anti-ESAT-6 IgA (**F**), anti-CFP-10 (culture filtrate protein-10) IgG (**G**), and anti-CFP-10 IgA (**H**) serodiagnosis assays for the comparison of 65 active tuberculosis (ATB), 53 latent tuberculosis infection (LTBI), and 62 non-infected individuals (NI). Each dot represents the values obtained from individual subjects, and horizontal bars indicate the average values. Groups were compared by one-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

2.3.3. Serum Levels of IgG and IgA in Subgroup Analysis

Figure 2-2 shows the IgG and IgA responses to each antigen when ATB patients were classified into acid-fast bacilli (AFB)-negative and AFB-positive TB groups. The levels of IgG against Ag85B and CFP-10 showed significant differences when comparing subgroups as follows: NI versus AFB-negative TB ($p < 0.001$), NI versus AFB-positive TB ($p < 0.001$), LTBI versus AFB-negative TB ($p < 0.001$), LTBI versus AFB-negative TB ($p < 0.001$), and LTBI versus AFB-negative TB ($p < 0.001$). Levels increased from NI to LTBI to AFB-negative and finally AFB-positive TB (Figure 2-2C, 2-2G). The IgA levels against ESAT-6 and CFP-10 were significantly higher in the AFB-positive TB group than in the NI group, however, there was no significant difference between the NI and AFB-negative TB groups (Figure 2-2F, 2-2H).

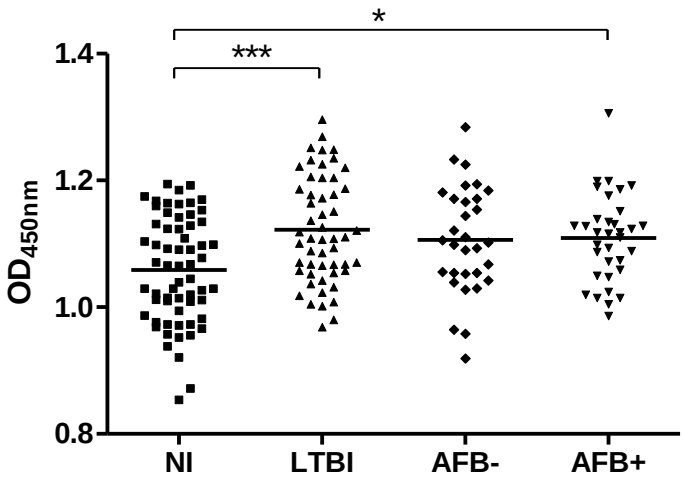
Similarly, when ATB patients were classified into minimal and advanced TB groups according to their radiological severity, the levels of IgG against Ag85B and CFP-10 showed significant differences between most groups as follows: NI versus minimal ($p < 0.001$), NI versus advanced ($p < 0.001$), LTBI versus minimal ($p < 0.001$), and LTBI versus advanced ($p < 0.001$). The values tended

to increase from NI, LTBI, minimal, and finally to advanced TB (Figure 2-3C, 2-3G). The levels of IgA against Ag85B were also significantly higher in the minimal TB group ($p < 0.001$) and the advanced TB group ($p < 0.001$) than in the NI group, and were significantly higher in the advanced TB group compared to the LTBI group ($p = 0.008$), however, there was no significant difference between the LTBI and minimal TB groups (Figure 2-3D). The levels of IgA against ESAT-6 and CFP-10 were significantly higher in the advanced TB group than in the NI group, however, there was no significant difference between the NI and minimal TB groups (Figure 2-3F, 2-3H).

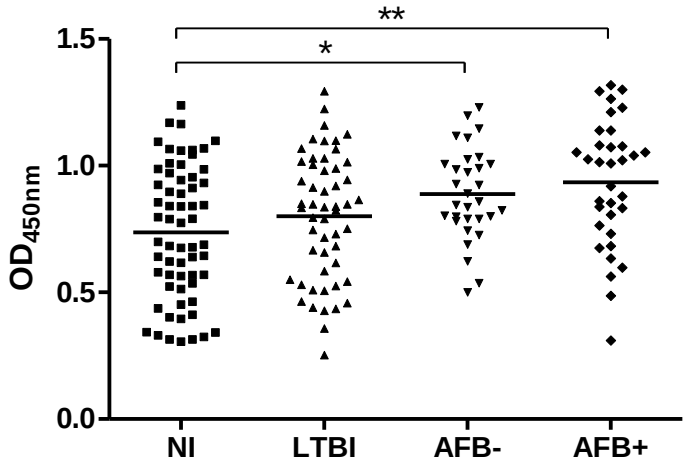
Figure 2-4 shows the IgG and IgA responses to each antigen when ATB patients were divided into new patients and retreatment groups. For the levels of IgG against Ag85B and CFP-10, no significant difference was observed between the new patient and retreatment groups, however, there were significant differences between all other groups as follows: NI versus new patient ($p < 0.001$), NI versus retreatment ($p < 0.001$), LTBI versus new patient ($p < 0.001$), and LTBI versus retreatment ($p < 0.001$). Furthermore, the antibody levels tended to increase in the following order: NI, LTBI, new patient, and retreatment (Figure 2-4C, 2-4G). IgA levels for TBCM and CFP-10 were significantly higher in new

patients (p = 0.031) and retreatment (p < 0.001) groups than in the NI group. There were no significant differences between the LTBI group and the new patients or retreatment groups, or between the new patients and retreatment groups (Figure 2-4B, 2-4H). There was also no significant difference in AFB positivity, radiological severity, or comorbidities between the new patient and the retreatment groups.

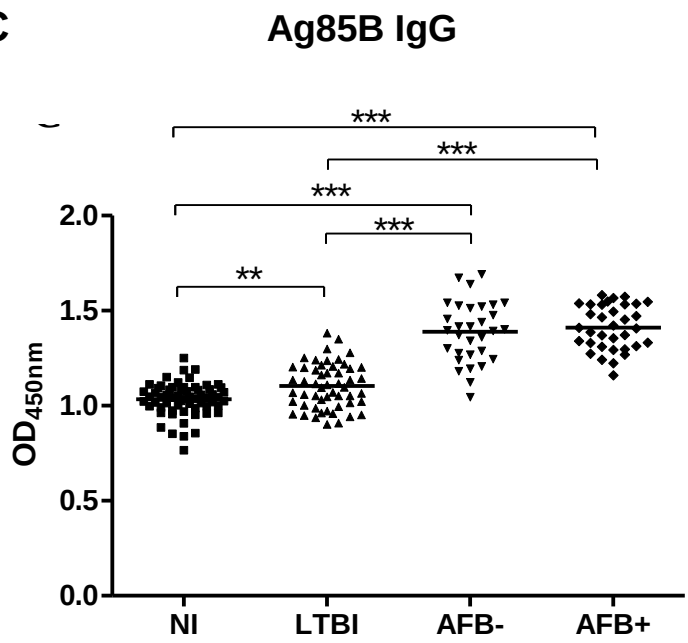
A


TBCM IgG
*
***
1.4
1.2
1.0
0.8
OD450nm
NI
LTBI
AFB-
AFB+

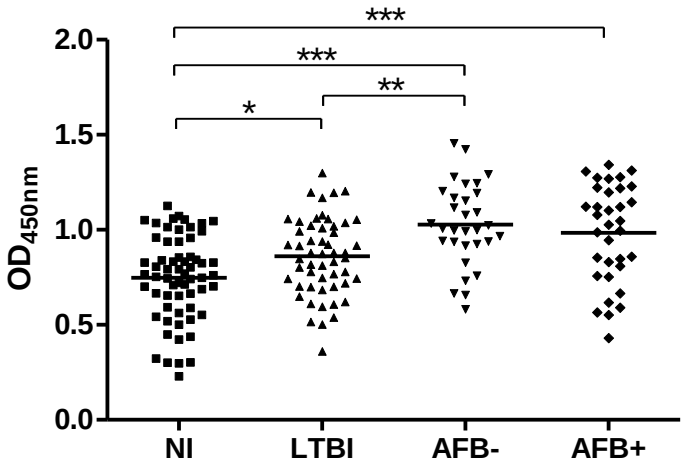
B


TBCM IgA
**
*
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
AFB-
AFB+

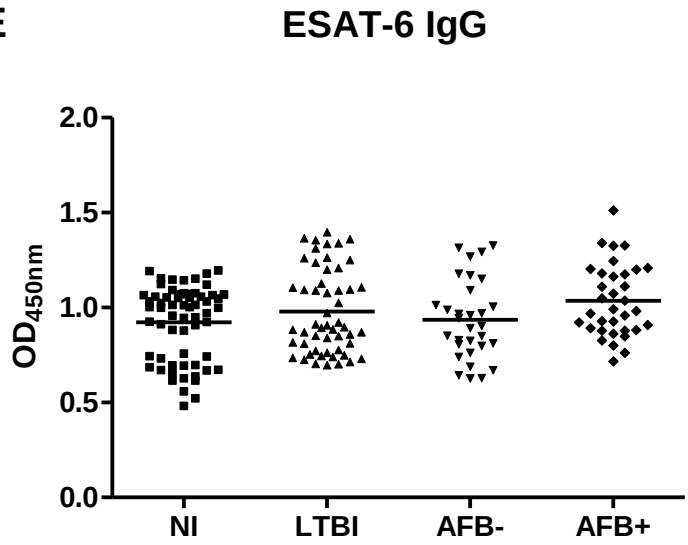
C


Ag85B IgG
***
***
***
***
**
2.0
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
AFB-
AFB+

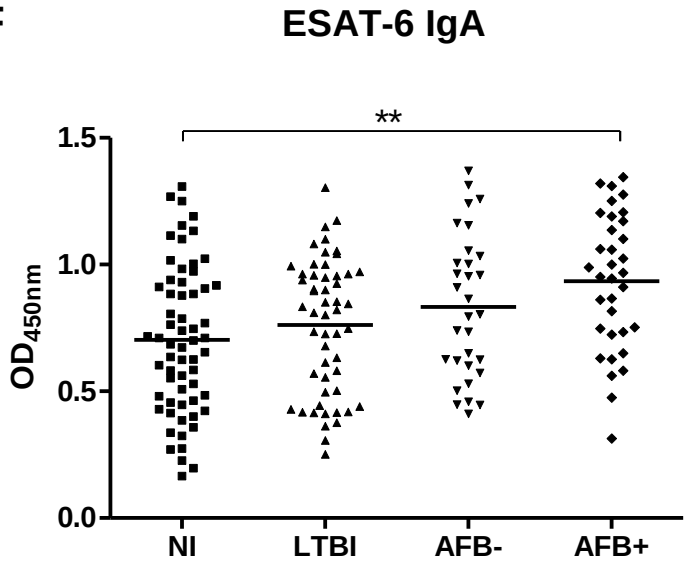
D


Ag85B IgA
***
***
**
*
2.0
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
AFB-
AFB+

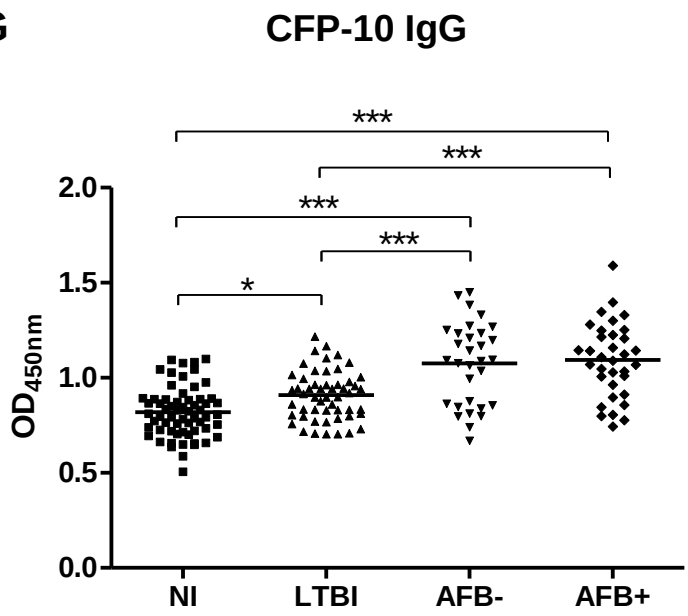
E


ESAT-6 IgG
2.0
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
AFB-
AFB+

F


ESAT-6 IgA
**
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
AFB-
AFB+

G


CFP-10 IgG
***
***
***
***
*
2.0
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
AFB-
AFB+

H

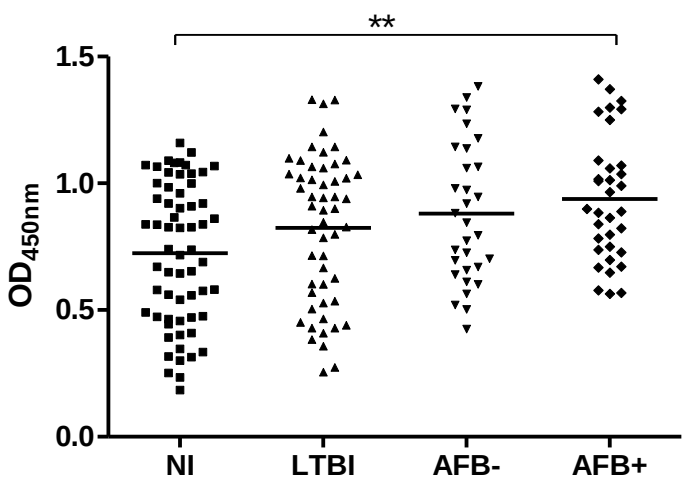
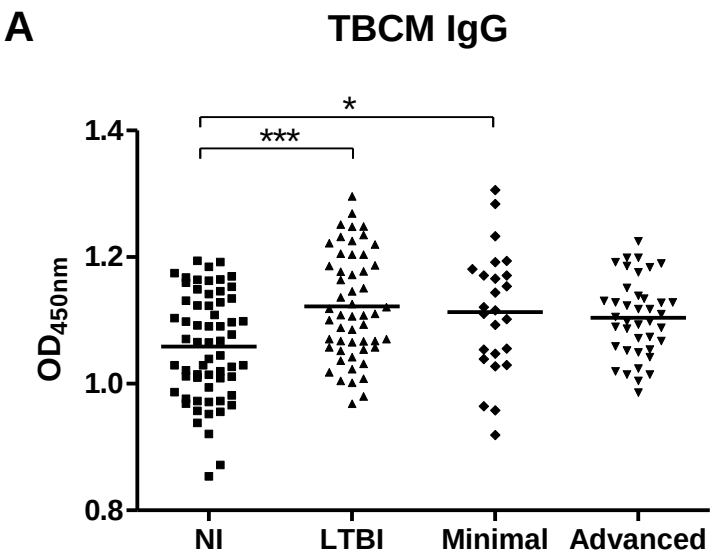
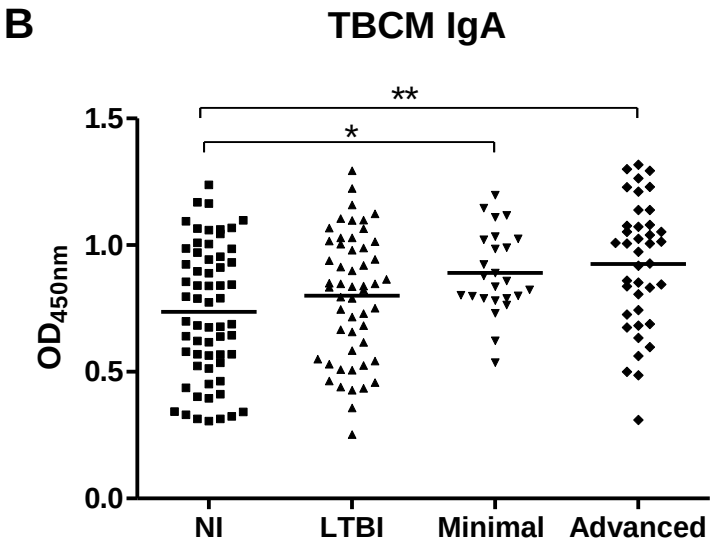

CFP-10 IgA
**
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
AFB-
AFB+

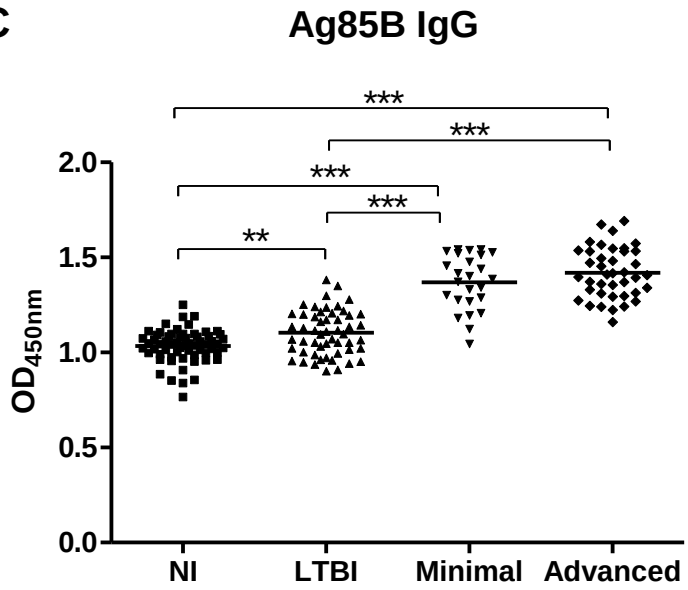
Figure 2-2. IgG and IgA responses to TB antigens in subgroups according to AFB smear results.

Optical density (OD) values of the anti-TBCM IgG (**A**), anti-TBCM IgA (**B**), anti-Ag85B IgG (**C**), anti-Ag85B IgA (**D**), anti-ESAT-6 IgG (**E**), anti-ESAT-6 IgA (**F**), anti-CFP-10 IgG (**G**), and anti-CFP-10 IgA (**H**) in serodiagnosis assays for the comparison of 31 acid-fast bacilli (AFB)-negative tuberculosis (AFB-), 34 AFB-positive tuberculosis (AFB+), 53 latent tuberculosis infection patients (LTBI), and 62 non-infected individuals (NI). Each dot represents the values obtained from individual subjects, and horizontal bars indicate the average values. Groups were compared by one-way ANOVA with Bonferroni's multiple comparisons. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

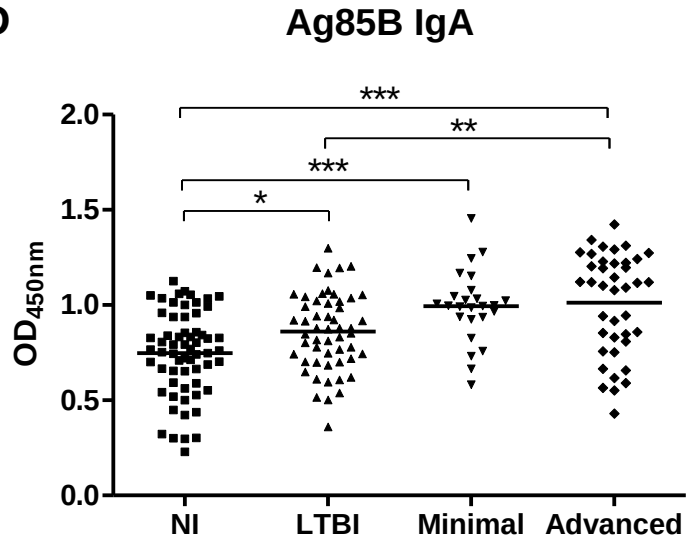

A
TBCM IgG
*
***
1.4
1.2
1.0
0.8
OD450nm
NI
LTBI
Minimal
Advanced


B
TBCM IgA
**
*
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
Minimal
Advanced

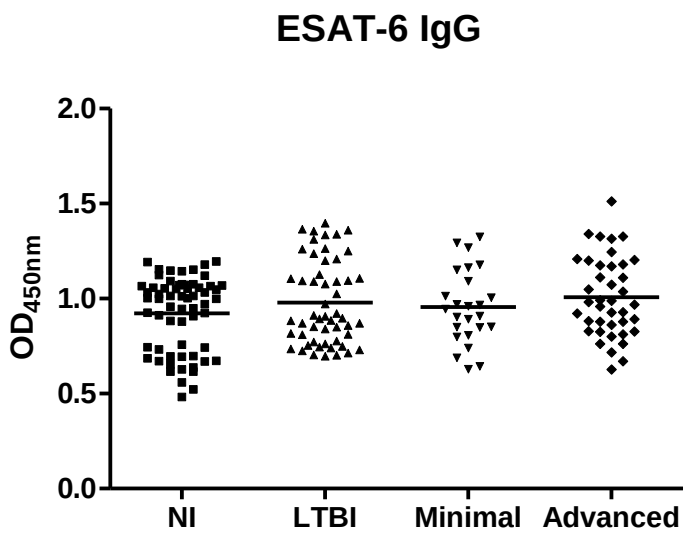
C


Ag85B IgG
***
***
***
***
**
2.0
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
Minimal
Advanced

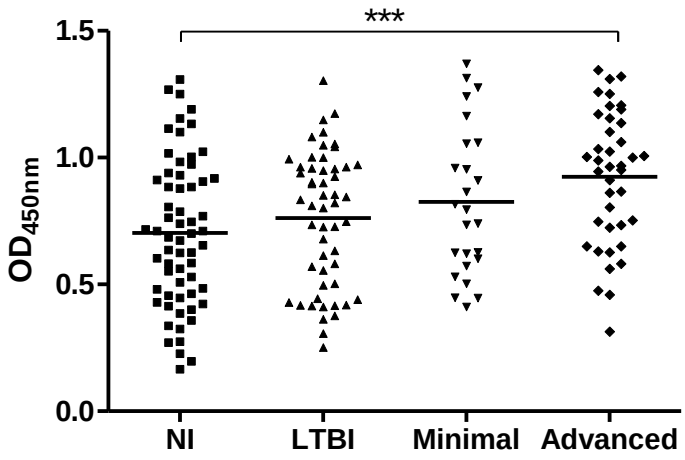
D


Ag85B IgA
***
**
***
*
2.0
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
Minimal
Advanced

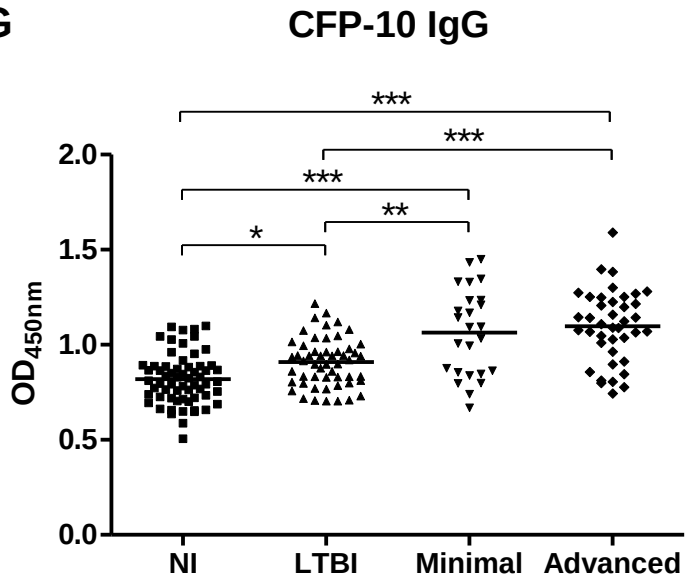
E


ESAT-6 IgG
2.0
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
Minimal
Advanced

F


ESAT-6 IgA
***
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
Minimal
Advanced

G


CFP-10 IgG
***
***
***
**
*
2.0
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
Minimal
Advanced

H

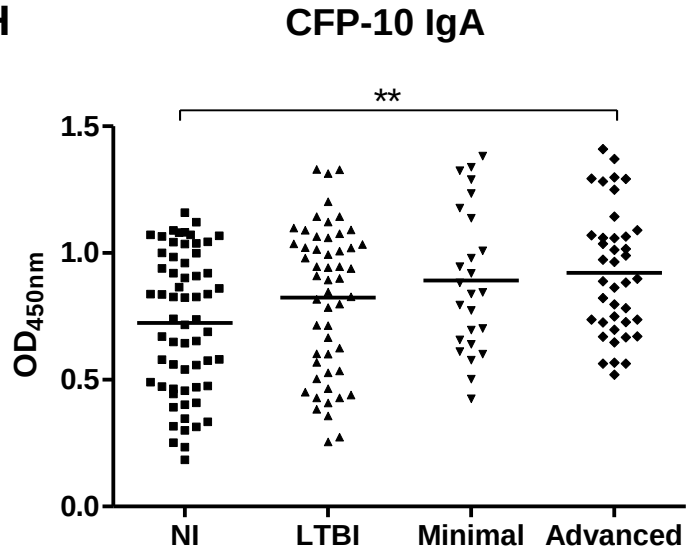
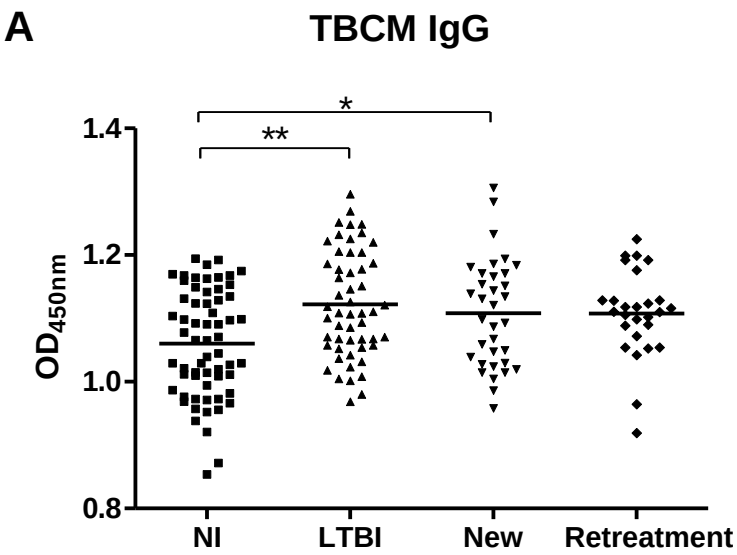
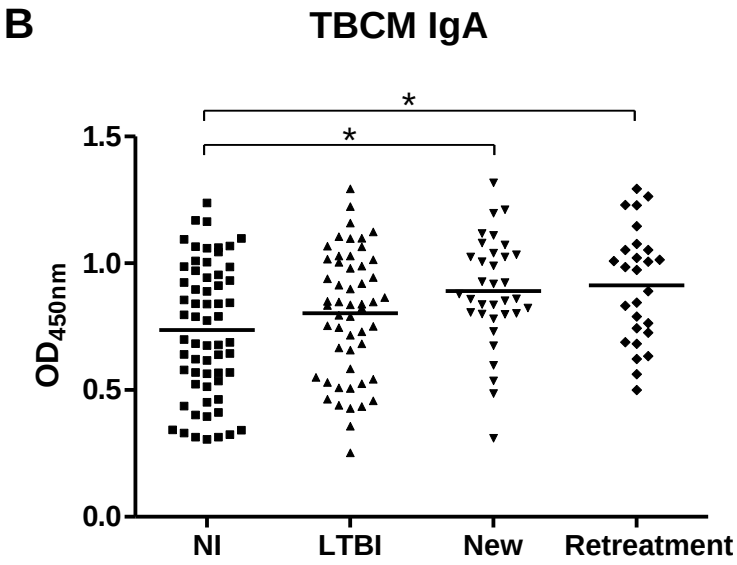

CFP-10 IgA
**
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
Minimal
Advanced

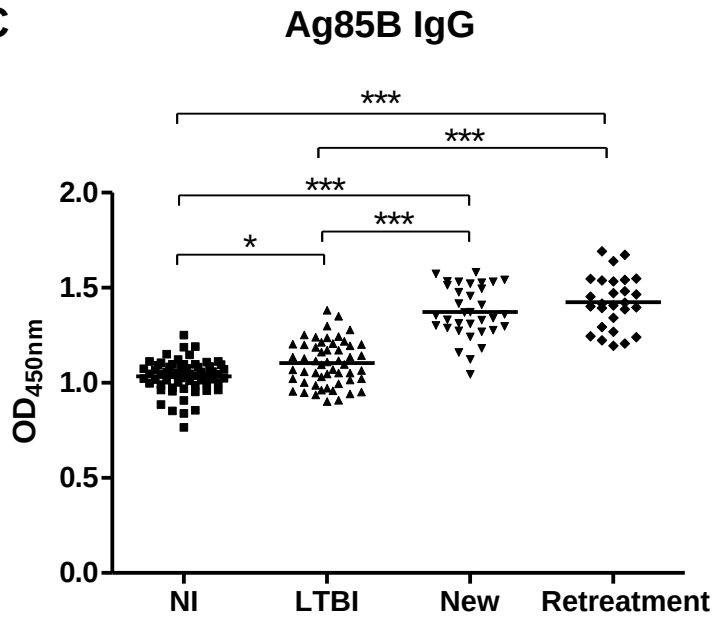
Figure 2-3. IgG and IgA responses to TB antigens in subgroups according to radiological severity.

Optical density (OD) values of the anti-TBCM IgG (**A**), anti-TBCM IgA (**B**), anti-Ag85B IgG (**C**), anti-Ag85B IgA (**D**), anti-ESAT-6 IgG (**E**), anti-ESAT-6 IgA (**F**), anti-CFP-10 IgG (**G**), and anti-CFP-10 IgA (**H**) in serodiagnosis assays for the comparison of 25 minimal tuberculosis, 40 advanced tuberculosis, 53 latent tuberculosis infection patients (LTBI), and 62 non-infected individuals (NI). Each dot represents the values obtained from individual subjects, and horizontal bars indicate the average values. Groups were compared by one-way ANOVA with Bonferroni's multiple comparisons. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

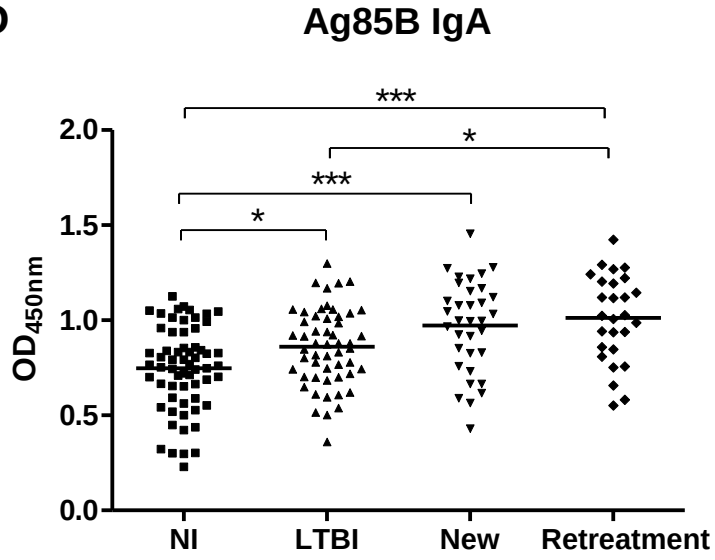

A
TBCM IgG
*
**
1.4
1.2
1.0
0.8
OD450nm
NI
LTBI
New
Retreatment

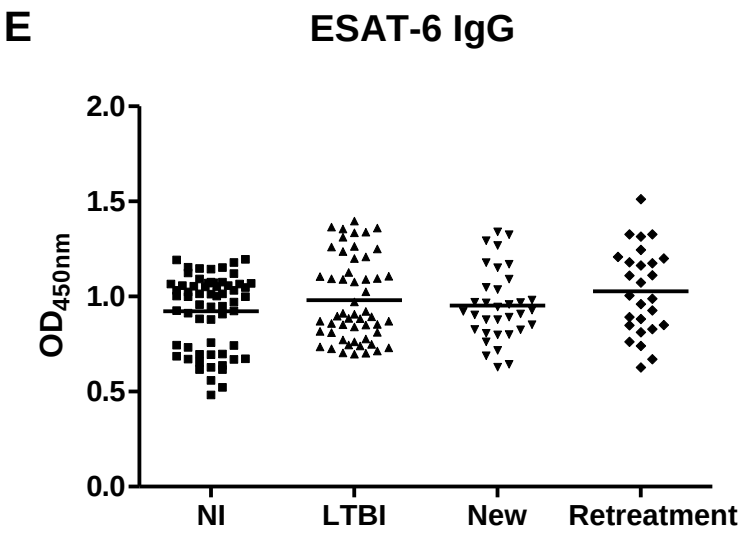

B
TBCM IgA
*
*
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
New
Retreatment

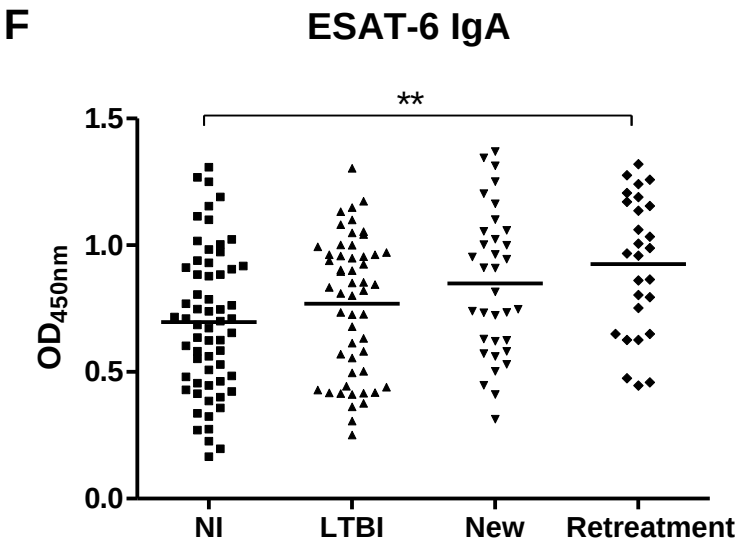
C


Ag85B IgG
***
***
***
***
*
2.0
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
New
Retreatment

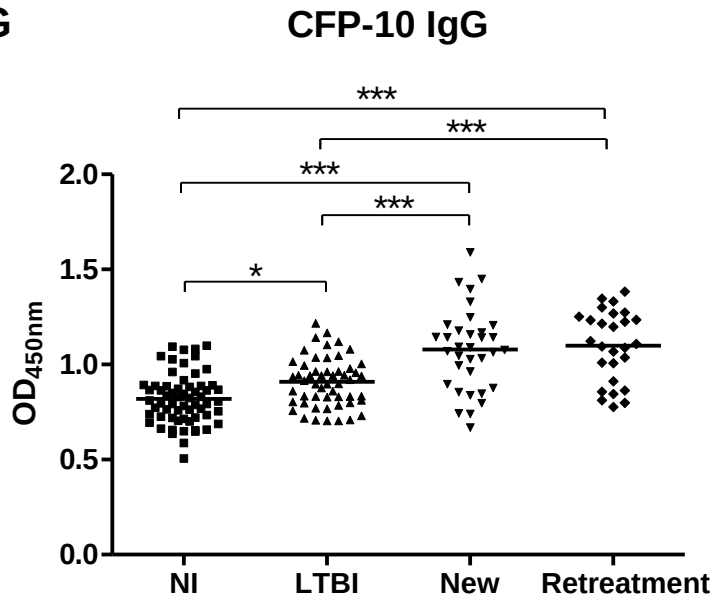
D


Ag85B IgA
***
*
***
*
2.0
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
New
Retreatment


E
ESAT-6 IgG
2.0
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
New
Retreatment


F
ESAT-6 IgA
**
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
New
Retreatment

G


CFP-10 IgG
***
***
***
***
*
2.0
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
New
Retreatment

H

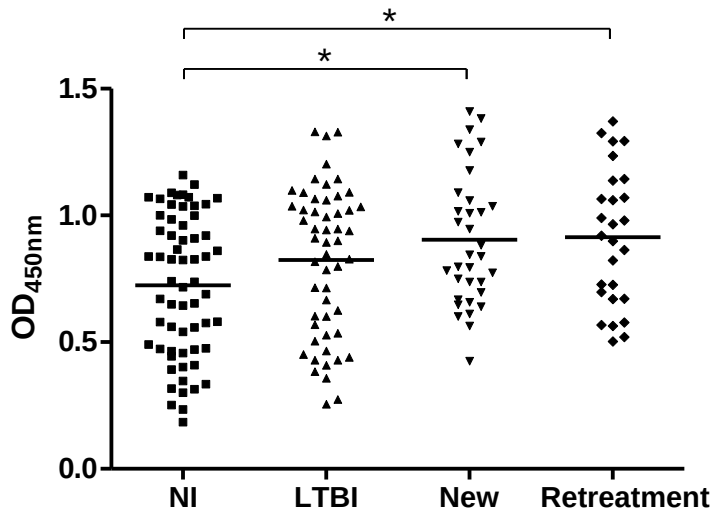
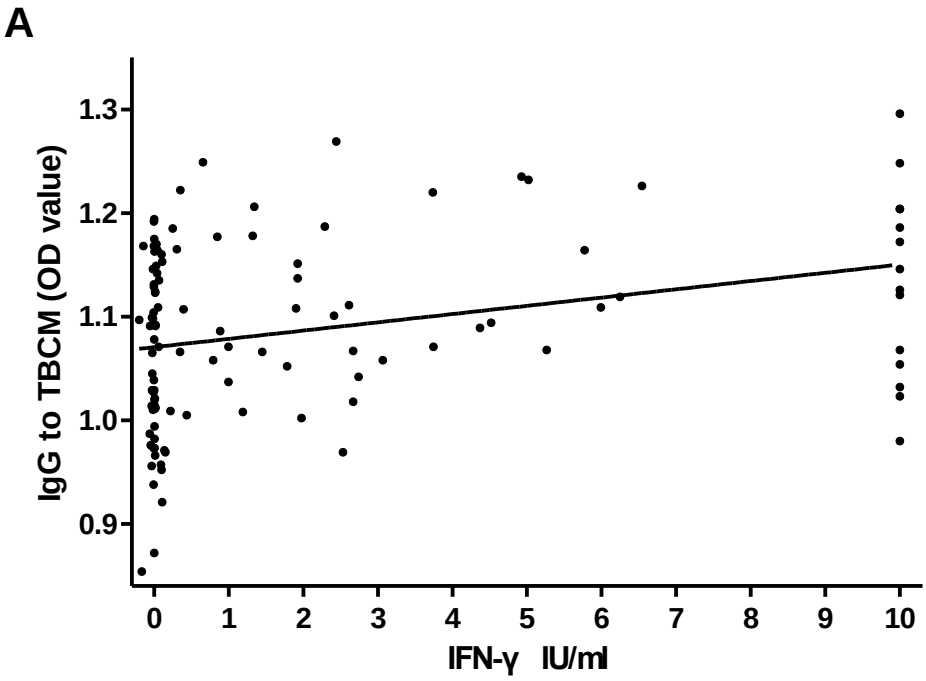

CFP-10 IgA
*
*
1.5
1.0
0.5
0.0
OD450nm
NI
LTBI
New
Retreatment

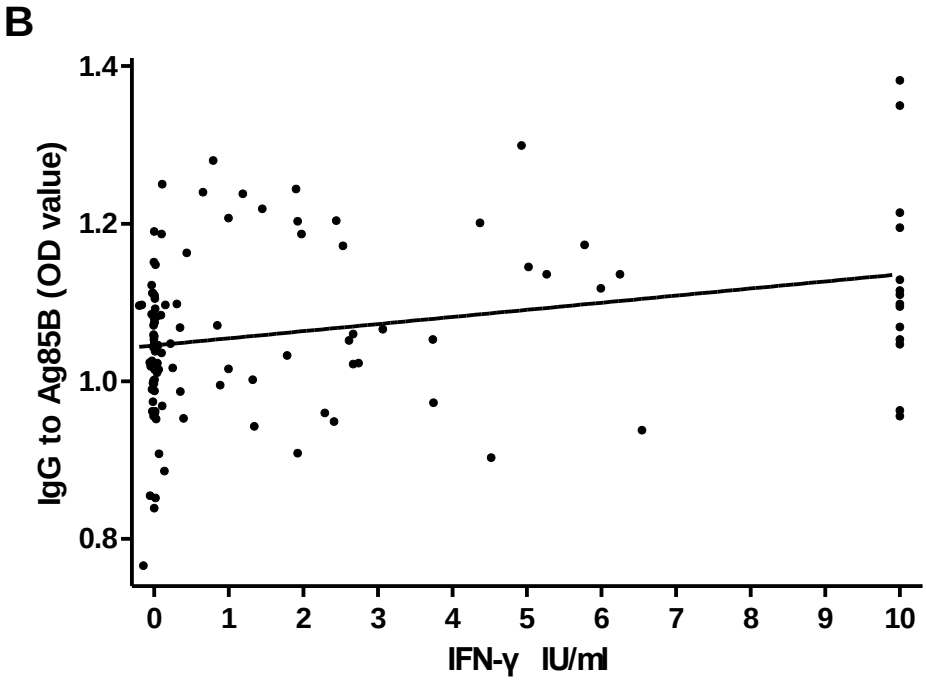
Figure 2-4. IgG and IgA responses to TB antigens in subgroups according to past TB treatment history.

Optical density (OD) values of the anti-TBCM IgG (**A**), anti-TBCM IgA (**B**), anti-Ag85B IgG (**C**), anti-Ag85B IgA (**D**), anti-ESAT-6 IgG (**E**), anti-ESAT-6 IgA (**F**), anti-CFP-10 IgG (**G**), and anti-CFP-10 IgA (**H**) in serodiagnosis assays for the comparison of 34 new patients, 27 retreatment patients, 53 latent tuberculosis infection patients (LTBI), and 62 non-infected individuals (NI). Each dot represents the values obtained from individual subjects, and horizontal bars indicate the average values. Groups were compared by one-way ANOVA with Bonferroni's multiple comparisons. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

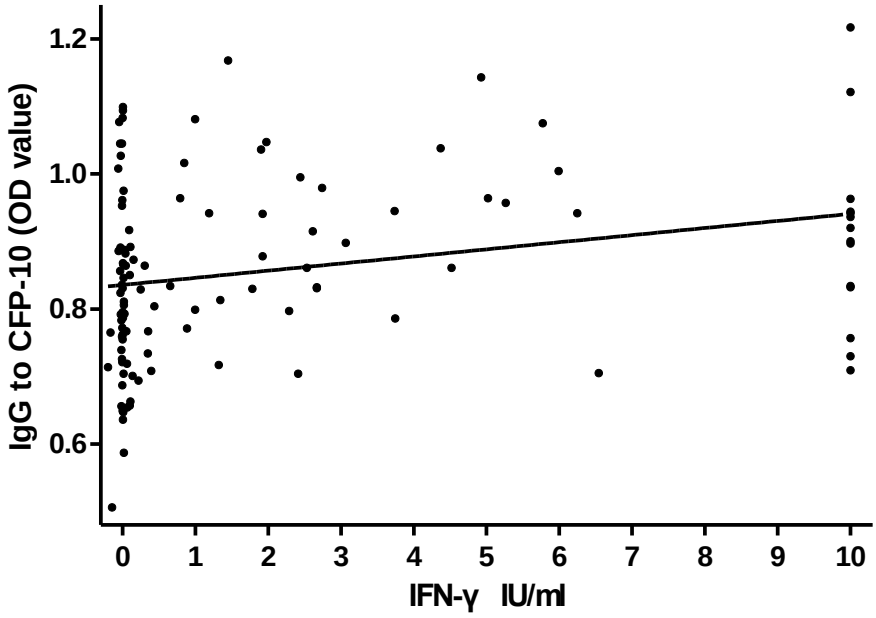
2.3.4. Correlations Among Serum Levels of IgA, IgG, and IFN-γ

Weak positive correlations were observed between the level of IFN-γ induced by the QFT-GIT test and the OD values of serum IgG against Ag85B (r = 0.286, p = 0.002), TBCM (r = 0.303, p = 0.001), and CFP-10 (r = 0.259, p = 0.005) and IgA against Ag85B (r = 0.193, p = 0.039; Figure 2-5).


A
IgG to TBCM (OD value)
1.3
1.2
1.1
1.0
0.9
0
1
2
3
4
5
6
7
8
9
10
IFN-γ IU/ml


B
IgG to Ag85B (OD value)
1.4
1.2
1.0
0.8
0
1
2
3
4
5
6
7
8
9
10
IFN-γ IU/ml

C


IgG to CFP-10 (OD value)
1.2
1.0
0.8
0.6
0
1
2
3
4
5
6
7
8
9
10
IFN-γ IU/ml

D

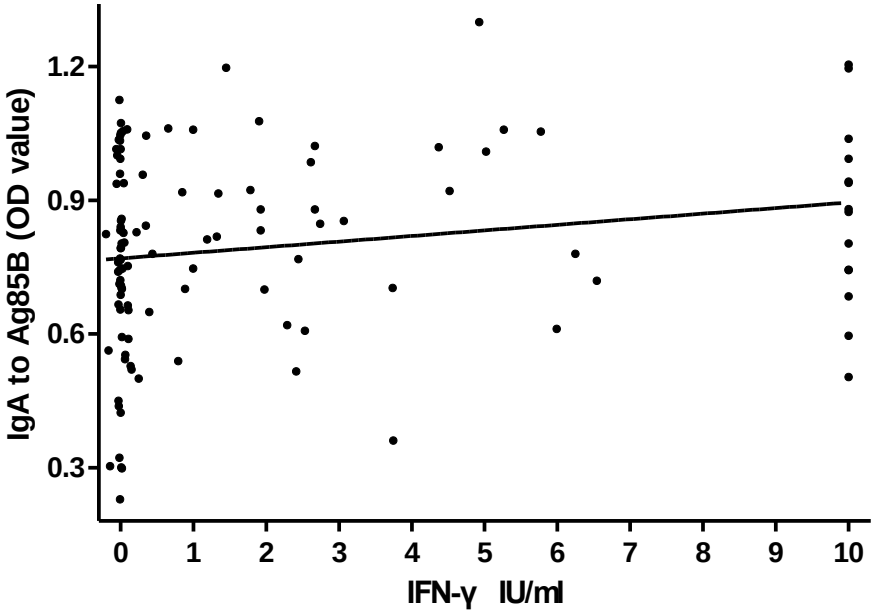
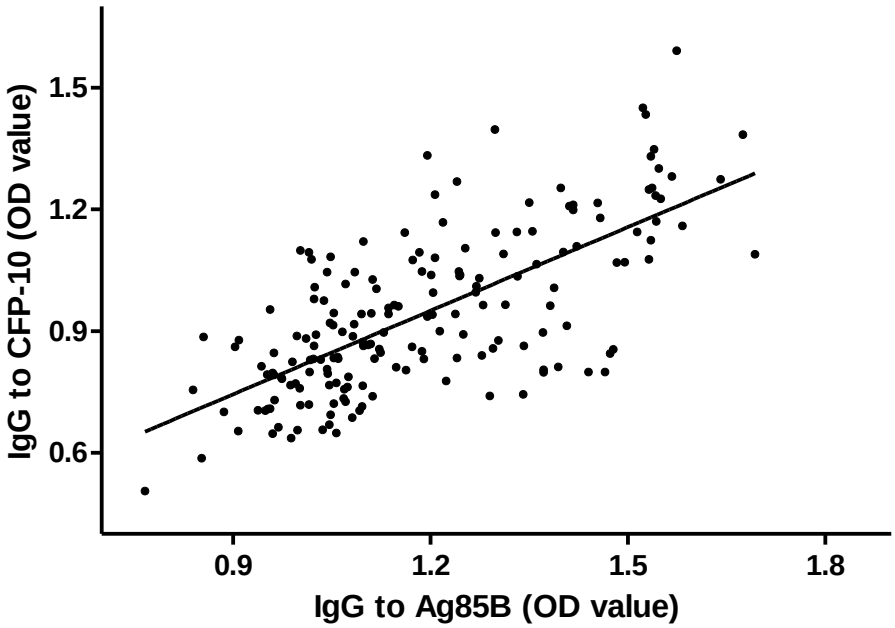

IgA to Ag85B (OD value)
1.2
0.9
0.6
0.3
0
1
2
3
4
5
6
7
8
9
10
IFN-γ IU/ml

Figure 2-5. Correlations among TB antigen-specific antibodies and interferon-gamma levels.

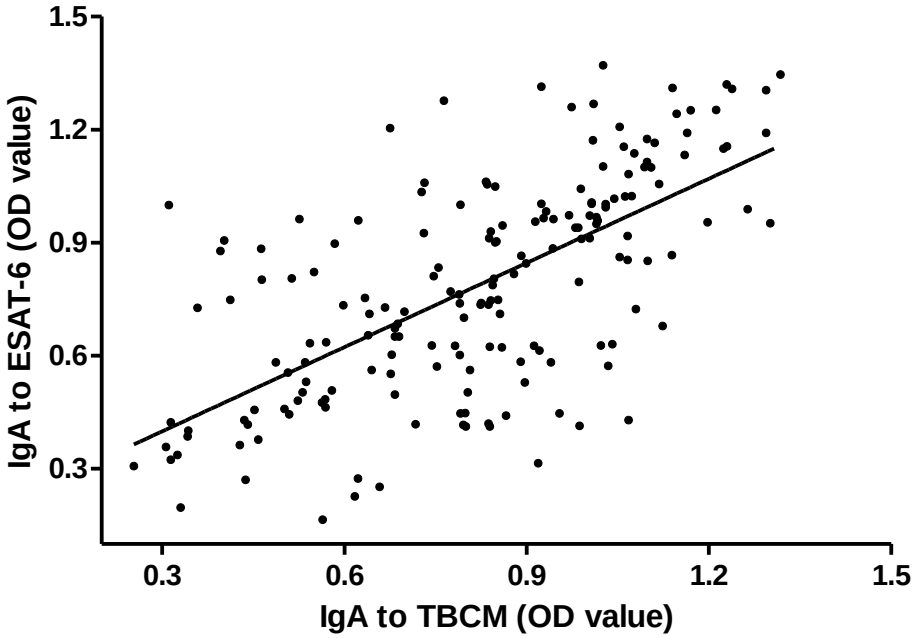
Correlation between the level of interferon-gamma and the level of serum IgG against TBCM (r = 0.303, p = 0.001) (**A**), IgG against Ag85B (r = 0.286, p = 0.002) (**B**), IgG against CFP-10 (r = 0.259, p = 0.005) (**C**), and IgA against Ag85B (r = 0.193, p = 0.039) (**D**).

A strong positive correlation was observed between the serum IgA levels against CFP-10 and ESAT-6 (r = 0.874, p < 0.0001). Meanwhile, moderate positive correlations were observed between serum IgA levels against CFP-10 and TBCM (r = 0.682, p < 0.0001), and Ag85B (r = 0.647, p < 0.0001), as well as between the serum IgA levels against ESAT-6 and TBCM (r = 0.653, p < 0.0001), and Ag85B (r = 0.634, p < 0.0001). Similarly, there were moderate positive correlations between serum IgG levels against CFP-10 and Ag85B (r = 0.699, p < 0.0001) in study subjects (Figure 2-6).

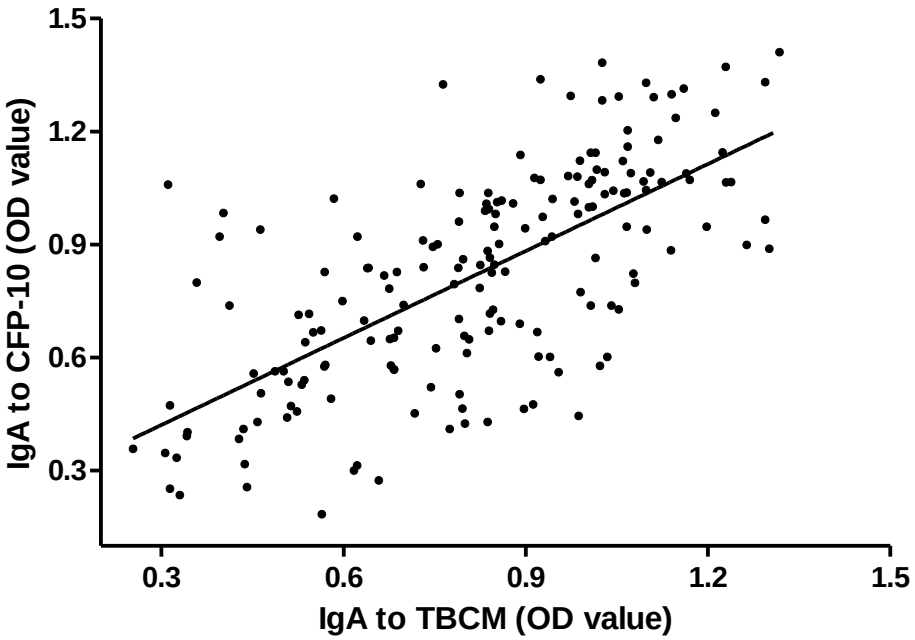
A


1.5
1.2
0.9
0.6
IgG to CFP-10 (OD value)
0.9
1.2
1.5
1.8
IgG to Ag85B (OD value)

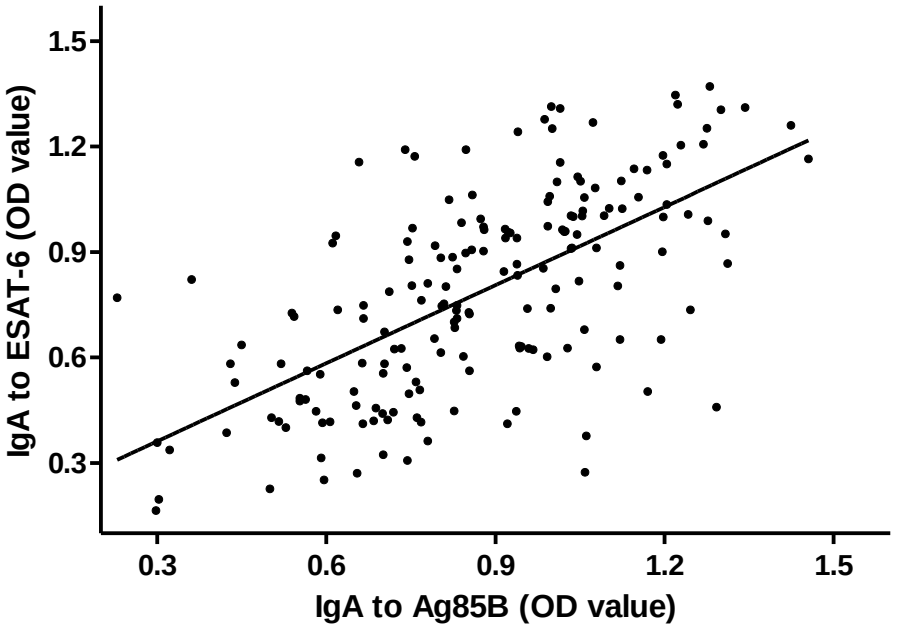
B


1.5
1.2
0.9
0.6
0.3
IgA to ESAT-6 (OD value)
0.3
0.6
0.9
1.2
1.5
IgA to TBCM (OD value)

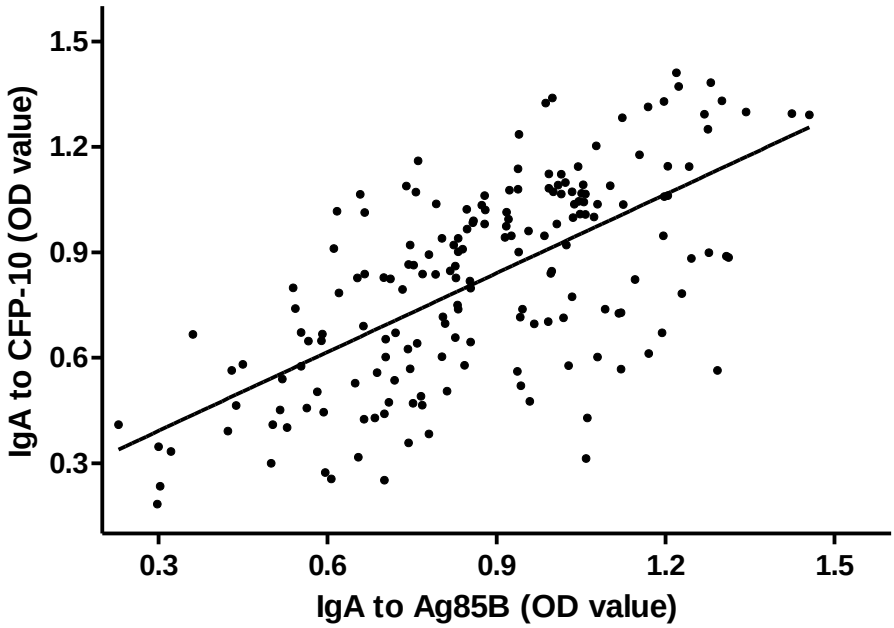
C


1.5
1.2
0.9
0.6
0.3
IgA to CFP-10 (OD value)
0.3
0.6
0.9
1.2
1.5
IgA to TBCM (OD value)

D


1.5
1.2
0.9
0.6
0.3
IgA to ESAT-6 (OD value)
0.3
0.6
0.9
1.2
1.5
IgA to Ag85B (OD value)

E


1.5
1.2
0.9
0.6
0.3
IgA to CFP-10 (OD value)
0.3
0.6
0.9
1.2
1.5
IgA to Ag85B (OD value)

F

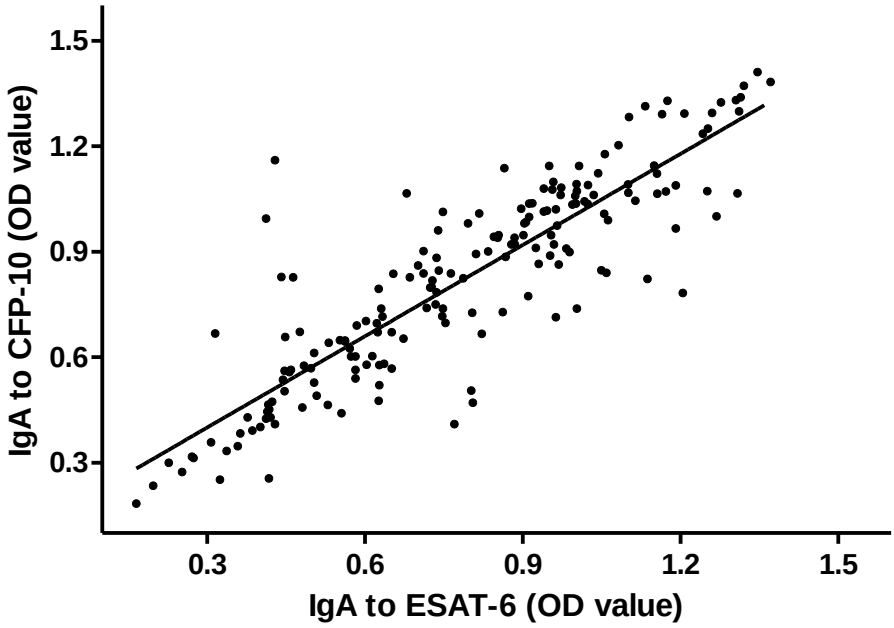

1.5
1.2
0.9
0.6
0.3
IgA to CFP-10 (OD value)
0.3
0.6
0.9
1.2
1.5
IgA to ESAT-6 (OD value)

Figure 2-6. Correlations among TB antigen-specific antibody levels.

Correlation between serum IgG levels against Ag85B and CFP-10 (r = 0.699, p < 0.0001) (A); IgA against TBCM and ESAT-6 (r = 0.653, p < 0.0001) (B); IgA against TBCM and CFP-10 (r = 0.682, p < 0.0001) (C); IgA against Ag85B and ESAT-6 (r = 0.634, p < 0.0001) (D); IgA against Ag85B and CFP-10 (r = 0.647, p < 0.0001) (E); and IgA against ESAT-6 and CFP-10 (r = 0.874, p < 0.0001) (F).

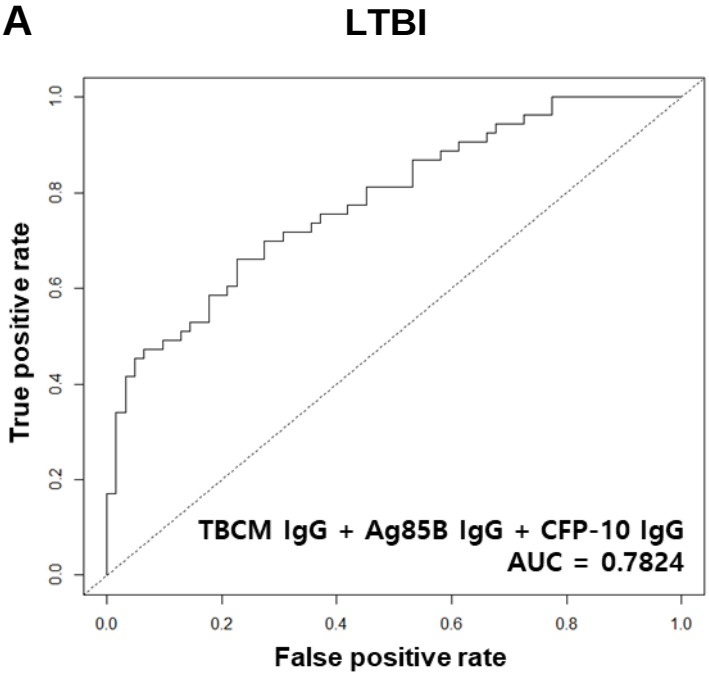
2.3.5. Diagnostic Performance of Antibodies Against *Mtb* Antigens for Diagnosis of LTBI or ATB

To investigate independent variables significantly related to LTBI diagnosis, multivariate logistic regression analysis including significant variables from the univariable analysis was performed, and additional models including age and sex were generated. Results show that IgG levels against TBCM, Ag85B, and CFP-10 were independently related to LTBI diagnosis. ROC curve analysis was performed for LTBI diagnostic performance, and the AUC combining each antibody value was highest with a value of 0.7824 (Figure 2-7A), compared to the AUC value of each IgG against TBCM, Ag85B, and CFP-10. When adjusting for age and sex, IgG against TBCM and CFP-10 and age were independently associated with LTBI diagnosis. When the ROC analysis was performed including these markers, the AUC value was 0.8597 (Figure 2-7B), which increased the predictive power compared to the model without age.

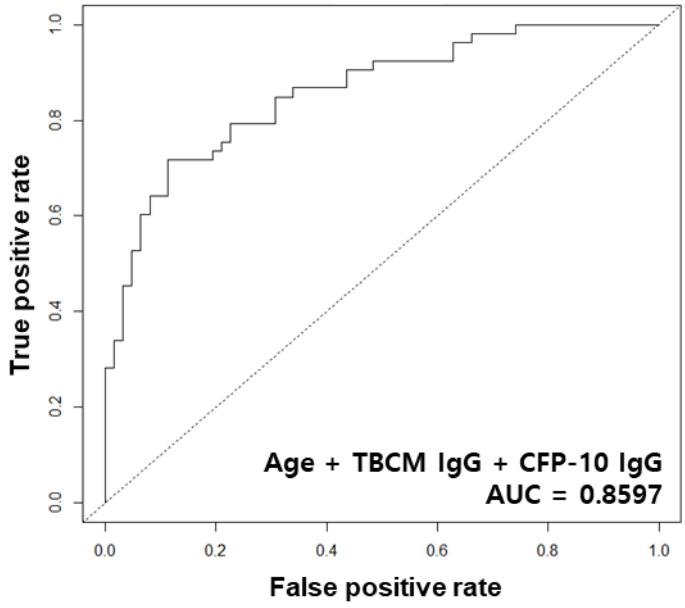
In the multivariate analysis related to ATB diagnosis, the IgG level against Ag85B was independently significantly related to the diagnosis of ATB, and the ROC analysis showed a strong diagnostic performance of 0.9885 (Figure 2-7C). When age and sex were

adjusted, IgG against Ag85B and age were independent significant factors, and in this model, the AUC was 0.9938 (Figure 2-7D), showing increased predictive power compared to the model without age. In the multivariate analysis for the differential diagnosis of LTBI and ATB, only IgG against Ag85B was an independent significant marker, showing great differentiating power (AUC: 0.9453) in the ROC analysis (Figure 2-7E). When adjusting for age and sex, IgG against Ag85B and age were two independent significant variables. In this model, the AUC was 0.9527 (Figure 2-7F), with higher predictive power than the model without age. The sensitivity and specificity of IgG against Ag85B in the diagnosis of ATB and LTBI are summarized in Table 2-2.

A

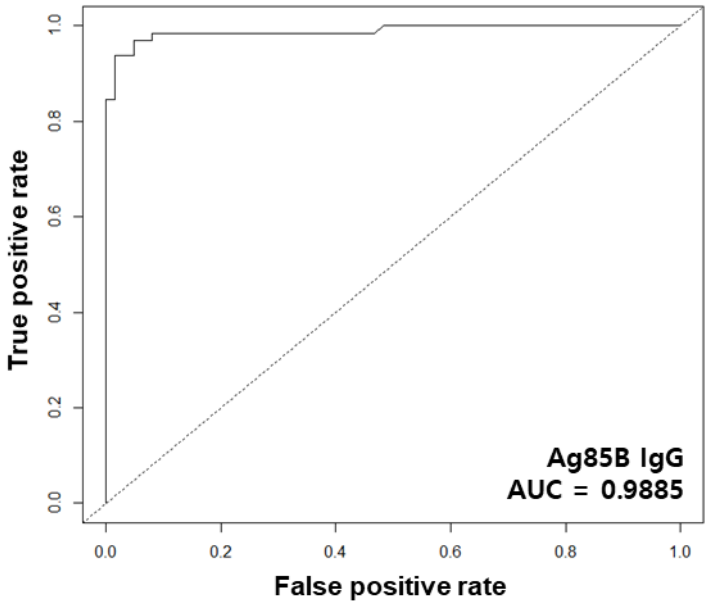

LTBI
True positive rate
False positive rate
0.0
0.2
0.4
0.6
0.8
1.0
TBCM IgG + Ag85B IgG + CFP-10 IgG
AUC = 0.7824

B


LTBI
True positive rate
False positive rate
0.0
0.2
0.4
0.6
0.8
1.0
Age + TBCM IgG + CFP-10 IgG
AUC = 0.8597

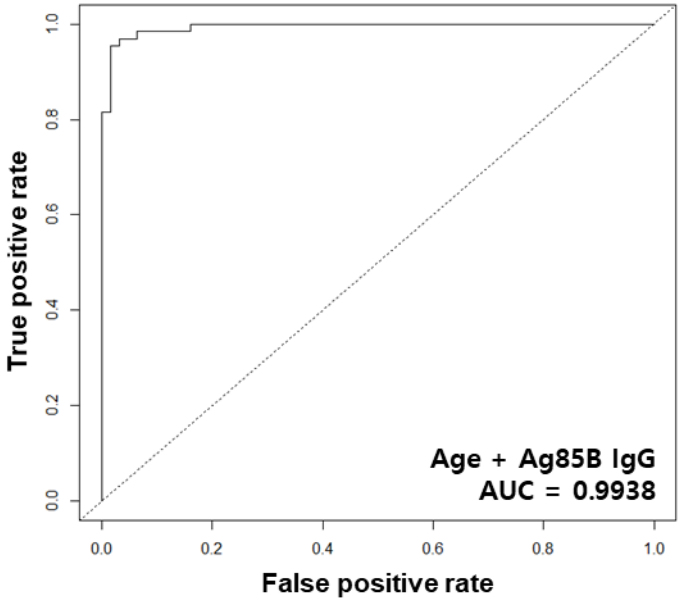
C

ATB


True positive rate
False positive rate
0.0
0.2
0.4
0.6
0.8
1.0
Ag85B IgG
AUC = 0.9885

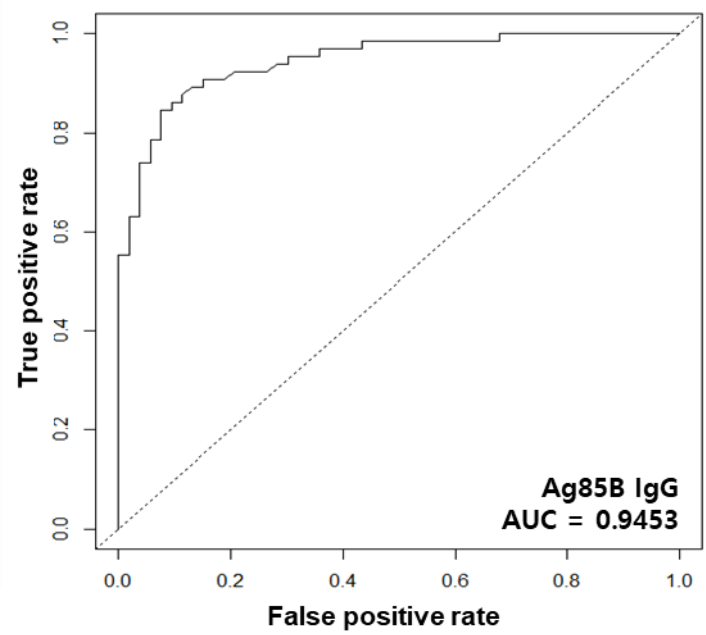
D

ATB


True positive rate
False positive rate
0.0
0.2
0.4
0.6
0.8
1.0
Age + Ag85B IgG
AUC = 0.9938

E

LTBI vs ATB



F

LTBI vs ATB

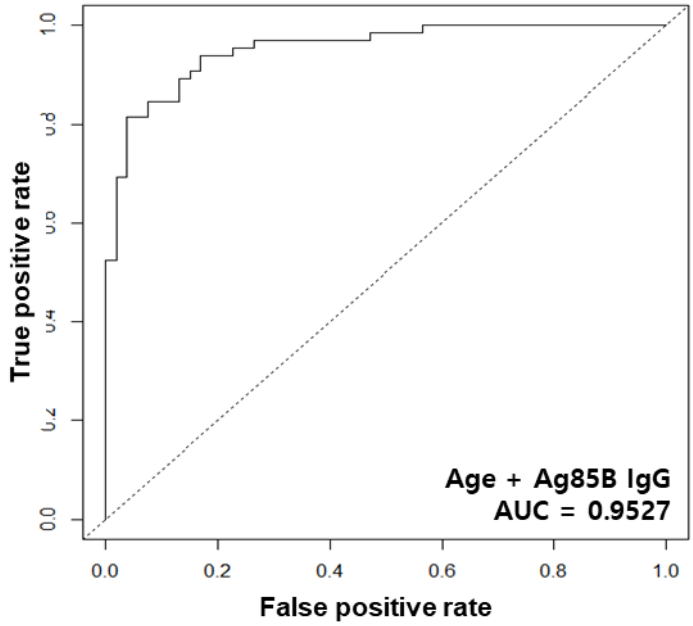


Figure 2-7. Receiver operating characteristic (ROC) curves for TB antigens-specific antibody responses.

ROC curve analysis and logistic regression models were used to evaluate the discriminatory power of biomarkers between non-infected (NI) and latent tuberculosis infection (LTBI) (A, B), NI and active tuberculosis (ATB) (C, D), and LTBI and ATB (E, F). The following biomarkers were used for each model configuration: IgG levels against TBCM, Ag85B, and CFP-10 (A); age and IgG levels against TBCM and CFP-10 (B); IgG against Ag85B (C); age and IgG against Ag85B (D); IgG against Ag85B (E); age and IgG against Ag85B (F).

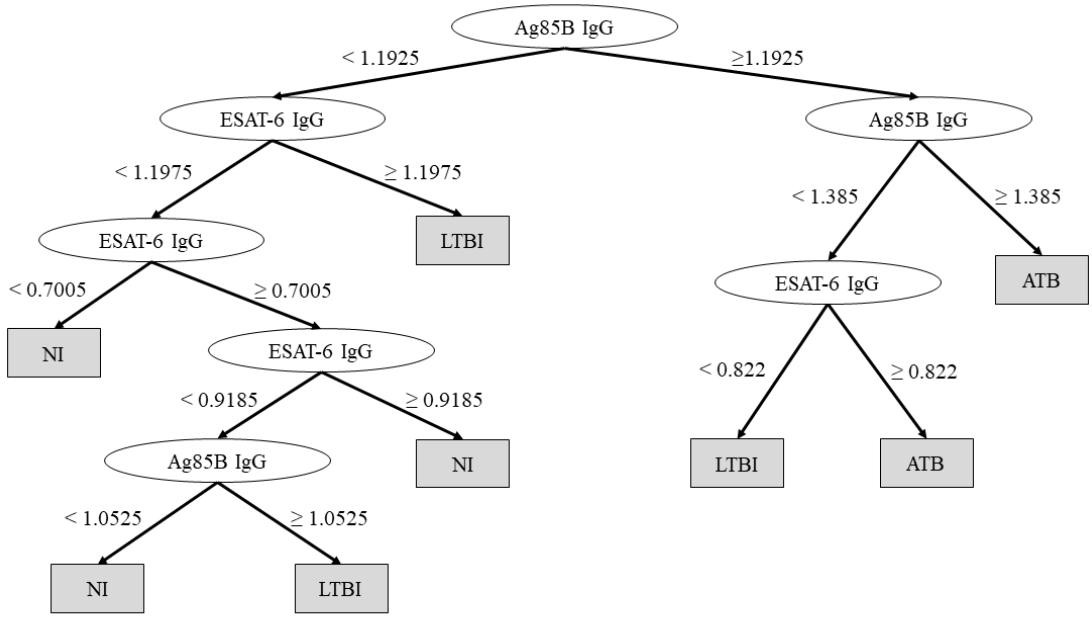
Table 2-2. Efficiency of IgG against Ag85B as a diagnostic marker for tuberculosis

Groups	AUC (*p* value)	Cutoff (OD)	Sensitivity (%)	Specificity (%)
NI vs LTBI	0.6593 (0.003326)	> 1.0595	60.40	62.90
NI vs ATB	0.9885 (< 0.0001)	> 1.156	96.90	95.20
LTBI vs ATB	0.945 (< 0.0001)	> 1.2415	87.70	88.70

AUC, area under the curve; OD, optical density; NI, non-infected; LTBI, latent tuberculosis infection; ATB, active tuberculosis

Additionally, a decision tree model using IgG against Ag85B and ESAT-6 was created to comprehensively predict the diagnosis of ATB or LTBI. The accuracy had a significant diagnostic value of 0.789 (Figure 2-8A). Moreover, when age was included in the model, this value was improved to 0.8269 (Figure 2-8B).

A


Ag85B IgG
< 1.1925
≥1.1925
ESAT-6 IgG
Ag85B IgG
< 1.1975
≥ 1.1975
< 1.385
≥ 1.385
ESAT-6 IgG
LTBI
ESAT-6 IgG
ATB
< 0.7005
≥ 0.7005
NI
ESAT-6 IgG
< 0.822
≥ 0.822
< 0.9185
≥ 0.9185
Ag85B IgG
NI
LTBI
ATB
< 1.0525
≥ 1.0525
NI
LTBI

B

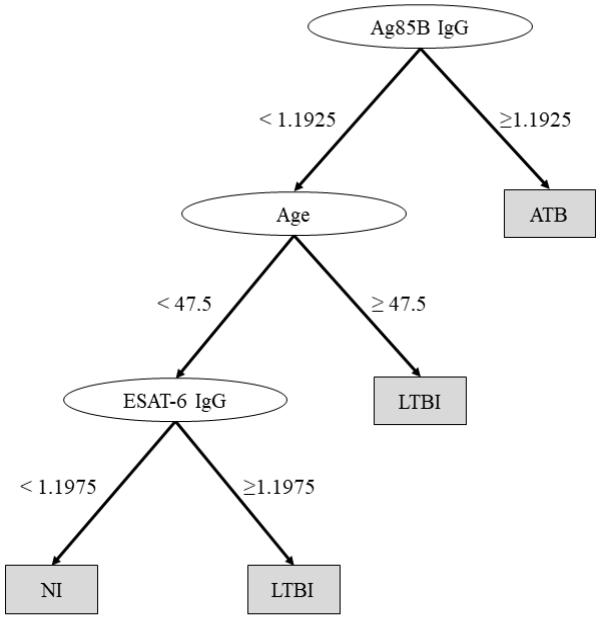

Ag85B IgG
< 1.1925
≥1.1925
Age
ATB
< 47.5
≥ 47.5
ESAT-6 IgG
LTBI
< 1.1975
≥1.1975
NI
LTBI

Figure 2-8. Decision tree for the prediction of TB.

Decision tree models to comprehensively predict the diagnosis of active tuberculosis (ATB) or latent tuberculosis infection (LTBI), using IgG against Ag85B and ESAT-6 (accuracy = 0.789) (A), or using age and IgG against Ag85B and ESAT-6 (accuracy = 0.8269) (B).

2.4. Discussion

In the present study, IgG and IgA responses to TB antigens TBCM, Ag85B, ESAT-6, and CFP-10 in NI, LTBI, and ATB subject groups were compared. The levels of IgG and IgA against Ag85B, and of IgG against CFP-10, were significantly higher in ATB patients, followed by LTBI patients, and were lowest in NI subjects. The IgG levels against TBCM was significantly higher in LTBI subjects than in NI subjects. When age and sex were adjusted in the multivariate analysis, IgG against TBCM and CFP-10 were independently related to LTBI diagnosis. Additionally, IgG against Ag85B was a significant marker, independently related to both ATB diagnosis and discrimination between ATB and LTBI.

These results suggest that levels of IgA and IgG correlate with bacillary load, which is consistent with the results of previous studies. Abebe et al. reported that the levels of IgA and IgG for Rv2031 were significantly higher in untreated TB patients, the next highest in generation contactors, and the lowest in the control cohort demonstrating that the IgA and IgG levels are strongly correlated with bacillary load [29]. Similarly, Kunnath-Velayudhan et al. reported that antibody levels were correlated with bacterial load when patients with suspected TB were classified according to

sputum smear and bacterial load [14]. When the burden of the bacilli is low, such as in LTBI or minimal TB cases, the concentration of membrane-associated proteins derived from the bacilli is also low. However, when the burden of the bacilli increases, such as in severe ATB, the activated bacilli secrete proteins, which is accompanied by an increase in antibody responses. Thus, these antibody response titers might reflect the status of TB disease or progression of infection. In the present study, the levels of IgG against Ag85B and CFP-10 could significantly discriminate specific TB subgroups, even when the ATB group was classified based on radiological severity, AFB smear results, and past treatment history. These results suggest that the antibody responses to specific TB antigens have diagnostic potential for use in discriminating the detailed spectrum of TB associated with bacillary load.

IgG against TBCM, a novel antigen, was an independent significant marker for LTBI diagnosis. The diagnostic model including IgG against TBCM in both models, with and without age, demonstrated good predictability. In addition, weak positive correlations were observed between the level of IFN-γ induced by the QFT-GIT test and the serum level of IgG against TBCM in LTBI and NI subjects. These results suggest that IgG against TBCM could potentially complement current diagnostic methods for

LTBI. Previous studies have also shown that TBCM is active in acidic environments, such as infected macrophages, and is essential for TB survival by regulating intermediates that are important for the biosynthesis of a wide range of compounds [30]. As *Mtb* could be starved due to nutrient deficiency inside macrophages in latent stages [31, 32], the metabolism of *Mtb* in LTBI might have a significant relationship with survival. In addition, since TB could present different gene expression patterns depending on the stage of infection, differences in immune responses to stage-specific antigens might occur [33]. The results from the present study suggest that TBCM could be associated with the survival of *Mtb* in the latent stage of the disease.

Herein, levels of IgG against Ag85B and CFP-10 were significantly different between LTBI and AFB-negative- or minimal TB, showing excellent discriminatory power between the detailed spectrum of TB. In particular, Ag85B IgG was not only highly predictive in ATB diagnosis, as well as in discriminating between LTBI and ATB, regardless of age in the multivariate analysis diagnostic model, but also was a significant factor in the decision tree model. Therefore, these results can be used to compensate for limitations in the current LTBI diagnosis methods that cannot distinguish between LTBI and ATB.

Some of the first antigens described for TB were mixtures of *Mtb* constituents, such as purified protein derivatives, however, since then many new antigens, including A60, Ag85, 38 kDa, 16 kDa, MPT51, *Mtb*48, ESAT-6, CFP-10, 2,3-diacyltrehalose (DAT), 2,3,6-triacyltrehalose (TAT), and lipoarabinomannan (LAM), have been developed to increase sensitivity and specificity [34]. However, the accuracy of serodiagnosis in TB varies widely between studies. In fact, a meta-analysis on 67 studies that performed commercial serological tests in pulmonary TB, reported sensitivity for each study ranging from 0 to 100%, with specificities from 31 to 100% [17, 35]. Previous studies using Ag85B, ESAT-6, and CFP-10 also showed varying results in terms of diagnostic accuracy. Kumar et al. reported that the sensitivity of IgG against Ag85 in the diagnosis of ATB was higher (84.1%) than that of IgG to CFP-10 (66%) and ESAT-6 (64.9%) [36]. In another study, the sensitivity of IgG against Ag85 in the diagnosis of ATB was 67.5%, higher than that of IgA and IgM against Ag85 [37]. However, another study reported the highest AUC of IgG against ESAT-6 when evaluating the value of IgG against Ag85B, ESAT-6, and CFP-10 in the diagnosis of ATB [38]. Meanwhile, the results of the present study demonstrated higher diagnostic accuracy than that of previous studies using similar antigens. These differences may be

attributed to the purity of the antigens or differences in the study populations. In contrast to the absence, or low number, of LTBI cases in other studies, the present study included a relatively large number of LTBI patients, diagnosed with the QFT-GIT assay. In addition, it was confirmed that the QFT-GIT assay results were negative for the healthy control group.

Previous studies have used immunoglobulin isotopes to highlight differences in immune responses. In several studies, IgG has been shown to have value in the serodiagnosis of TB and has been reported to be more sensitive than IgA or IgM [39-43]. Although IgA is relatively less established, some studies have reported it to have higher sensitivity and specificity than IgG and is, thus, of diagnostic importance [13, 43, 44]. Considering that *Mtb* invades and multiplies in the respiratory tract, it is feasible that the production of IgA could be stimulated during contact between TB antigens and the mucosal surface [45]. Alternatively, IgM is reportedly associated with non-specific reactions and is not correlated with TB severity [43, 46]. IgM is primarily produced in the early stages of infection. Therefore, the value of IgM in the diagnosis of TB might be relatively low since IgG, rather than IgM, increases as time passes after infection or following reactivation of TB [47, 48]. Meanwhile, another study reported that IgG and IgM

are both elevated during infection, which indicates conflicting results [49]. Similar to previous studies, the antibody responses to different antigens in the present study varied by isotype, and the IgG response appeared to be more discriminatory than IgA.

The present study has several limitations. Firstly, when the ATB group was divided into subgroups, the number of samples in each subgroup was reduced resulting in insufficient power to prove statistical significance among the subgroups. There were also differences in age and sex between NI, LTBI, and ATB groups and although each variable was adjusted through multivariate analysis, if a larger number of samples were obtained, the diagnostic usefulness of each marker in the age- and sex-matched comparison groups could be confirmed.

2.5. Conclusion

In conclusion, results of this study demonstrate that IgG antibody responses to TBCM, Ag85B, and CFP-10 have potential for diagnosing TB. Further, these responses could significantly discriminate between NI, LTBI, and ATB individuals. These results suggest that TB antigen-specific antibodies could be used to develop reliable ELISA tests for the diagnosis of TB.

Chapter 3. Usefulness of IgA and IgG Responses to Macrophage Migration Inhibitory Factor for Diagnosing Tuberculosis

3.1. Introduction

Macrophage migration inhibitory factor (MIF) was first described as a cytokine secreted from activated T cells that inhibits macrophage migration, and enhances its ability to kill intracellular parasites or tumor cells [50, 51]. Reportedly, MIF is also secreted from endothelial cells and fibroblasts [52, 53] and mediates cell growth, T cell activation, and various inflammatory and immune responses [54]. In particular, it induces the synthesis of inflammatory cytokines and antagonizes the inhibition of cytokine production by glucocorticoids [55].

Recent studies have shown that MIF expression is increased in TB, as well as in several immune-related, and inflammatory diseases. Das et al. reported reduced expression of the MIF gene causes decreased cytokine and reactive oxygen production, thereby inhibiting the killing of mycobacteria and increasing the risk of TB. When both aerosolized and i.v. models of mycobacterial infection were studied, the results showed that the survival rate after infection with TB was significantly lower in MIF-deficient mice than that in wild type mice. In addition, MIF-deficient mice showed more accumulated *Mtb* in the lungs and exhibited decreased production of innate cytokines including tumor necrosis factor-

alpha (TNF-α), interleukin (IL)-12, and IL-10. As such, MIF appears to play an important, protective role in infection control [56].

The presence of naturally occurring autoantibodies against cytokines in healthy individuals and patients has been documented in many reports [57, 58]. In a study evaluating the prevalence of anti-cytokine autoantibodies in 15 healthy individuals, all individuals tested had autoantibodies for several different cytokines. In particular, autoantibodies to IL-2, IL-8, TNF-α, vascular endothelial growth factor, and granulocyte colony-stimulating factor (G-CSF) were observed in all subjects. These results showed that anti-cytokine autoantibodies are ubiquitous in healthy individuals [57]. Antibody responses to cytokines appear to play an important role in immune regulation [57, 59]. Several studies have reported that immunoglobulin A (IgA) and immunoglobulin G (IgG) respond to various cytokines, including interferon-gamma (IFN-γ), G-CSF, and tumor necrosis factor-alpha (TNF-α) in *Mycobacterium* infection, HIV infection, alveolar protein syndrome, and autoimmune diseases, differently depending on the disease state [58, 60-62]. Previous studies have shown several examples of how cytokine antibodies act on hematopoietic and host defense in the context of hematopoietic and normal function. Granulocyte-

macrophage colony-stimulating factor (GM-CSF) autoantibodies are present in low concentrations in healthy conditions. However, above the concentration sufficient for completely neutralizing the biological activity of GM-CSF due to an increase in antibody concentration, the bone marrow cell function regulated by GM-CSF decreased to the lowest level and the risk of pulmonary alveolar proteinosis was increased [63]. Similarly, it has been reported that erythropoietin autoantibodies induce pure red-cell aplasia [64], and G-CSF autoantibodies induce neutropenia in Felty's syndrome [65]. In addition, high levels of IL-8 autoantibody complexes are known to increase chemotactic activity of attracting neutrophils to the lungs, which can likely cause acute respiratory distress syndrome [66]. Meanwhile, IL-6 autoantibodies can increase the risk of bacterial infection [67]. In addition, IFN-γ autoantibodies have also been reported to cause infections against intracellular pathogens such as TB and NTM [68, 69]. Conversely, it has been reported that some cytokine autoantibodies may weaken disease severity. Joint destruction in arthritis was found to be milder in IL-1α autoantibody positive patients than in IL-1α autoantibody negative patients [70]. In addition, TNF-α autoantibodies can reduce the activity of systemic lupus erythematosus [71].

Considering the regulatory role of autoantibodies during neutralization, their levels may show a trend opposite to that of cytokines. Therefore, it may be hypothesized that in response to increased MIF levels in TB, antibodies against MIF are lower in TB patients than in healthy individuals. However, to date, no studies have been conducted on the response of antibodies against MIF in TB.

In the present study, the IgA and IgG responses to MIF were analyzed in the sera of patients with ATB and LTBI, as well as in that of non-infected (NI) individuals. In addition, it was determined whether the antibody responses to MIF are valuable for rapid diagnosis of TB.

3.2. Materials and Methods

3.2.1. Study Subjects

Participants were enrolled as described previously [72]. Briefly, 180 individuals including 65 ATB patients, 53 LTBI patients, and 62 NI individuals were enrolled at the National Medical Center, Seoul, Republic of Korea. ATB was diagnosed in patients with positive sputum or bronchoscopy specimens according to a TB nucleic acid amplification test or a mycobacterial culture. LTBI was diagnosed based on a positive result from the QFT-GIT assay, for those without chest radiographic abnormalities and clinical symptoms. Individuals were considered NI in case of negative results from the QFT-GIT assay. Five milliliters of peripheral blood were obtained at the time of enrollment. Both laboratory and clinical data were collected at the time of usual hospital visits. All participants were HIV-negative. The median age was 33 years (interquartile range 29-33 years) in the NI group, 49 years (33-57 years) in the LTBI group, and 60 years (51-67 years) in the ATB group. Of the 180 participants, 21 (33.9%) in the ATB group, 21 (39.6%) in the LTBI group, and 56 (86.2%) in the ATB group were male.

3.2.2. Preparation of MIF

Recombinant MIF protein was purified from *Escherichia coli* as previously described [27, 28]. Briefly, BL21 *E. coli* strains (RBC Bioscience, Taipei City, Taiwan) were transformed with pET28a-MIF to express and purify the fusion protein. The bacterial cultures were induced with 0.4 mM isopropyl β-D-thiogalactoside (IPTG, Duchefa Biochemie, Haarlem, Netherlands). Next, the bacterial cultures were sonicated for 10 min at 4 ° C, and centrifuged at 1600 × *g* for 20 min at 4 ° C. Pellets that contained His-MIF were resuspended in binding buffer containing 4 M urea (Sigma Aldrich, St. Louis, MO, USA). His-MIF was purified using Ni-NTA His•Bind Resin (Merck, Darmstadt, Germany) and subsequently eluted using elution buffer (300 mM NaCl, 50 mM sodium phosphate buffer, and 250 mM imidazole) that also contained 4 M urea. The MIF protein was then dialyzed to remove the imidazole, residual salts, and urea.

3.2.3. Enzyme-Linked Immunosorbent Assays (ELISA)

Antibody isotypes, IgA and IgG, serum levels were assessed using ELISA as previously described with minor modifications [72].

Briefly, corning 96-well EIA/RIA plates (Corning Inc., Kennebunk, ME, USA) were coated overnight at 4 ° C in a solution with 5 μg/mL MIF that was diluted in 0.05 M carbonate-bicarbonate coating buffer. The plates were washed three times with phosphate-buffered saline (PBS) that contained 0.05% Tween-20 and were subsequently blocked with 5% BSA in PBS at RT for 1 h. Next, 100 μL of the samples, which were diluted (1:10) in PBS, were added to each well, and then incubated at RT for 2 h. After washing, the indicated secondary antibody was diluted at 1:500 in 5% BSA, and 100 μL was added to each well of the respective plates for 1 h at RT. The secondary antibodies were as follows: anti-human IgG (H+L), HRP conjugate (W4038, Promega, Madison, WI) or anti-human IgA HRP-conjugated antibody (PA1-74395, Invitrogen, Rockford, IL, USA). Next, the plates were washed and 100 μL of 3,3′,5,5′-Tetramethylbenzidine (BD OptEIA substrate; BD Biosciences, San Diego, CA, USA) was added to each well. The reaction was quenched after 10 min with 50 μL of 1 N sulfuric acid. The plates were analyzed at 450 nm.

3.2.4. Data Analysis

Antibody levels, presented as OD values, and one-way ANOVA with Bonferroni's multiple comparisons were used to compare IgA

and IgG responses among the different groups. A binary logistic regression was performed to assess the biomarkers to predict either LTBI or ATB. The AUC of the ROC curve was calculated to compare each biomarker and its predictive potential. Correlations among IgA and IgG serum levels in addition to IFN-γ, age, BMI, sex, and drug resistance were examined with Pearson's correlation coefficient. Both SPSS Statistics 17.0 (SPSS Inc., Chicago, IL, USA) and Prism 5.0 (GraphPad Software, La Jolla, CA, USA) were used for data analyses. A p value of < 0.05 was regarded as statistically significant.

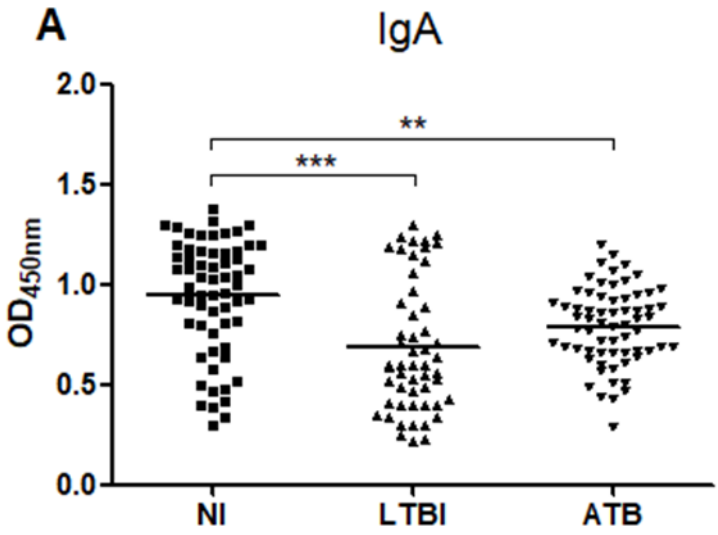
3.2.5. Ethics Statement

All subjects provided written, informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the institutional review board of National Medical Center (IRB no. H-1811-096-002) and Seoul National University Hospital (H-2006-090-1132).

3.3. Results

3.3.1. Serum Levels of IgA and IgG against MIF

Figure 3-1 shows the IgA and IgG responses to MIF for each participating group. IgA levels against MIF were significantly lower in the LTBI ($p < 0.0001$) and ATB groups ($p = 0.006$) compared with the NI group. However, there was no significant difference between the ATB and LTBI groups (Figure 3-1A). Levels of IgG against MIF were significantly lower in the LTBI group than in the NI group ($p = 0.011$). There was no significant difference in IgG levels among the ATB group and the LTBI and NI groups (Figure 3-1B).


A
IgA
2.0
1.5
1.0
0.5
0.0
OD450nm
**
***
NI
LTBI
ATB

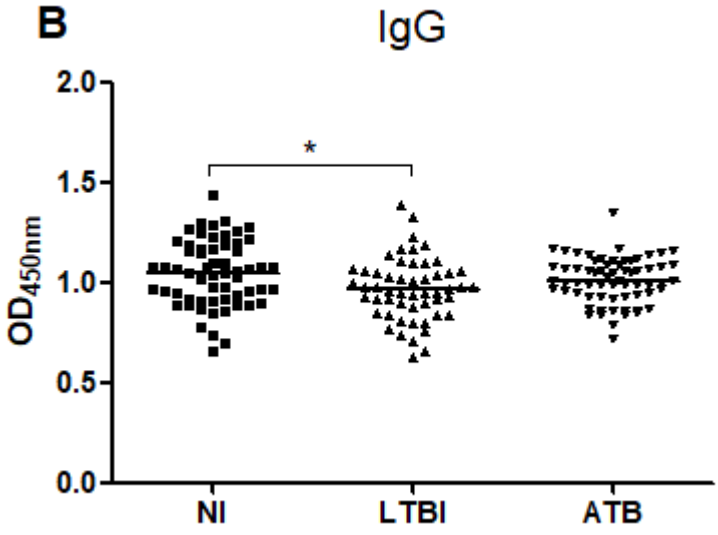

B
IgG
2.0
1.5
1.0
0.5
0.0
OD450nm
*
NI
LTBI
ATB

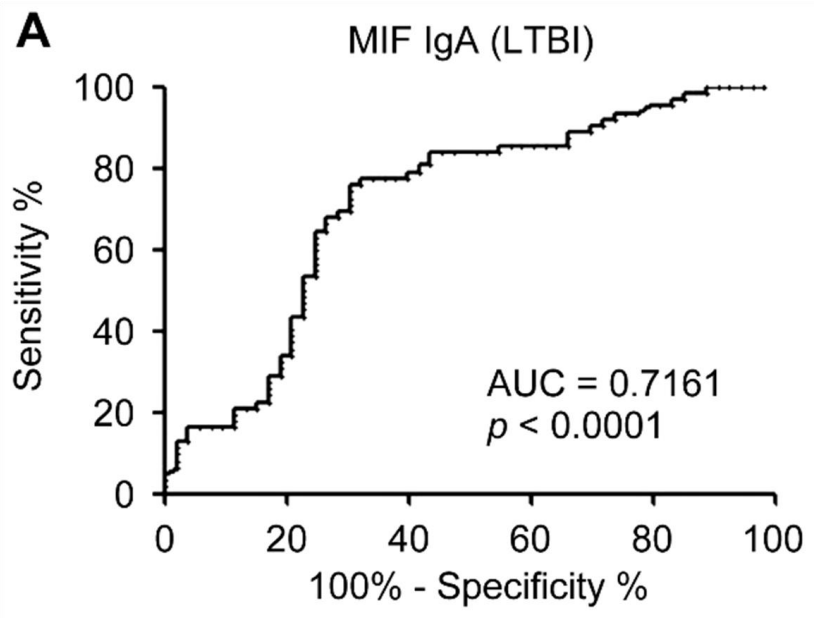
Figure 3-1. IgA and IgG responses to macrophage migration inhibitory factor (MIF).

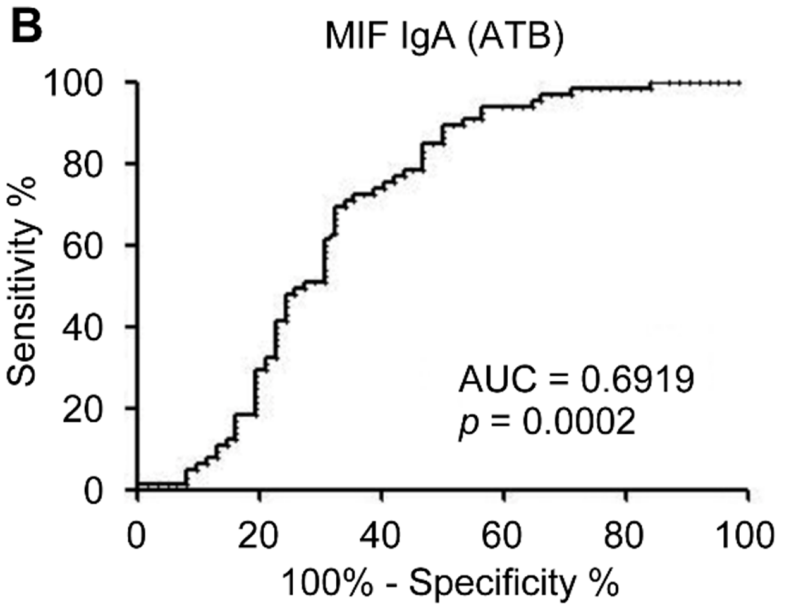
Optical density (OD) values of the anti-MIF immunoglobulin A (IgA) (A), and anti-MIF immunoglobulin G (IgG) (B) serodiagnosis assays for the comparison of 65 active tuberculosis (ATB) patients, 53 latent tuberculosis infection (LTBI) patients, and 62 non-infected (NI) individuals. Each dot represents the values obtained from individuals, and the horizontal bars indicate the mean values. Groups were compared using one-way ANOVA with Bonferroni's multiple comparisons. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

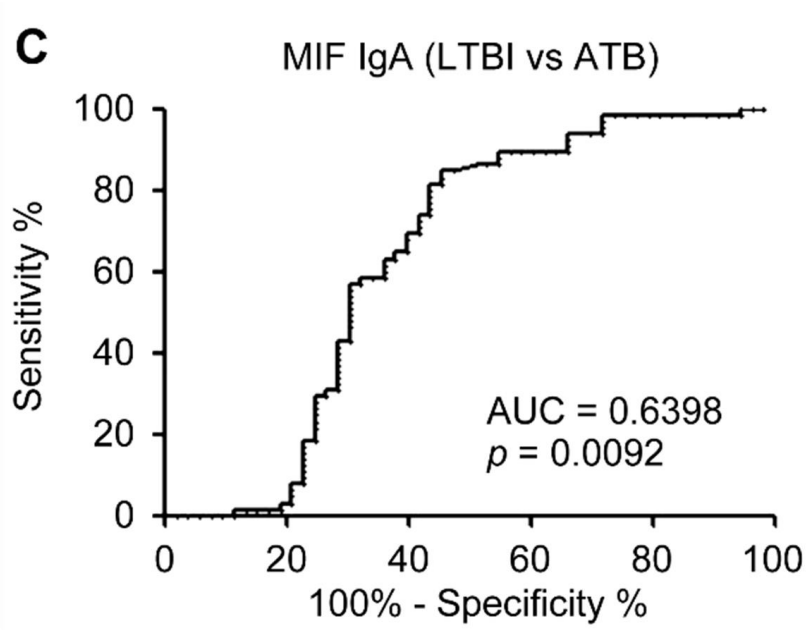
In the logistic regression analysis, the level of IgA against MIF was significantly related to diagnosis of both LTBI ($p < 0.0001$) and ATB ($p = 0.001$), as well as to the differential diagnosis of LTBI and ATB ($p = 0.029$).

The AUC for IgA responses to MIF was 0.7161 ($p < 0.0001$) in the diagnosis of LTBI (Figure 3-2A), 0.6919 ($p < 0.0002$) in the diagnosis of ATB (Figure 3-2B), and 0.6398 ($p = 0.0092$) in the differential diagnosis of either LTBI or ATB (Figure 3-2C). IgG levels against MIF were significantly related to LTBI diagnosis ($p = 0.011$) with an AUC of 0.6397 ($p = 0.0100$) in the diagnosis (Figure 3-2D). However, the IgG response to MIF was not significantly related to ATB diagnosis or differential diagnosis.

The sensitivity and specificity of IgA and IgG against MIF in the diagnosis of ATB and LTBI are summarized in Table 3-1.


A
MIF IgA (LTBI)
Sensitivity %
100
80
60
40
20
0
0
20
40
60
80
100
100% - Specificity %
AUC = 0.7161
p < 0.0001


B
MIF IgA (ATB)
Sensitivity %
100
80
60
40
20
0
0
20
40
60
80
100
100% - Specificity %
AUC = 0.6919
p = 0.0002


C
MIF IgA (LTBI vs ATB)
Sensitivity %
100
80
60
40
20
0
0
20
40
60
80
100
100% - Specificity %
AUC = 0.6398
p = 0.0092

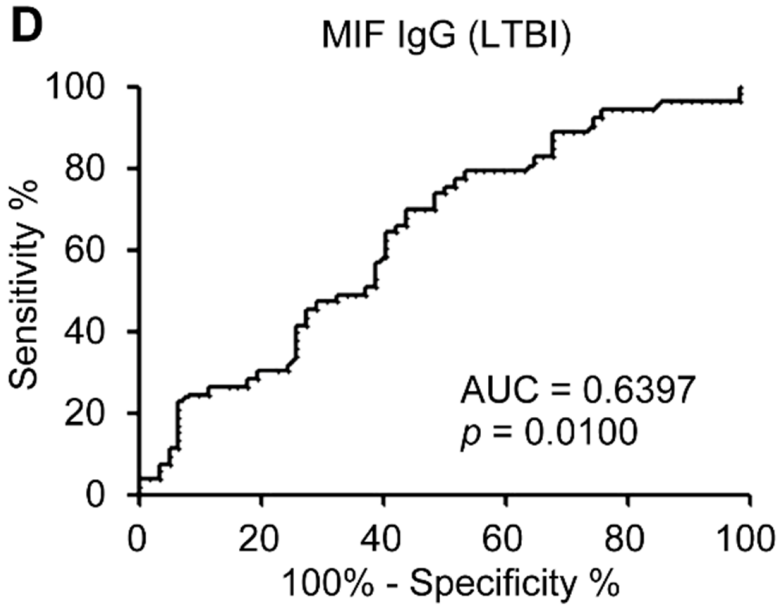

D
MIF IgG (LTBI)
Sensitivity %
100
80
60
40
20
0
0
20
40
60
80
100
100% - Specificity %
AUC = 0.6397
p = 0.0100

Figure 3-2. Receiver operating characteristic (ROC) curves for macrophage migration inhibitory factor-specific antibody responses.

ROC curves were used to evaluate the discriminatory power of anti-MIF IgA between non-infected (NI) and latent tuberculosis infection (LTBI) (A), NI and active tuberculosis (ATB) (B), as well as LTBI and ATB (C), and anti-MIF IgG between NI and LTBI (D). The AUCs and *p* values are indicated on the graphs.

Table 3-1. Efficiency of IgA and IgG against MIF as diagnostic markers for tuberculosis

Groups and antibody	AUC (*p* value)	Cutoff (OD)	Sensitivity (%)	Specificity (%)
IgA response to MIF				
NI vs. LTBI	0.7161 (< 0.0001)	0.7825	69.8	75.8
NI vs. ATB	0.6919 (< 0.001)	0.8835	69.2	67.7
LTBI vs. ATB	0.6398 (< 0.01)	0.7075	64.6	62.3
IgG response to MIF				
NI vs. LTBI	0.6397 (< 0.05)	1.0175	64.2	59.7

MIF, macrophage migration inhibitory factor; AUC, area under the curve; OD, optical density; NI, non-infected; LTBI, latent tuberculosis infection; ATB, active tuberculosis

3.3.2. IgA and IgG Serum Levels in Subgroup Analysis

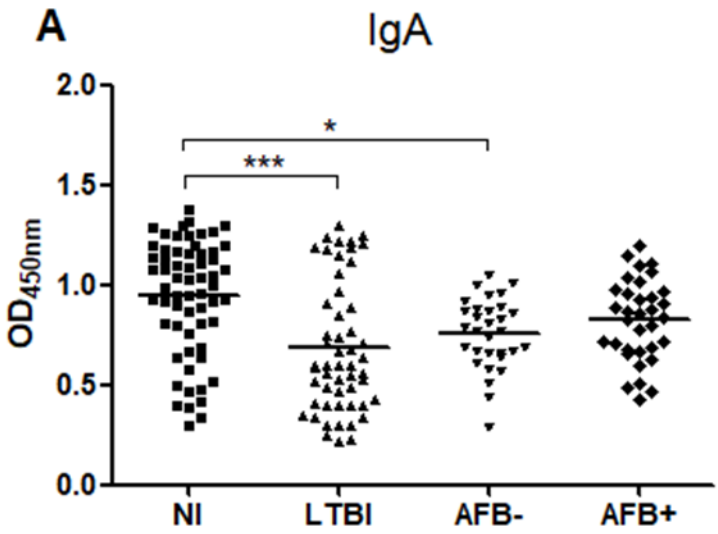
Figure 3-3A and 3-3B show the IgA and IgG responses to MIF when patients with ATB were classified into either AFB-negative or AFB-positive TB groups. The levels of IgA against MIF were significantly lower in the AFB-negative TB group than in the NI group (p = 0.011). However, no significant difference was observed between the NI and AFB- positive TB groups, or among the LTBI and both AFB-positive and AFB-negative TB groups (Figure 3-3A).

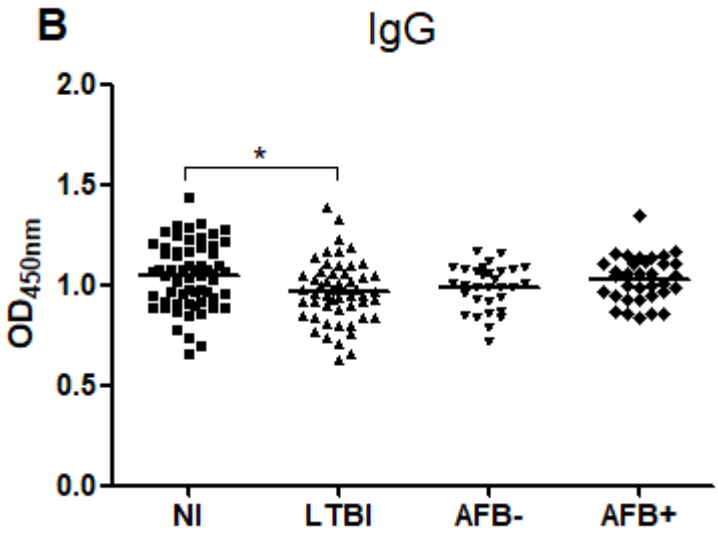
The levels of IgG against MIF showed no significant differences among the LTBI group or both the AFB-negative and AFB-positive TB groups, nor among the NI group and both the AFB-negative and AFB-positive TB groups (Figure 3-3B).

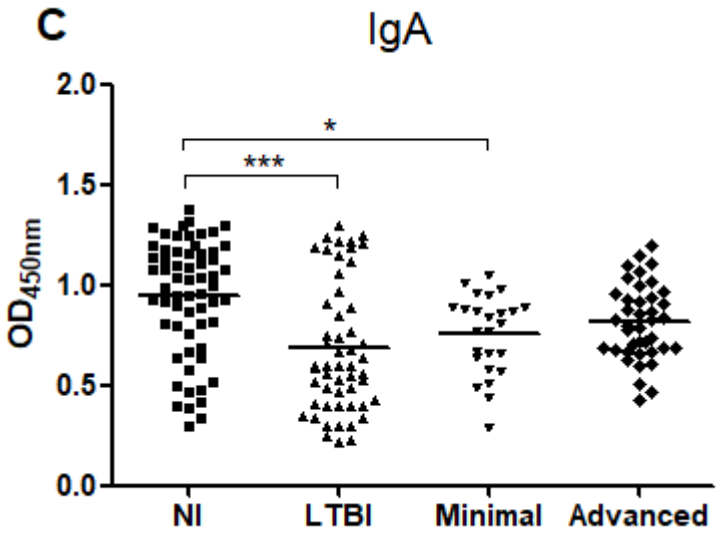
Moreover, when patients with ATB were categorized into minimal and advanced TB groups due to their radiological severity, IgA levels against MIF were found to be significantly lower in minimal TB groups than in the NI group (p = 0.021; Figure 3-3C). There was no significant difference in IgG levels against MIF between each subgroup of ATB and both the LTBI and NI groups (Figure 3-3D). The AFB positive rate was significantly higher at

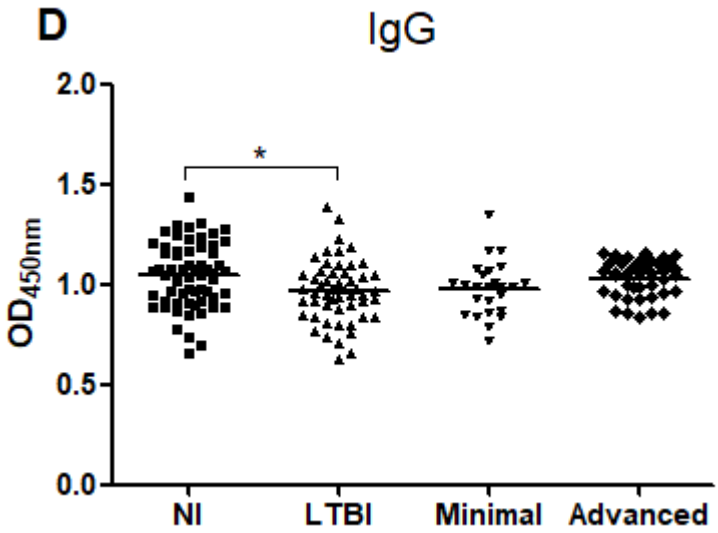
75.0% (n = 30/40) in the advanced TB group compared with 16.0% (n = 4/25) in the minimal TB group ($p < 0.0001$).

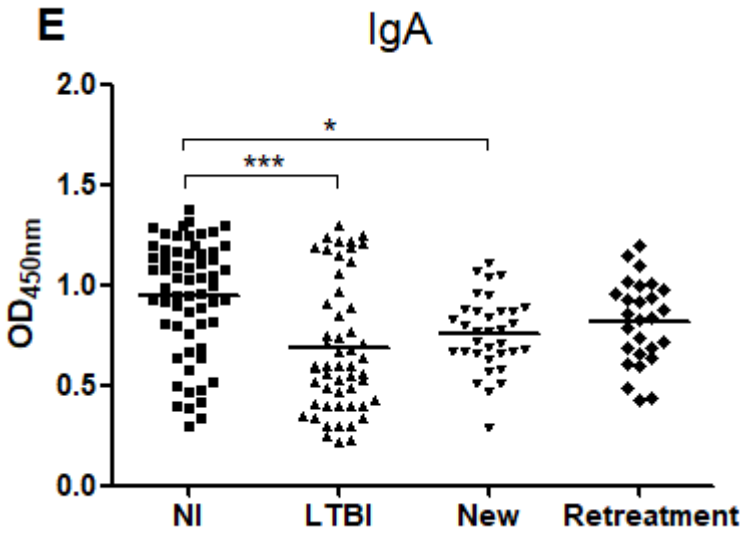
Figure 3-3E and 3-3F show the IgA and IgG responses to MIF when the patients with ATB were subdivided into either new patients or retreatment groups. IgA levels against MIF were significantly lower in new patients ($p = 0.010$) than in the NI group (Figure 3-3E). Further, there was no significant difference in IgG levels against MIF when comparing the other groups and both the new patients and retreatment groups (Figure 3-3F). There was also no significant difference in the AFB positive rate or radiological severity between the new patients and retreatment groups (data not shown).


A
IgA
2.0
1.5
1.0
0.5
0.0
OD450nm
*
***
NI
LTBI
AFB-
AFB+


B
IgG
2.0
1.5
1.0
0.5
0.0
OD450nm
*
NI
LTBI
AFB-
AFB+


C
IgA
2.0
1.5
1.0
0.5
0.0
OD450nm
*
***
NI
LTBI
Minimal
Advanced


D
IgG
2.0
1.5
1.0
0.5
0.0
OD450nm
*
NI
LTBI
Minimal
Advanced


E
IgA
2.0
1.5
1.0
0.5
0.0
OD450nm
*
***
NI
LTBI
New
Retreatment

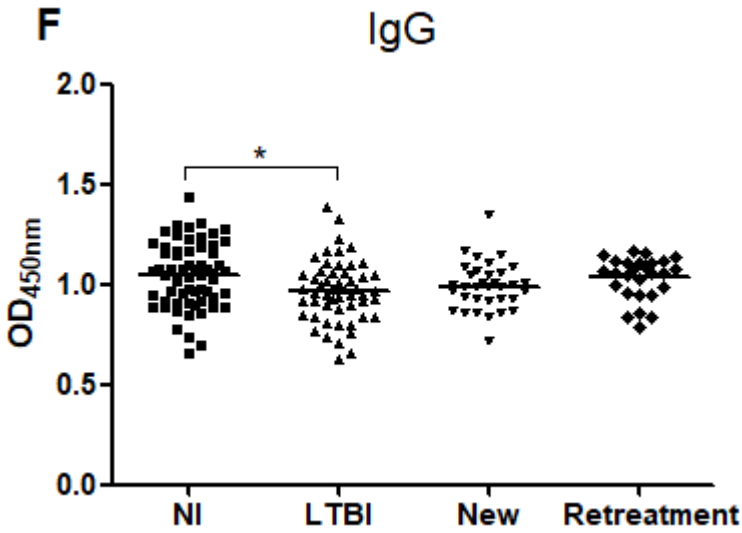

F
IgG
2.0
1.5
1.0
0.5
0.0
OD450nm
*
NI
LTBI
New
Retreatment

Figure 3-3. IgG and IgA responses to macrophage migration inhibitory factor in subgroups according to AFB smear results, radiological severity, and past TB treatment history.

Optical density (OD) values of the anti-MIF IgA (A) and anti-MIF IgG (B) serodiagnosis assays for the comparison of 31 acid-fast bacilli (AFB)-negative tuberculosis (AFB-) patients, 34 AFB-positive tuberculosis (AFB+) patients, 53 latent tuberculosis infection (LTBI) patients, and 62 non-infected (NI) individuals. OD values of the anti-MIF IgA (C) and anti-MIF IgG (D) serodiagnosis assays for the comparison of 25 minimal tuberculosis patients, 40 advanced tuberculosis patients, 53 LTBI patients, and 62 NI individuals. The OD values of the anti-MIF IgA (E) and anti-MIF IgG (F) serodiagnosis assays for the comparison of 34 new patients, 27 retreatment patients, 53 LTBI patients, and 62 NI individuals. Each dot represents the values obtained from individual subjects, and horizontal bars indicate the average values. Groups were compared using

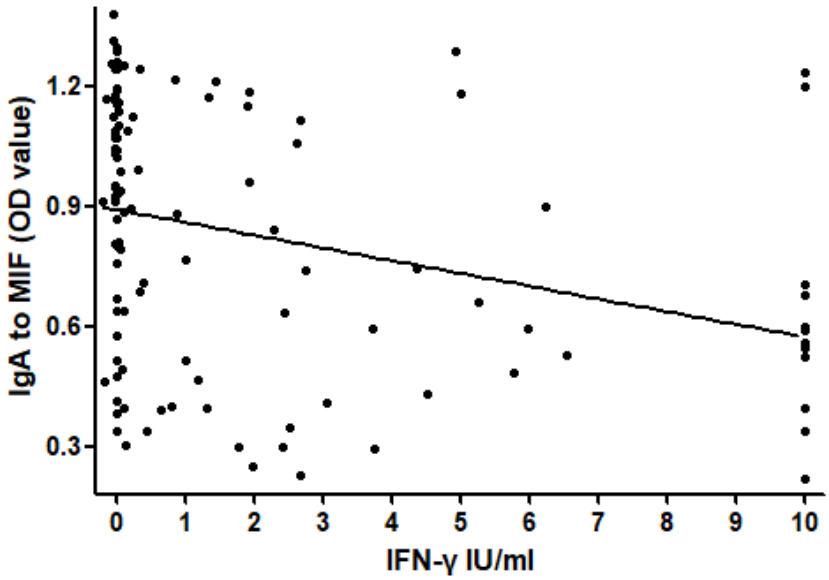
one-way ANOVA with Bonferroni' s multiple comparisons.

$*p < 0.05$, $***p < 0.001$.

3.3.3. Correlations Between IgA and IgG Serum Levels, and IFN-γ, Age, BMI, Sex, and Drug Resistance

A moderate significant positive correlation was observed between the serum IgA and IgG levels against MIF (r = 0.564, p < 0.0001). Meanwhile, weak negative correlations were observed between the level of IFN-γ induced in the QFT-GIT test and the OD values of serum IgA (r = − 0.325, p < 0.0001) and IgG (r = − 0.295, p = 0.001) against MIF (Figure 3-4).

A


IgA to MIF (OD value)
1.2
0.9
0.6
0.3
0
1
2
3
4
5
6
7
8
9
10
IFN-γ IU/ml

B

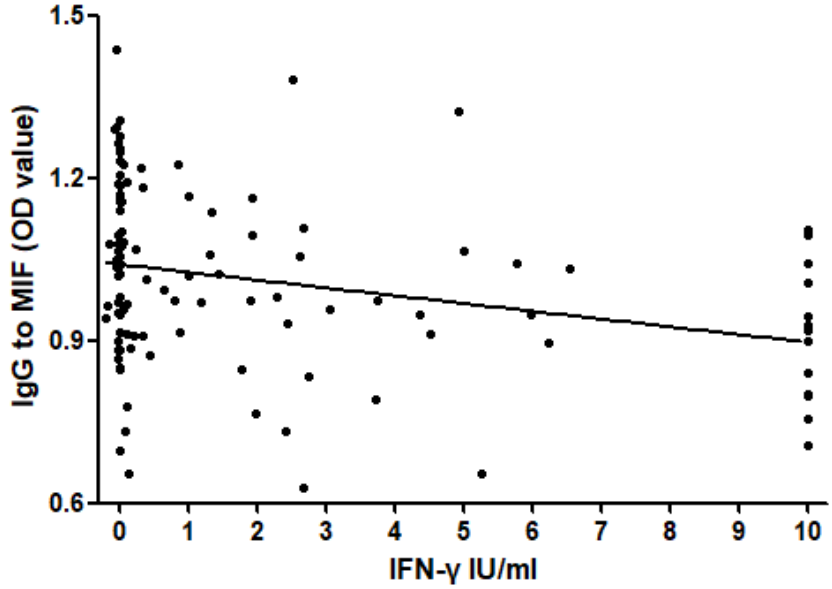

IgG to MIF (OD value)
1.5
1.2
0.9
0.6
0
1
2
3
4
5
6
7
8
9
10
IFN-γ IU/ml

Figure 3-4. Correlations between anti-macrophage migration inhibitory factor antibody and interferon-gamma levels.

Correlation between the level of interferon-gamma and the levels of serum IgA ($r = -0.325$, $p < 0.0001$) (A) and IgG ($r = -0.295$, $p = 0.001$) (B) against MIF.

Moreover, serum levels of both IgA (r = −0.422, p < 0.0001) and IgG (r = −0.254, p = 0.001) against MIF had a significant negative correlation with age. In addition, there was a significant negative correlation between BMI and IgG response against MIF (r = −0.201, p = 0.009), however, no significant correlation was observed between BMI and IgA response against MIF (r = −0.111, p = 0.154). There were also no significant differences in IgA and IgG levels against MIF based on sex and drug resistance.

3.4. Discussion

In this study, IgA and IgG responses were compared to MIF in ATB, LTBI, and NI groups. The IgG response against MIF was significantly lower in patients with LTBI than in NI individuals. The anti-MIF IgA levels were significantly lower in patients with both LTBI and ATB than in NI individuals. The level of IgA against MIF also showed significant predictive potential in the diagnosis of ATB and LTBI, according to ROC analysis, suggesting that mucosal immunity to MIF may be associated with TB pathogenesis. Individually, IgG and IgA antibodies against MIF could not achieve good sensitivity and specificity, respectively, to replace conventional diagnostics. In previous studies, higher predictive power could be achieved when tests of multiple markers were combined [37, 73, 74]. Because statistically significant differences in anti-MIF antibody levels existed between study groups, it may be possible to increase the predictive power of the existing method by combining it with other known markers.

Previous studies have reported increased MIF levels in TB [56, 75]. Meanwhile, in contrast to the protein level, the antibody levels against MIF decreased in TB and LTBI patients compared to those in NI individuals in the current study. MIF is expected to modulate

the induction or maintenance of Th1 responses in TB [76]. Therefore, it is inferred that low levels of MIF antibodies may be associated with elevated levels of MIF and macrophage activity in TB.

However, the results of this study showed no significant difference in IgA and IgG levels between ATB and LTBI. In addition, the antibody levels against MIF tended to increase in ATB, advanced TB, and AFB-positive TB patients compared to those in LTBI, minimal TB, and AFB-negative TB patients, respectively, although the increase was not significant. These results are consistent with previous studies, which reported that circulating MIF levels were not necessarily higher in patients with moderate or advanced TB compared to patients with minimal TB [75]. MIF is secreted from T lymphocytes and macrophages, as well as from anterior pituitary cells, in response to lipid polysaccharide stimulation [77]. Since the level of MIF can be regulated through both the immune and endocrine systems, it is possible that various regulatory mechanisms are involved in the regulation of levels of MIF, as well as the antibodies against MIF.

In the present study, both IgA and IgG responses to MIF showed a significant negative correlation with IFN-γ level, suggesting that IgA and IgG responses against MIF can complement

current methods used to diagnose LTBI. It has also been reported that IFN-γ regulates both the synthesis and secretion of MIF [78]. Meanwhile, MIF secretion from macrophages is reportedly induced by TNF-α and IFN-γ [79].

Although currently there are few reports on anti-MIF antibodies, anti-IFN-γ antibodies have been previously described in patients with TB, NTM, HIV, and African trypanosomiasis [68, 80, 81]. Anti-IFN-γ autoantibodies appear to be associated with inhibition of downstream cytokine induction. Patel et al. found that autoantibodies against IFN-γ antibodies existed at high titers in severe nontuberculous mycobacterial infections [68]. In addition, Madariaga et al. demonstrated the presence of anti-IFN-γ autoantibodies in the serum of TB patients [80]. They hypothesized that as the disease progresses, the level of autoantibody production increases, and that suppression of the Th1-type immune response contributes to progression of the disease [80].

Autoantibodies against cytokines have been reported in several previous studies, however, their physiological and pathophysiological significance is largely unknown [58]. In some studies, it was assumed that cytokine autoantibodies regulate biological activity by neutralizing cytokines [82], and serve as reservoirs for cytokines by extending their half-life through

cytokine-autoantibody complexes [83]. Therefore, it is speculated that the antibody response to MIF observed in the current study is likely to be related to the antagonistic effect on MIF expression induced via IFN-γ. Previous studies showed that autoantibodies against GM-CSF [63], IL-2 [84], IL-6 [85], and IFN-γ [62] neutralized the respective cytokines in vitro. The levels of anti-IL-6 Ab were quantified in 4,230 blood donors, and high anti-IL-6 Ab titers were associated with IL-6 deficiency in vivo [85]. Considering these previous results, it would be supposed that low levels of anti-MIF autoantibodies in patients with tuberculosis might be associated with modulation of MIF activation. To clarify this mechanism, it would be necessary to measure the levels of anti-MIF autoantibodies and perform MIF neutralization assays in future studies.

Certain limitations were noted in the current study. First, the production of MIF was not quantified. In addition, it was not confirmed whether anti-MIF autoantibodies were affected by other cytokines, the levels of which may increase in TB infection. Hence, further studies are needed to confirm the functional relationship between anti-MIF autoantibodies and MIF.

3.5. Conclusion

Collectively, this is the first study to analyze responses to MIF in NI, LTBI, and ATB groups. Further, IgA antibody responses to MIF could be a potential target for diagnosing LTBI. Although none of the antibodies could achieve high diagnostic predictive power individually, these findings suggest that antibodies to MIF have the potential to be used in the development of rapid assays to complement the existing TB diagnostic methods.

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국문초록

혈청학적 결핵 진단법의 유용성에 관한 연구

이지연
서울대학교 대학원
의학과 미생물학전공
지도교수 국윤호

배경: 공공 보건의 중대한 문제인 결핵을 효과적으로 관리하기 위해서는 조기 진단이 필수적이지만, 기존 검사법들은 검사 시간, 비용, 정확성 및 재현성을 포함한 많은 제한점이 있다. 혈청학적 검사는 기존 방법의 한계를 개선할 수 있는 잠재력을 제공하므로, 본 연구에서는 혈청학적 결핵 진단에 유용한 새로운 지표들을 개발하고자 하였다.

방법: 결핵균 항원 (TBCM, Ag85B, ESAT-6, CFP-10) 및 대식세포 이동저지인자 (macrophage migration inhibitory factor, MIF)에 대한 항체 반응을 65 명의 활동성 결핵, 53 명의 잠복 결핵 및 62 명의 비감염 군에서 측정하였다. 효소결합면역흡착검사를 사용하여 연구 참가자의 혈청에서 IgG 및 IgA 수준을 측정하였다. 잠복 결핵 진단에는 QuantiFERON-TB Gold In-Tube 검사를 사용하였다.

결과: TBCM에 대한 IgG 수준은 비감염군에 비해 잠복 결핵군에서 유의하게 높았다. Ag85B에 대한 IgG 및 IgA 수준과 CFP-10에 대한 IgG 수준은 활동성 결핵 군에서 가장 높았고, 다음으로 잠복 결핵, 비감염군 순이었다. 활동성 결핵군을 객담 항산균 도말 검사 결과, 영상학적 중증도 및 결핵 치료 과거력에 따라 세분화하였을 때, Ag85B 및 CFP-10에 대한 IgG 수준은 잠복 결핵군 및 비감염군에 비하여 활동성 결핵군의 각 하위 군에서 유의하게 높았다. Ag85B, TBCM 및 CFP-10에 대한 IgG 수준과 Ag85B에 대한 IgA 수준은 잠복 결핵 및 비감염군에서 IFN-γ 수준과 유의한 양의 상관 관계를 보였다. 연령 및 성별을 보정한 모델에서 TBCM 및 CFP-10에 대한 IgG는 잠복 결핵 진단과 독립적으로 유의한 연관성을 보였으며, Ag85B에 대한 IgG는 활동성 결핵 진단 및 잠복결핵과 활동성 결핵의 감별진단과 독립적인 연관성을 보였다.

MIF에 대한 IgA 수준은 비감염군보다 잠복 결핵 및 활동성 결핵군에서 유의하게 낮았으며, 잠복 결핵 진단, 활동성 결핵 진단 및 잠복결핵과 활동성 결핵의 감별진단과 유의한 연관성을 보였다. MIF에 대한 IgG 수준은 비감염군보다 잠복 결핵군에서 유의하게 낮았으며 잠복 결핵 진단과 유의한 연관성이 있었다. MIF에 대한 IgA 수준은 항산균 도말 음성 활동성 결핵, 영상학적 경증 활동성 결핵 및 결핵 신환자에서 각각 비감염군에 비하여 유의하게 낮았다. MIF에 대한 IgA 및 IgG 수준은 IFN-γ 수준과 유의한 음의 상관 관계를 나타내었다.

결론: TBCM, Ag85B 및 CFP-10에 대한 IgG 항체 반응은 활동성 결핵, 잠복 결핵 및 비감염 군을 유의하게 구분하였으며, MIF에 대한 IgA 항체 반응은 잠복 결핵 및 활동성 결핵의 진단과 유의한 연관성을 보여 결핵의 새로운 혈청학적 진단법 개발을 위한 활용 가능성을 보여준다.

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주요어 : 결핵, 혈청학, 진단, 생물표지자, 대식세포이동저지인자, 사이토카인

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