PPE 59 immunoreactivity in tuberculosis patients: IgA immunodominance to that IgG and IgM

Ana Carla de Paulo Mulinari
Fundacao Oswaldo Cruz
Isabela Gama Sardella
Fundacao Oswaldo Cruz
Vania Maria C. da Silva
Universidade Federal do Rio de Janeiro Faculdade de Medicina
Anna Cristina C. Carvalho
Fundacao Oswaldo Cruz
Alberto Matteelli
Universita degli Studi di Brescia
Maria Helena Féres Saad (saad@ioc.fiocruz.br)
Fundacao Oswaldo Cruz https://orcid.org/0000-0002-7882-3977

Research article

Keywords: Mycobacterium tuberculosis, tuberculosis, antibody, serology, PPE59 (rv3429), IgA

Posted Date: March 10th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-16530/v1

Abstract

Background: The role of protein families PE/PPE remains relatively enlightening. The gene rv3429, coding the PPE59 protein, is one of the 12 members of the PPE subfamily, and it is absent in all BCG strains. Although elicits low T cell response, there are no clues for the ability to induce a humoral response.

Methods: We cloned, expressed, and analyzed by ELISA IgG, IgM and IgA anti PPE59 in 212 sera. Of them, 69 were from tuberculosis patients' residents in Italy (TB^{IT}, 12 native and 67 immigrants), the remaining 133 are Brazilians citizens (BR). Pulmonary (pTB^{IT}) or extrapulmonary (eTB^{IT}) clinical forms were 54 or 25. The pTB^{BR} were 52 and 81 non-TB patients, including 10 non-tuberculous mycobacteria infected (NTM).

Results: Keeping the specificity at 97%, IgA sensitivity decreased from pTB^{BR} (53%) to pTB^{IT} (38%) and eTB^{IT} (28%), with an overall sensitivity of 42.7% and moderate accuracy (61.8 %), while IgG showed significant lower ($p<0.0001$) performance, 21%, 3%, 0%, 9% and 37%, respectively, at specificity of 83%. Groups were not discriminate by IgM. False-positive NTM IgG was high. Combination 16kDa IgG, previously performed only on Bazilians' sera, plus PPE 59 IgA results increased sensitivity to 71% at 87.4% specificity. The overall smear microscopy (SM) diagnosis was 70.8% and a combination of SM/IgA increased sensitivity to 74% ($p=0.01$), mainly among SM- cases. Among pTB^{BR}, all these rapid tests, including SM, sensitivity reach 86.5% ($p=0.001$). Positive IgA polarization was significant in extensive lung disease ($p=0.001$) and alcohol users ($p=0.04$).

Conclusion: Although PPE59 IgA independently has moderate accuracy on TB diagnosis, together with other biomarkers contributes to improving its detection. Moreover, the polarized reactivity deserves further investigation.

Background

Despite the availability of effective treatment for tuberculosis (TB), a disease caused by Mycobacterium tuberculosis bacilli (MTB), remains among the ten main causes of death globally and the leading cause of mortality from a single infectious agent, overcoming HIV/AIDS [1]. Diagnosis of active TB is based on the initial clinical suspicion and radiological findings, and subsequently on the laboratory confirmation [2]. Delay in TB diagnosis prevents early treatment, playing a role in the maintenance of TB transmission in the community [3]. The oldest immune-based tests for TB diagnosis, i.e, tuberculin skin test (TST) and serology, although relatively cheap, have low specificity because of cross-reactivity with antigens both present on vaccine bacilli Calmette Guérin (BCG) and non-tuberculous mycobacteria (NTM) species [4]. Respect to TST, serology has the potential of being used at point-of-care and can be performed at work field without onsite microscopy services, particularly useful when dealing with special populations, such as immigrants, indigenous, homeless [5; 6].

The protein members of PPE (Pro-Pro-Glu) family have not been fully explored; recent studies have suggested the involvement of PPE family in many aspects of pathogenesis, including bacterial attachment to host cells, immunomodulation, and ability to persist in granuloma [7]. The importance of the PPE proteins in the generation of antigenic variations has been shown. The PPE41 was reported to induce immune responses of B cells using a panel of human sera from patients with active TB and relapsing patients with extrapulmonary TB [8; 9]. The PPE Rv1168c was described as having strong specific immunoreactivity in sera of patients with active TB compared to that PPD, ESAT-6, and hsp60 [10]. On the other hand, PPE55 protein is differentially recognized by antibodies elicited during subclinical TB infection in animal models, as well as their peptide PPE-C in humans with clinical TB-HIV negative and months prior TB manifestation on HIV positive cases, suggesting been useful in different status of TB [11].

Therefore, the PPE proteins have the potential to be specific markers for the diagnosis of tuberculosis. The rv3429, encoding the protein PPE59, has low homology in the C-terminal region with other mycobacterial species and was described to elicit low T cell immune response [12], but there are no clues about its ability to induce a humoral response. The PPE 59 bioinformatics' analysis revealed seven B cell epitopes, belonging to the different region 11 that is absent in M. bovis BCG strains and in other mycobacterial species [12; 13; 14]. The aim of this study was to clone and express the rv3429 gene coding PPE59 and to screen immunoreactivity to IgG, IgM, and IgA in sera of TB patients using an in-house ELISA method.

Methods

Cloning, expression and purification of recombinant PPE 59 protein

The *rv3429* gene sequence encoding the PPE 59 protein was generated by PCR from the genomic DNA of H37Rv using upstream 5´GCG^GATCCATGCATCCAATGATA3´ and downstream 5´CA^AGCTTCTACCCGCCCCCGCCCCCGTA3´ primers, comprising BamHI and HindIII restriction endonucleases, respectively. The standard conditions for thermal cycle amplification was 94°C for 5 min; 25 cycles of 1 min each of 94°C, 60. 6°C, and 72°C; final step of at 72°C for 5 min. The cloning, expression and purification were performed as described previously with minor modifications on protein purification [15]. Briefly, the transformed expression system containing the pQE80L-*rv3429* were better isopropyl-beta-D-thiogalactopyranoside (IPTG) 1mM induced at 37°C for 4h. The recombinant proteins were mainly present in the insoluble fraction, and therefore, the purification of His tag fusion protein was carried out under denaturing condition with 8M urea lyses buffer. The over expressed recombinant proteins were confirmed by probing with anti-His tag monoclonal antibody and SDS-PAGE analyzed. Solubilized protein was loaded into HisLink Protein Purification Resin (Promega, USA) and eluted at different concentrations (5mM, 20mM, 100mM and 250mM) of imidazole (Quiagen, EUA) buffer and of urea (8M e 6M).

Subjects

pTB^{BR} or pTB^{IT}: confirmed pulmonary TB; eTB^{IT}: confirmed extrapulmonary TB; ORD^{BR}: other respiratory diseases (different etiologies); NTM: non-tuberculous mycobacteria infection; HD^{BR}: individuals with no history or contact with TB.

The study was performed according to the code of Ethics of the World Medical Association (Declaration of Helsinki) and was approved by the Fiocruz Ethics Committee in Research (No. 560-10) and the Clinical Ethics Committee di Malattie Infettive - Spedali Civili of the Institute of Infectious and Tropical Diseases of University of Brescia, Italy (N. 196/03 -11). As depicted in figure 1, the group I comprised 133 Brazilians (BR) subjects, 52 presenting the pulmonary form (pTB^{BR}) and 81 subjects were added as control, of which 55 patients with other respiratory diseases (ORD^{BR}): asthma, bronchitis, sinusitis, pneumonia, Wegener's granulomatosis, chronic obstructive pulmonary disease (COPD) and cancer (PD/CA), individuals that experienced recent contact with active TB patients (rCt), 10 NTM infected patients with pulmonary infection by *M. avium*, *M. avium-intracellulare, M.fortuitum, M. abscessus, M. kansasii* or *M. szulgai (*kindly donated by R. Olmo, LAHAN/IOC/FIOCRUZ); and 16 healthy donors (HD^{BR}) with unknown history of TB. Brazilian subjects were prospectively recruited in a previous study [16], in one year period (2003-2004), and had their diagnosis done at a primary health care center (Centro de Saúde Municipal Heitor Beltrão), in Rio de Janeiro, a Brazilian endemic area for TB. The group II with 79 TB patients born (n=12) or immigrants in Italy (n=67) (IT), 54 presenting the pulmonary form (pTB^{IT}) and 25 were extrapulmonary (eTB^{IT}). Immigrants enrolled are Africans (n=22), of which 10 Senegalese, 7 Marroquin and one, each, from Gambia, Ghana, Kenia, Liberia and Nigeria; Asians (n=34), of which 20 from India, 12 from Pakistan and 2 Philippines; and 10 Europeans (4 Romanian, 3 Moldavian, 2 from Kosovo and 1 Russian), and 1 was Brazilian. Body sites of eTB^{IT} included lymphnode (n=10), bone (n=3), vertebra (n=2), pleural, intestinal, adrenal or epididymitis (one, each), and multiple tissue (n=6). These patients had TB diagnosed and treated at Infectious and Tropical Diseases Clinic of Azienda Ospedaliera Spedali Civili di Brescia, Italy, in the period from 2011 to 2012. All individuals were ≥18 years and HIV negative. TB diagnosis were based on clinical, radiographic and microbiological methods. Sputum microscopy (SM) performed using the hot Ziehl-Neelsen method and semi quantitative acid-fast bacilli analyzed as positive or negative. Chest X ray (CXRs) changes classified as low (normal or suggestive of pulmonary disease), intermediate (infiltrates) and high probability for TB (infiltrates and/or cavities) [17]. Patients who had negative microbiological tests but clinical and laboratory response to anti-TB treatment were diagnosed with probable TB. TB^{IT} patients were tested by interferon-gamma release assay (IGRA) using Quantiferon Gold Tube® for *M. tuberculosis* infection detection.

Enzyme Linked Immunosorbent Assay (ELISA)

In order to analyze the individual immune response of IgG, IgM and IgA to the PPE59 protein, ELISA was standardized for each isotype using a pool of sera from 10 pTB^{BR} and 16 HD^{BR}, half-positive or negative TST. The tests were performed in microplates with 96 wells-flat bottom (Nunc, USA) coated with antigen at the 0.5 and 1.0µg/ml prepared in coating buffer

carbonate/bicarbonate, pH=9.6 and incubated at 37°C for 2h. The test was performed as described previously [15] with minor modification. The 1:1000 diluted HRP-conjugate goat anti-human IgG and -IgM (Thermo Fisher Pierce, USA) and the 1:2500 diluted anti-human IgA were used. Sera aliquots used did not experience the freezer-thaw cycle before testing. Individual response of all samples was blind tested (the operator had no knowledge on samples characteristics). Taking advantage of the Brazilian sera previously were IgG tested with other mycobacterial antigens [16], we analyzed the results combining with those obtained for PPE59 IgG, IgM or IgA.

Data analysis

Diagnostic codes were open at the end of all tests, and healthy donors and ORD^{BR} were used as reference control groups for receiver operating characteristic curve analysis (ROC), defining the cut off value for sensitivity and specificity calculation. Antibodies levels were analyzed as a function of medium absorbance and minimum and maximum. GraphPad Prism 7 software (San Diego, CA, USA) was applied for statistical analysis. As the data was not normally distributed, non-parametric Kruskal Wallis was performed to analyze significant differences in all groups and Mann-Whitney test was used for pairwise comparisons, while the Fisher exact test and Chi-Square test with Yates's correction was used to evaluate the significance of the difference between the numbers of positives of different serology tests. Accuracy was calculated over the total of truly positive and truly negative, relative to the sample studied, formulae: (true positive + true negative) / total number of populations studied. Having as indicators of accuracy the values between 0 - 40 % said as low accuracy, 50% - 70% moderate and 80% - 100% high accuracy. Odds ratios (OR) and logistic regression analyzes was used in the uni and multivariable analyzes, p values ≤ 0.05 were considered significant with their respective 95% confidence intervals. SPSS Statistics 18 (IBM Corporation) was applied for data analyze.

Results

Characteristics of study subjects

The clinical, epidemiological and laboratory characteristics of the adults' participants are shown in table 1. The group I, HD^{BR} participants was younger than the other patients were (p=0.01) and most of the patients were males, for both groups. Opposite to group II, the group I was significantly alcohol users (p=0.004). Contrary to the group I (65.3%), the majority of group II (88.6%) patients were diagnosed through positive SM and/or culture; only seven pTB^{BR} and five pTB^{IT} had probable TB. Approximately 60% of the pTB^{BR} showed CXR findings of high probability for TB, while in the Italians/immigrants' cases intermediary changes predominated (p=0.03). In group I, the presence of BCG scar was high among HD^{BR} (100%), followed by ORD^{BR} (49%) and pTB^{BR} (38%), while the frequency of positive TST performed in the way back: 50.0%, 61.8%, and 78.8%, respectively. Information on TST was not available to study group II, but Quantiferon® was performed in 53% of them, of which 44% eTB^{IT} and 48% pTB^{IT} were positive. Most of TB^{IT} patients (84.8%) were already on treatment with first-line TB drugs, while TB^{BR} were free of treatment, at blood withdrawn.

Cloning, expression, and purification of PPE 59 protein

The 534pb of rv3429 gene was successfully cloned into pQE80L and most of the expressed PPE 59 protein was present in the inclusion bodies of the pellet being purified under denaturing condition with urea 8M. The release of the target protein from the Ni-resin column with expected molecular weight (21 kDa) occurred in the eluate containing 6M urea and 100mM imidazole, and the concentration of the expressed protein was high, 700 µg/ml (figure 2). The protein proved to be stable under stored condition (4°C or 8°C), and the same batch was used for all immunological tests.

Purification process under denaturing conditions, incubated with mouse IgG anti-His. Lane 1, pre-stained protein molecular ladder; lane 2, positive control; line 3, overnight pellet; line 4, supernatant; lines 5 and 6, eluates of PPE59 (MW=21kDa).

Detection of humoral responses by quantitative IgA, IgG and IgM ELISA PPE59:

The pTB IgM class serum levels were not significantly different from HD^{BR} with positive or negative TST (p=0.17) and the results remained inconclusive even after testing the pools of sera individually, then it was not further analyzed. There were, however, significant differences for IgG and IgA responses (p=0.02) (Figure 3). For further analysis, 1:50 serum dilution was applied in individuals' tests.

Was used pools of sera from 20 Brazilians individuals with pulmonary tuberculosis (•) and 8 healthy donors positive (■) or negative (▲) TST, respectively.

After compilation of all ELISA results, patients' clinical forms were open and, in general, sera of TB participants showed IgA medians higher that IgG in all groups studied (p< 0.0001). The PPE59 specific IgA medians in pTB sera was highly significant related to all controls (ORD/HD, p<0.0001). Stratifying the population by geographic area of origin, medians of absorbance slightly decreased from Brazilians [1.039 (0.863-1.142)] through Africans [0.924 (0.515-1.277)] and Asians [0.890 (0.623-1.210)] to Europeans pTB patients [0.770 (0.651-1.182), p=0.89]. For eTB Asians had higher absorbance median [0.866 (0.532-1.155)], followed by Africans [0.609 (0.300-1.006)] and Europeans [0.593; (0.448-0.851); p=0.31], however not statistically significant to that pTB (p>0.0954). On the other hand, pTB^{BR} [0.435 (0.301-0.662)] and Europeans [0.334 (0.207-0.443)], although lower, elicited higher medians of PPE59-IgG compared to eTB^{IT}, except for Europeans [0.196 (0.159-0.380)], which median was lower to that ORD^{BR} [0.469 (0.351-0.630)] and HD^{BR} [0.241 (0.167-0.377)]. Contrary to IgA, NTM elicited significant higher IgG median (0.904 (0.806-1.107) compared with all other individuals (p< 0.001) (Table 2).

Humoral responses by qualitative IgA and IgG ELISA PPE59

Based on the differences observed in the quantitative analysis of the serologic test, we further performed a ROC curve analysis to evaluate its potential diagnosis. The value of the area under the curve (AUC) was higher for IgA (0.823) than IgG (0.467) as well as the cut-off of 1.030 (with LR of 21.81) and 0.732 (with LR of 1.043), respectively (Figure 4). The predicted sensitivity at the high specificity was variable, which denote heterogeneity of antibodies response according to population tested (table 3). The pTB^{BR} ELISA IgA sensitivity was 53.3%, decreasing toward pTB^{IT} (38.2%) and eTB^{IT} (28%), with an overall detection of 42.7%. The IgG sensitivity was 21.3%, 3%, and 0%, respectively, with an overall detection of 9%, at a lower specificity (83.1%) (Table 3). IgG false positive was high among NTM and rCt (Figure 5). The overall accuracy of ELISA PPE59 IgA to detect TB was moderate (61.8%) with higher positive predictive valor (PPV=96.5%) but low negative one (NPV=47.9%), while the low for IgG. A combination of positive scores obtained by both isotypes tested did not improve the accuracy, because only slightly increase the sensitivity jeopardizing the specificity (Figure 4).

Area under the curve (AUC) was calculated with 52 TB patients and 81 controls. ROC (95% CI). PPV: positive predictive value; NPV: negative predictive value; A: accuracy.

Dotted line: Cut-off point 1.030 for PPE59-IgA and 0.732 for PPE59-IgG. Light grey: Stratified ORD by asthma, bronchitis, sinusitis, pneumonia, Wegener's granulomatosis, cancer and chronic obstructive pulmonary disease (PD/CA), recent contact of active TB patient (rCt) and NTM: nontuberculous mycobacteria pulmonary infection. 5A and 5B: ELISA PPE59-IgA and IgG dot plot, respectively.

TB Detection value of IgA and IgG PPE59 combined with results of five other antigens previously tested

Taking advantage of the Brazilians sera previously tested with other mycobacterial antigens [16], we analyzed the results combining with those for the present PPE59 IgA and/or IgG (Table 3). In the former study lower ELISA sensitivity for the single antigens ESAT-6 (22.5%), 16kDa (37.1%), MT10.3 (23.6%), MPT-64 (36%) or 38kDa (33.7%) IgG, with specificity ≤ 92%. However, the combination of positive results of PPE59 IgA plus ESAT-6 or 38 kDa IgG increased the sensitivity (55.8% or 59.6%), without jeopardizing the specificity (90.2% or 94.4%). The combination of PPE59 IgA with 16kDa-IgG reach the best sensitivity (71.1%), maintaining good specificity (87.4%). The other results of IgA and/or IgG combinations did not perform better (Table 4).

ELISA performance compared to smear microscopy results

Typically, although microscopy is not powerful enough when used solely as the sole screening and / or diagnostic method for TB it performed easily, rapidly and at a low cost. Thus, we combine both methodologies to improve the detection of patients with active TB. The SM plus ELISA PPE59-IgA results increased overall TB detection to 74%, while SM detects 70.8% (p=0.01). ELISA PPE59 IgG showed no improvement, performed lower to that SM alone (p=0.69). Stratifying the TB groups, the increase was significant in the Groups I for positive SM/IgA, improving sensitivity to 80.7% (p=0.01), once serology detects 12 negative SM pTB^{BR} patients or test not applied (n=3). While in Group II slightly increased sensitivity detecting four of the negative SM eTB^{IT} (p=0.26) and one pTB^{IT} (p=0.82). Combining SM, PPE59 IgA and 16kDa IgG ELISA results (available only to Brazilians), there was an improvement in sensitivity, as those not detected by PPE59 IgA (n=10, of which 6 SM- and 4 SM not done) 16kDa IgG recognized 3 subjects (two SM- and one not done), bringing the sensitivity to 86.5% (p=0.001) (Table 5).

Uni and multivariate analysis based on IgA and IgG ELISA PPE59

In tables 6 and 7, we present the association of the PPE59 reactivity and clinical and demographic characteristic of Group I patients. In the univariate analysis, PPE59 IgA positivity was associated with being male, alcohol and tobacco user, TST reactor, having positive sputum SM and abnormal CXR ($p<0.003$). On the other hand, patients aged ≥40 years were more likely of having a positive IgG result (OR=2.8; 95% CI: 1.0-7.9; p=0.04) as well as alcoholic (OR=3.2; 95% CI: 1.1-8.8; p = 0.02) and those with CXR suggestive findings for high-probability TB (OR=3.6; 95% CI 1.3-9.9; p=0.00). However, in the multivariate model, the variables independently associated with a positive PPE59 IgA result were male sex (OR=2.9; 95% CI 1.0-8.0; p=0.03), alcohol user (OR=2.7; 95% CI: 1.0-7.2; p=0.04) and CXR findings highly suggestive of TB (OR=27.8; 95% CI: 2.7-281.2; p=0.00). Alcohol use remained independently associated with PPE59 IgG positivity (OR= 3.0; 95% CI 0.9-9.6; p=0.05). In the studied Group II there were no statistically significant associations between clinical and laboratorial variables and positivity for both IgA and IgG PP59 ELISA (p>0.30).

Discussion

The rv3429c gene, which encodes the PPE59 protein, is located in the RD11, a region absent from all BCG strains [18; 15] and corresponds to 5 ORFs in the Mycobacterium tuberculosis genome, coding also for the TB 7.7 antigen [18], which is used in the commercial interferon-gamma release assay for TB/latent infection identification [19; 20]. Moreover, the predicted location is the cellular periplasm, between the inner membrane and the cytoplasmic, and the successful cloning and high expression of the PPE59 protein described here justify its evaluation.

Does IgA PPE59 is a potential biomarker to compose a TB diagnostic tool? Over the decades, several mycobacterial antigens studied have revealed the potential for TB diagnostic testing including the use of multiple antigens, as recently 57 field-based multiplexed serological assays of M. tuberculosis, and reference assay based on 132 antigens applied to screen patients with suspected TB symptoms on two continents [26]. As a result, IgG performed similarly for three or multiple antigens, with a sensitivity of 35% at 90% specificity. However, our study, in response to a single antigen, achieved an overall result of 42.7%, performing better for the IgA than IgG, with higher specificity (97.5%) even involving sera of TB patients from different geographic areas and clinical forms of TB.

Combination of IgA PPE59 with other biomarkers improves sensitivity? It is noteworthy the sensitivity improvement in ELISA IgA results when combinations of PPE59 IgA plus ESAT-6 or 38 kDa IgG were analyzed, maintaining better or similar specificity to Broger et al (2017) study. Variability in the median levels of reactivity found by these authors, among Vietnamese and Peruvian samples, was also observed in our study, where the absorbance was higher in the Brazilian cohort, decreasing toward Europeans but without achieve statistical significance, except for IgG. However, it is important to note that the lack of proper control from a non-endemic area may limit the interpretation of our results. Our findings confirm that due to heterogeneity in the recognition of antigens by serum antibodies in TB, multi-cocktails of antigens or their peptides remain the best strategy to potentiate a diagnostic test accuracy; the major challenge is to find the best antigens and isotypes combination [13; 27]. Note that combination of PPE 59 IgA plus 16kda IgG bring the sensitivity to 74%, and adding the results of other rapid tests (SM = 66%) the sensitivity reaches 86.5% (Sp = 87.4%), making this combination a potential target product profile triage test in TB high burden area. However, this data based only on the Brazilian population requires further evaluation. In addition, the

sensitivity of SM was significantly variable (53–78.2%, p = 0.002), however despite ELISA PPE59 was not powerful enough to equally diagnosing the TB, serology improved case finding, mainly among negative SM cases of pulmonary or extrapulmonary clinical form, based on IgA PPE 59 single antigen or multiple antigens and isotypes. Thus, could be explained by Ab production that may not dependent on bacillary load and produced in the body site of infection. Therefore, serology may be an adjuvant tool detecting TB paucibacillary cases and PPE 59 IgA advantage is the high specificity.

Does the antibodies' isotypes choice important in a serological test? Increased IgA levels were mainly found among patients' control group with COPD, smokers, and rCt. COPD in some studies is considered a risk factor for the development of pulmonary TB, and both diseases have common risk factors, such as smoking and low socioeconomic status [28]. Moreover, the two ORD individuals who were positive for PPE59 IgA had a history of recent contact with active TB patients; therefore, these participants could have been infected by M. tuberculosis and potentially progress for active TB. Contrary to PPE59 IgA, NTM IgG cross-reactivity was higher; corroborating that beside antigens, the isotypes choice can be a big deal as TB biomarker.

To date, the actual function of PPE59 protein in metabolism and its evolutionary role in M. tuberculosis is still unknown. The role of PPE59 protein as an inducer of cell-mediated response by IFN-γ and interleukin-1 was previously described [14; 15], but as a result of this present study, the dominance of IgA reactivity and lower-IgG in the sera of patients with TB was uncovered. In a study carried out in China and India, the PPE 57 (rv3425) and PPE 17 (rv1168) proteins, respectively, were recognized by IgG antibodies with high sensitivity and specificity (100%), even in extrapulmonary TB cases [11; 29]. However, opposite to our study, the control group was smaller (n = 20) and did not comprise ORD patients. These results may also suggest that PPE59 and PPE57 bacteria expression may changes under different host conditions since recognition occurs by different antibodies isotypes.

Comparing with other serological biomarkers associated with risk factor for TB. Studies with different antigens showed that IgG ESAT6/16 kDa/HBHA was increased in active TB (declining after treatment), and in latently infected contact, suggesting that specific antibodies were associated with the bacillary load [30]. While IgA-HrpA levels correlated significantly with active TB, the IgA-16 kDa/ESAT6 was significantly associated with the nutritional status of the participants. Others reported IgA anti-mycobacterial antigens HrpA and MDP1 higher response among controls than in TB patients suggesting that different stress on bacterium and host modulates proteomic expression, and the IgA immune response may be protective [31]. Williams et al (2004) reported IgA against the 16 kDa α-cristalin mediated some protection against TB, as evidenced by reduced mycobacterial burden in the lungs [32]. However, IgA PPE 59 reactivity was associated with the presence of chest radiographic alteration of high probability of TB. Considering that in Brazil TB patients are most detected in the spontaneous demand of health care units and are usually diagnosed when the disease is more advanced, it is possible that PPE59 is preferentially expressed in later phases of the clinical evolution of TB disease [33]. On the other hand, TB patients alcohol user (both Brazilian and Italian/immigrants) are ≥ 2.7 times more likely to be positive to PPE59 IgA or IgG. Similar results were observed for 38 kDa and MPT-64 IgG responses tested previously in the same Brazilian population [16]. Louw et al. (2016), evaluating the quality of life among individuals with pulmonary TB in Africa, identified the harmful effect of alcohol use among TB patients, since excess alcohol ingestion negatively influence the cellular immune response, although little is known about the mechanisms involved on it [34; 35]. However, our study was not designed to specifically address the effect of alcohol in humoral response of TB patients, more studies are needed to elucidate these serological findings. Moreover, lower positive IgA PPE 59 frequency in IT patients may be related to treatment, as recently described for LppZ IgA low response after 2 months of anti-TB treatment [38], however, our study was not designed to follow up the treated patients. No other demographic or clinical characteristic was associated with Ab positivity among Italian/immigrants, especially for IgG, which is mainly due to the low number of individuals who were positive for the test.

Suggested PPE 59 IgA function

The IgA main function is to exert an effector action on the mucosal immune system and is an important defense against pathogens that invade the host via mucosa, such as M. tuberculosis [13; 36]. IgA exerts a variety of protective functions between which it interferes with pathogen adherence in mucosal cells (immune exclusion), promotes intracellular neutralization and excretion of the pathogen. Its monomeric form, present in the serum, exerts a complementary action, eliminating pathogens that have crossed the mucosal barrier and still interact with the Fc receptors of cytotoxic cells-dependent antibody

[37]. Therefore, the presence of detectable PPE59 IgA in TB patients may be exerting a protective action, containing the bacilli that escaped the mucosal barrier, whose immune response best controls bacillary multiplication.
Limitations. Limitation of this study includes heterogeneity of specific treatment between study groups, no availability of proper control in the group II, not enough TB^{IT} sera to test the other antigens and lack of follow-up immune response of treated patients. Further studies to evaluate the dynamic of PPE59 IgA during TB disease/treatment must be accomplished.

Conclusion

This is the first study showing PPE59-IgA immunodominance than IgG and no IgM among TB patients of different geographic areas at high specificity and slightly increased sensitivity when in combination with ESAT-6, or 38 kDa or 16 kDa IgG assays lowering specificity. Notably, the combination of both rapid teste SM and ELISA PPE59 IgA (and 16kDA IgG) sensitivity and specificity, although imperfect, fit for triage test. Moreover, significant PPE59 IgA reactivity was strongly associated with the presence of CXR alterations of high TB probability ($p = 0.001$) and with alcohol use ($p = 0.04$). Although PPE59 IgA is not enough accurate to be used independently, this biomarker may be an adjuvant tool for detect symptomatic TB suspects and together with other antigens and/or isotypes and SM tests may be prospectively evaluated as a potential target in high TB burden areas. Additionally, it deserves further investigation on the positive polarization of immune response.

Declarations

Ethics approval and consent to participate

The study was approved by the Fiocruz Ethics Committee in Research (No. 560-10), Rio de Janeiro, Brazil and by the Ethical Committee of Azienda Ospedaliera Spedali Civili di Brescia, Italy (N. 196/03 -11). Before recruiting patients, a detailed explanation was given and written informed consent was obtained from them.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing Interest

The authors declare not have competing interests.

Funding

FAPERJ (Fundação para o Amparo à Pesquisa no Rio de Janeiro, Brasil), is the agency to promote science, technology and innovation in the State of Rio de Janeiro. Linked to the State Secretariat of Science and Technology, the agency aims to stimulate activities in the scientific and technological areas and to broadly support projects and programs of academic and research institutions based in the State of Rio de Janeiro. Fundação Oswaldo Cruz, Instituto Oswaldo Cruz (POM, PAEF), RJ, is the largest research entity in Brazil. Its mission is to produce, disseminate and share knowledge and technologies aimed at strengthening and consolidating the Unified Health System (SUS) and contributing to the promotion of health and quality of life for the Brazilian population. Resources from these two institutions were used to purchase the necessary material for the study. CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brasil), The Coordination for the Improvement of Higher Education Personnel (CAPES), is linked to the Ministry of Education (MEC), plays a fundamental role in the expansion and consolidation of stricto sensu postgraduate courses (master's and doctorate) in all states of the Federation, and supported the master's student involved in the study.

Authors' contributions

All listed authors are in agreement with the data and writing of this manuscript. ACPM: developed all the tests and analyzed the results and wrote the manuscript, IGS: assistance in cloning and ptn expression, VMCS: provided Brazilian clinical and demographic information, ACCC: provided patients clinical and demographic information and wrote the manuscript, AM: coordinate Italians samples and information, MHFS: designed the study, analyzed the data, wrote and reviewed the manuscript.

Acknowledgements

We would like to thank Dr Roberta Olmo of the Oswaldo Cruz Institute for making available the NTM samples used in this study.

Abbreviations

BCG: bacilli Calmette Guérin; TB: tuberculosis; HIV: human immunodeficiency virus; AIDS: acquired immunodeficiency syndrome; TST: tuberculin skin test; MTB: *Mycobacterium tuberculosis*; TNF-α: tumor necrosis factor alpha; IL-6: Interleukin-6; PPD: Purified protein derivative (PPD skin test for tuberculosis); PCR: polymerase chain reaction; IPTG: isopropyl β-D-1-thiogalactopyranoside; 8M: 8 molar; 5mM: 5 milimolar; COPD: chronic obstructive pulmonary disease; PD/CA: other non-tuberculous lung diseases; rCt: recent contact with active TB patients; NTM: nontuberculous mycobacteria pulmonary infection; CXRs: Chest X ray; IGRA: Interferon Gamma Release Assay; ROC: receiver operating characteristic; OR: odds ratio; AFB: Acid-Fast Bacillus; ELISA: enzyme-linked immunosorbent assay; AUC: area under the ROC curve; kDa: KiloDaltons; PPV: positive predictive value; NPV: negative predictive value; A: accuracy; CI: confidence interval; RD11: region of difference 11; ORF: open reading frame; LTBI: latent tuberculosis infection.

References

[1] WHO, Global Tuberculosis Report 2018, 2018, http://www.who.int/tb/publications/global_report/en/.

[2] Lewinsohn DM, Leonard MK, LoBue PA, et al. Official American Thoracic Society/Infectious Diseases Society of America/Centers for Disease Control and Prevention Clinical Practice Guidelines: Diagnosis of Tuberculosis in Adults and Children. Clin Infect Dis. 2017;64(2):111-115. doi:10.1093/cid/ciw778.

[3] Chan ED, Heifets L, Iseman MD. Immunologic diagnosis of tuberculosis: a review. Tuber Lung Dis. 2000;80(3):131-40

[4] Baghaei P, Tabarsi P, Sabour H, et al. Detection of Antibodies Against 6, 16 and 38 kDa Antigens of *Mycobacterium tuberculosis* as a Rapid Test for Diagnosis of Tuberculosis. Tanaffos. 2011;10(4):17-22.

[5] Anochie PI, Onyeneke EC, Ogu AC, et al. Recent advances in the diagnosis of *Mycobacterium tuberculosis*. Germs. 2012;2(3):110-120. doi:10.11599/germs.2012.1021.

[6] Feng X, Yang X, Xiu B, et al. IgG, IgM and IgA antibodies against the novel polyprotein in active tuberculosis. BMC Infectious Diseases. 2014;14: 336. doi:10.1186/1471-2334-14-336.

[7] Sampson SL. Mycobacterial PE/PPE Proteins at the Host-Pathogen Interface. Clinical and Developmental Immunology. 2011; 2011:497203. doi:10.1155/2011/497203.

[8] Li Y, Miltner E, Wu M, et al. A *Mycobacterium avium* PPE gene is associated with the ability of the bacterium to grow in macrophages and virulence in mice. Cell Microbiol. 2005 Apr;7(4):539-48.

[9] Chakhaiyar P, Nagalakshmi Y, Aruna B et al. Regions of high antigenicity within the hypothetical PPE major polymorphic tandem repeat open-reading frame, Rv2608, show a differential humoral response and a low T cell response in various categories of patients with tuberculosis. J Infect Dis. 2004 Oct 1;190(7):1237-44.

[10] Khan N, Alam K, Nair S, Valluri VL, Murthy KJR, Mukhopadhyay S. Association of Strong Immune Responses to PPE Protein Rv1168c with Active Tuberculosis. Clinical and Vaccine Immunology: CVI. 2008;15(6):974-980. doi:10.1128/CVI.00485-07.

[11] Singh KK, Dong Y, Patibandla SA, McMurray DN, Arora VK, Laal S. Immunogenicity of the *Mycobacterium tuberculosis* PPE55 (Rv3347c) Protein during Incipient and Clinical Tuberculosis. Infection and Immunity. 2005;73(8):5004-5014. doi:10.1128/IAI.73.8.5004-5014.2005.

[12] Chen J, Su X, Zhang Y et al. Novel recombinant RD2- and RD11-encoded *Mycobacterium tuberculosis* antigens are potential candidates for diagnosis of tuberculosis infections in BCG-vaccinated individuals. Microbes Infect. 2009 Sep;11(10-11):876-85. doi: 10.1016/j.micinf.2009.05.008

[13] Parkash O, Singh BP, Pai M. Regions of differences encoded antigens as targets for immunodiagnosis of tuberculosis in humans. Scand J Immunol. 2009 Oct;70(4):345-57. doi: 10.1111/j.1365-3083.2009.02312.x.

[14] Al-Attiyah R, Mustafa AS. Characterization of Human Cellular Immune Responses to Novel *Mycobacterium tuberculosis* Antigens Encoded by Genomic Regions Absent in *Mycobacterium bovis* BCG. Infection and Immunity. 2008;76(9):4190-4198. doi:10.1128/IAI.00199-08.

[15] Sardella IG, Mulinari ACP, Fonseca LS et al. Cloning, Expression and Characterization of Fusion Proteins Based on Peptides of Rv1980c Disrupting Rv3019c Sequence and Evaluation of its Potential Immunoreactivity in Pulmonary Tuberculosis Sera. Mycobact Dis 2015. 5:183. doi: 10.4172/2161-1068.1000183

[16] Silva VM, Sardella IG, Luiz RR et al. Immunoreactivity of five antigens of *Mycobacterium tuberculosis* in patients attending a public health care facility in an area with high endemicity for TB. Microbiol Immunol. 2008 Nov;52(11):544-50. doi: 10.1111/j.1348-0421.2008.00072.x.

[17] American Thoracic Society. (2000) Diagnostic standards and classification of tuberculosis in adults and children. Am J Respir Crit Care Med 161: 1376–95.

[18] Mustafa AS, Al-Saidi F, El-Shamy AS et al. Cytokines in response to proteins predicted in genomic regions of difference of *Mycobacterium tuberculosis*. Microbiol Immunol. 2011 Apr;55(4):267-78. doi: 10.1111/j.1348-0421.2011.00307.x.

[19] Kim SY, Park MS, Kim YS et al. The responses of multiple cytokines following incubation of whole blood from TB patients, latently infected individuals and controls with the TB antigens ESAT-6, CFP-10 and TB7.7. Scand J Immunol. 2012 Dec;76(6):580-6. doi: 10.1111/j.1365-3083.2012.02776.x.

[20] Losi M, Knights AJ, Mariani F et al. QuantiFERON-TB performance enhanced by novel *Mycobacterium tuberculosis*-specific antigens. Eur Respir J. 2016 Feb;47(2):660-4. doi: 10.1183/13993003.01015-2015.

[21] Tortora GJ, Funke BR, Case CL 2012. Microbiologia. Doenças Microbianas do trato respiratório. Arthmed. Brasil. 674-704pp.

[22] Karboul A, Mazza A, Gey van Pittius NC, Ho JL, Brousseau R, Mardassi H. Frequent Homologous Recombination Events in *Mycobacterium tuberculosis* PE/PPE Multigene Families: Potential Role in Antigenic Variability. Journal of Bacteriology. 2008;190(23):7838-7846. doi:10.1128/JB.00827-08.

[23] Akhter Y, Ehebauer MT, Mukhopadhyay S et al. The PE/PPE multigene family codes for virulence factors and is a possible source of mycobacterial antigenic variation: perhaps more? Biochimie. 2012 Jan;94(1):110-6. doi: 10.1016/j.biochi.2011.09.026.

[24] McEvoy CRE, Cloete R, Müller B, et al. Comparative Analysis of *Mycobacterium tuberculosis* pe and ppe Genes Reveals High Sequence Variation and an Apparent Absence of Selective Constraints. Neyrolles O, ed. PLoS ONE. 2012;7(4): e30593. doi: 10.1371/journal.pone.0030593.

[25] Vordermeier HM, Hewinson RG, Wilkinson RJ, et al. Conserved Immune Recognition Hierarchy of Mycobacterial PE/PPE Proteins during Infection in Natural Hosts. Sechi LA, ed. PLoS ONE. 2012;7(8): e40890. doi: 10.1371/journal.pone.0040890.

[26] Broger T, Basu Roy R, Filomena A, et al. Diagnostic Performance of Tuberculosis-Specific IgG Antibody Profiles in Patients with Presumptive Tuberculosis from Two Continents. Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America. 2017;64(7):947-955. doi:10.1093/cid/cix023.

[27] Gutlapalli R, Sykam A, Tenali SP, Chandran P, Suneetha S, Suneetha LM. Detection of Tuberculosis in HIV Co-infected Individuals: Use of Multiple ELISA Responses to 38kDa, Lipoarabinomannan and ESAT– 6 of *M. tuberculosis*. Journal of Clinical and Diagnostic Research: JCDR. 2016;10(2): KC01-KC04. doi:10.7860/JCDR/2016/16559.7322.

[28] Liaquat A, Iram S, Hussain S, Yusuf NW, Azeem H. Concomitant presence of culture-proven active pulmonary tuberculosis in patients with chronic obstructive pulmonary disease - A hospital-based study. Pakistan Journal of Medical Sciences. 2015;31(6):1344-1348. doi:10.12669/pjms.316.8166.

[29] Zhang H, Wang J, Lei J et al. PPE protein (Rv3425) from DNA segment RD11 of *Mycobacterium tuberculosis*: a potential B-cell antigen used for serological diagnosis to distinguish vaccinated controls from tuberculosis patients. 2007 Feb;13(2):139-45.

[30] Abebe F, Belay M, Legesse M, K. L. M. C. F, Ottenhoff THM. IgA and IgG against *Mycobacterium tuberculosis* Rv2031 discriminate between pulmonary tuberculosis patients, *Mycobacterium tuberculosis*-infected and non-infected individuals. Neyrolles O, ed. PLoS ONE. 2018;13(1): e0190989. doi: 10.1371/journal.pone.0190989.

[31] Niki M, Suzukawa M, Shunsuke A. et al. Evaluation of Humoral Immunity to *Mycobacterium tuberculosis*-Specific Antigens for Correlation with Clinical Status and Effective Vaccine Development. Journal of Immunology Research. Volume 2015, Article ID 527395, 13 pages. http://dx.doi.org/10.1155/2015/527395

[32] Williams A, Reljic R, Naylor I, et al. Passive protection with immunoglobulin A antibodies against tuberculous early infection of the lungs. Immunology. 2004;111(3):328-33.

[33] Kunnath-Velayudhan S, Salamon H, Wang H-Y, et al. Dynamic antibody responses to the *Mycobacterium tuberculosis* proteome. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(33):14703-14708. doi:10.1073/pnas.1009080107.

[34] Louw JS, Mabaso M, Peltzer K 2016. Change in Health-Related Quality of Life among Pulmonary Tuberculosis Patients at Primary Health Care Settings in South Africa: A Prospective Cohort Study. PLoS One 11: e0151892.

[35] Mertens JR, Flisher AJ, Ward CL, Bresick GF, Sterling SA, Weisner CM. Medical Conditions of Hazardous Drinkers and Drug Users in Primary Care Clinics in Cape Town, South Africa. J Drug Issues. 2009;39(4):75796776.

[36] Chin ST, Ignatius J, Suraiya S, Tye GJ, Sarmiento ME, Acosta A, Norazmi MN, Lim TS 2015. Comparative study of IgA V H 3 gene usage in healthy TST − and TST + population exposed to tuberculosis: deep sequencing analysis. Immunology 144: 302–311.

[37] Achkar JM, Casadevall A. Antibody-Mediated Immunity against Tuberculosis: Implications for Vaccine Development. Cell host & microbe. 2013;13(3):250-262. doi: 10.1016/j.chom.2013.02.009.

[38] Xiao J, Xiong Y, Chen Y, et al. Determination of Lipoprotein Z-Specific IgA in Tuberculosis and Latent Tuberculosis Infection. Frontiers in Cellular and Infection Microbiology. 2017; 7:495. doi:10.3389/fcimb.2017.0049

Tables

Table 1: Characteristics of the study subjects.

	Study group I (n=133) (%)				Study group II (n=79) (%)	
Characteristics	*pTB^{BR}* *n=52*	*ORD^{BR}* *n=55*	*NTM* *n=10*	*HD^{BR}* *n=16*	*pTB^{IT}* *n=54*	*eTB^{IT}* *n=25*
Age (M±SD)	(45.7±18.2)	(49.1±18.8)	-	(33.8 ±11.3)[1]	(39.4±16,9)	(41.9±15.6)
Male	35 (67.0)	25 (45.0)	4 (40)	6 (37.5)	42 (78.0)	12 (48.0)
Alcohol user	20 (38.4)[2]	8 (14.5)	-	0	7 (12.9)	0
No information	1 (1.9)	0	10 (100)	0	19 (35.1)	3 (12.0)
Tabaco user	22 (42.3)	12 (21.8)	-	0	14 (25.9)	6 (24.0)
No information	1 (1.9)	0	10 (100)	0	20 (37.0)	3 (12.0)
Presence BCG Scar	20 (38.4)	27 (49.0)	-	16 (100.0)	NA	NA
TST and/or IGRA						
Positive	41 (78.8)	34 (61.8)	-	8 (50.0)	26 (48.1)	11 (44.0)
Not done	7 (13.4)	0	-	-	24 (44.4)	13 (52.0)
AFB-SM and/or culture						
Positive	34 (65.3)	0	-	-	49 (90.7)	21 (84.0)
Not done	7 (13.4)	11 (20.0)	-	16 (100.0)	3 (5.5)	2 (8.0)
CXR changes of probability for TB						
High	31 (59.6)[2]	1 (1.81)	-	-	20 (37.0)	0 (0.0)
Intermediate	10 (19.2)	2 (3.63)	-	-	25 (46.2)[3]	6 (24)
Low/Normal	10 (19.2)	52 (94.5)	-	-	1 (1.85)	17 (68)
Not done	1 (1.92)	0 (0.0)	10 (100)	16 (100.0)	8 (14.8)	2 (8.0)
Anti-TB Therapy						
Free	52 (100.0)	55 (100.0)	-	NA	12 (22.0)	-
Treated	-	-	-	-	42 (78.0)	25 (100.0)

M± SD: mean ± standard deviation. pTB or eTB: pulmonary or extrapulmonary tuberculosis, ORD: other respiratory disorder, HD: healthy donor. BR: Brazilian, IT: Italian or immigrants in Italy. TST: tuberculin skin test (positive: ≥ 5 mm). IGRA: interferon gamma released assay (positive: ≥0,35 IU/ml) immigrants only performed. AFB-SM: acid-fast bacilli semi quantitative analysis by sputum microscopy. CXR: chest radiograph. NA: not applicable. [1] p=0.01. [2, 3] p=0.03.

Table 2. Median of Enzyme immunosorbent assay (ELISA) results of PPE59-IgA and IgG.

Patients' sera	ELISA-PPE59			
	N	IgA**	N	IgG**
pTB^{BR*}	52	1.039 (0.863-1.142)	47	0.435 (0.301-0.662)
pTB^{IT}	54	0.886 (0.621-1.206)	54	0.317 (0.237-0.409)
African	18	0.924 (0.515-1.277)	18	0.268 (0.249-0.473)
Asian	19	0.890 (0.623-1.210)	19	0.319 (0.234-0.393)
European	17	0.770 (0.651-1.182)	17	0.334 (0.207-0.443)
eTB^{IT}	25	0.663 (0.503-1.067)	25	0.220 (0.178-0.309)
African	4	0.609 (0.300-1.006)	4	0.211 (0.190-0.313)
Asian	15	0.866 (0.532-1.155)	15	0.267 (0.187-0.305)
European	6	0.593 (0.448-0.851)	6	0.196 (0.159-0.380)
p valor pTB^{BR} vs IT		*0.113*		*0.001*
p valor pTB vs eTB		*0.095*		*0.007*
All TB patients	131	0.931 (0.6510-1.155)	126	0.319 (0.232-0.447)
ORD^{BR*}	55	0.758 (0.487-0.916)	51	0.469 (0.351-0.630)
NTM	10	0.515 (0.450-0.600)	10	0.904 (0.806-1.107)
HD^{BR*}	16	0.525 (0.384-0.735)	16	0.241 (0.167-0.377)
p valor ORD vs HD^{BR}		*0.005*		*< 0.0001*
p valor NTM vs HD^{BR}		*0.032*		*< 0.0001*
All control	81	0.682 (0.475-0.823)	77	0.437 (0.293-0.598)
p valor pTB^{BR} vs ORD^{BR}		*<0.001*		*0.3863*
p valor pTB^{BR} vs HD^{BR}		*<0.001*		*<0.0001*
p *valor pTB vs All control*		*< 0.0001*		*< 0.0001*

Were tested serum from Brazilians and Italian/immigrants with pulmonary and extra pulmonary tuberculosis (pTB$^{BR/IT}$, eTB^{IT}), other respiratory disease (ORD^{BR}) and health donors (HD^{BR}) subjects.
* Kruskall Wallis pTB^{BR} x ORD^{BR} x HD^{BR}: IgA p<0.0001 and IgG p=0.0004.
** Man-Whitney IgA vs IgG p< 0.0001.

Table 3: Sensitivity and specificity of IgA and IgG ELISA based on the PPE59 antigen.

Sera	IgA (95% CI)			IgG (95% CI)			IgA+IgG (95% CI)		
	N+/T	Sensitivity	Specificity	N+/T	Sensitivity	Specificity	N+/T	Sensitivity	Specificity
pTB^{BR}	28/52	53.3 (39.5 to 67.8)		10/47	21.3 (10.7 to 35.6)		31/52	59.6 (45.1 to 73)	
pTB^{IT}	21/54	38.2 (26 to 53.1)		2/54	3 (1 to 12.7)		21/54	38.2 (26 to 53.1)	
eTB^{IT}	7/25	28 (12 to 49.4)		0/25	0 (0 to 13.7)		7/25	28 (12 to 49.4)	
All TB	56/131	42.7 (34.1 to 51.7)		12/126	9 (5 to 16)		65/131	49.6 (40.7 to 58.4)	
Control	2/81		97.5 (91.4 to 99.7)	13/77		83.1 (72.9 to 90.7)	14/81		82.7 (72.7 to 90.2)

The test was performed among Brazilians and Italians/immigrants with pulmonary (pTB$^{BR/IT}$) or extrapulmonary tuberculosis (eTB^{IT}). N+/T: number of sera testing positive/total of sera tested.

Table 4: Combinatory analysis results of different antigens* and PPE59.

ELISA combinatory results	Sensitivity % (Number of+)/52 [IC95%]	Specificity % (Number of+)/71 [IC95%]
PPE59-IgA + IgG-ESAT-6	55.8 (29) [41.3 to 69.5]	94.4 (4) [86.2 to 98.4]
PPE59-IgG + -ESAT-6	32.7 (17) [20.3 to 47.1]	83.1 (12) [72.3 to 90.9]
PPE59-IgA + IgG-16kDa	71.1 (37) [56.9 to 82.8]	87.4 (9) [77.3 to 94]
PPE59-IgG + 16kDa	48 (25) [34 to 62.3]	76.1 (17) [64.4 to 85.3]
PPE59-IgA + IgG-38kDa	59.6 (31) [45.1 to 72.9]	90.2 (7) [80.4 to 95.9]
PPE59-IgG + 38kDa	42.3 (22) [28.7 to 56.8]	81.7 (13) [70.7 to 89.9]
PPE59-IgA + IgG-MT10.3	59.6 (31) [45.1 to 72.9]	88.8 (8) [79 to 95]
PPE59-IgG + MT10.3	42.3 (22) [28.7 to 56.8]	76.1 (17) [64.4 to 85.3]
PPE59-IgA + IgG-MPT64	67.3 (35) [52.8 to 79.6]	88.8 (8) [79 to 95]
PPE59-IgG + MPT64	51.9 (27) [37.6 to 66]	76.1 (17) [64.4 to 85.3]
PPE59-IgA + ESAT-6 + 38kDa IgG	59.6 (31) [45.1 to 72.9]	87.4 (9) [77.3 to 94]
PPE59 + ESAT-6 + 38kDa IgG	40.3 (21) [27 to 55]	81.7 (13) [70.7 to 89.9]
PPE59 IgA + All antigens IgG	78.8 (41) [65.3 to 88.9]	74.7 (18) [62.9 to 84.2]
All antigens IgG	65.3 (34) [50.9 to 78]	66.2 (24) [54 to 77]
PPE59 IgA/IgG + all antigens IgG*	82.6 (43) [69.6 to 91.7]	64.8 (25) [52.5 to 75.7]

The analysis results of combination of positive IgG ELISA of different antigens* (ESAT-6, 16Kda, 38Kda, MT10.3, and MPT-64*) and IgA and/or IgG PPE59 from Brazilian patients with pulmonary tuberculosis and controls, comprising patients with other respiratory disorders and healthy donors.* Results previously obtained by Silva et al, 2008.

Tabel 5: Sensitivity of AFB-SM combined with sensitivity of IgA and IgG ELISA based on the PPE59.

	Number of positive (Total) % (95% CI)			
	pTB^{BR}	**pTB^{IT}**	**eTB^{IT}**	**All TB**
SM				
Positive	30 (45)	39 (49)	11 (19)	80 (113)
	66.6 (53 to 78.2)	79.5 (65.6 to 89.7)	57.9 (33.5 to 79.7)	70.8 (61.5 to 78.9)
Negative	15 (45)	10 (49)	8 (19)	33 (113)
	33.3 (20 to 48.9)	20.4 (10.2 to 34.3)	42.1 (20.2 to 66.5)	29.2 (21 to 38.5)
Not done	7 (52)	5 (54)	6 (25)	18 (131)
SM *vs* SM-	*0.002*	*0.0001*	*0.337*	*0.0001*
SM^{+}/IgA PPE59	42 (52)	40 (54)	15(25)	97 (131)
	80.7 (67.4 to 90.3)	74 (60.3 to 85.0)	60 (38.6 to 78.8)	74 (67.3 to 82.6)
SM vs SM/IgA	*p=0.01*	*p=0.82*	*p=0.26*	*p=0.02*
SM^{+}/IgG PPE59	32 (52)	39 (54)	11 (25)	82 (131)
	61.5 (47 to 74.70)	72 (58.3 to 83.5)	44 (24.4 to 0.65.0)	62.6 (53.72 to 70.89)
SM vs SM/IgG	*0.69*	*p=1.0*	*p=1.0*	*p=0.80*
SM^{+}/PPE59-IgA/ IgG-16kDa	45 (52) 86.5 (76.2 to 93.5)	NA	NA	NA
SM vs SM/IgA/IgG	*p=0.001*			

Results obtained among Brazilians and Italians/immigrants with pulmonary (pTB$^{BR/IT}$) or extrapulmonary tuberculosis (eTB^{IT}), as well as IgA ELISA PPE59 plus IgG 16kDa results among pTB^{BR}. χ^2 Pearson's chi-square, with Yates's correction; SM: acid fast bacilli by Ziehl Neelsen stained smear microscopy. NA: data not available.

Table 6: Univariate analysis based on positivity for ELISA IgA and IgG PP59.

Factors	Study Group I			
	IgA	p value	IgG	p value
Males	**3.8 (1.4-9.7)**	**0.00**	1.3 (0.5-3.4)	0.55
Female	1			
Age ≥ 40	1.1 (0.5-2.7)	0.66	**2.8 (1.0-7.9)**	**0.04**
Age ≤ 40	1			
Alcohol user	**3.9 (1.5-9.7)**	**0.00**	**3.2 (1.1-8.8)**	**0.02**
No alcohol user	1			
Tabaco user	**2.5 (1.0-6.1)**	**0.03**	2.0 (0.7-5.4)	0.14
No Tabaco user	1			
TST ≥5mm	**13.0 (1.6-100.7)**	**0.00**	4.3 (0.5-35.6)	0.13
TST ≤5mm	1			
CXR High	**5.5 (2.2-13.4)**	**0.00**	**3.6 (1.3-9.9)**	**0.00**
CXR intermediary	**22.7 (4.6-111.9)**	**0.00**	1.4 (0.3-5.8)	0.59
CXR normal	1			
Positive AFB/or C	**5.7 (2.0-15.7)**	**0.00**	4.2 (0.4-38.6)	0.16
Negative AFB/or C	1			

The univariate analysis uses the demographic, clinical and laboratory characteristics obtained in the ELISA IgA and IgG PP59 of Brazilian patients with pulmonary tuberculosis and controls, including patients with other respiratory disorders and healthy donors. CI: confidence interval. TST: tuberculin skin test (positive: ≥ 5 mm). CXR: Chest radiograph with changes of high and intermediate probability of TB. AFB: acid-fast bacilli. C: culture in solid medium.

Table 7: Logistic regression analysis associated with PPE59 IgA among Brazilian pulmonary tuberculosis patients (n = 52).

Factors	Study Group I			
	IgA	p value	IgG	p value
Males	**2.9 (1.0-8.0)**	**0.03**	1.0 (0.3-3.0)	0.98
Female	1			
Age ≥ 40	1.1 (0.3-4.2)	0.81	3.6 (0.8-15.9)	0.08
Age ≤ 40	1			
Alcohol user	**2.7 (1.0-7.2)**	**0.04**	**3.0 (0.9-9.6)**	**0.05**
Probability of CXR TB image				
Higher	**27.8 (2.7-281.2)**	**0.001**	5.4 (0.6-43.8)	0.11
intermediary	9.5 (0.6-132)	0.09	1.0 (0.0-18.4)	0.99
normal	1			

CI: confidence interval. TST: tuberculin skin test (positive: ≥ 5 mm). CXR: Chest X ray.

Figures

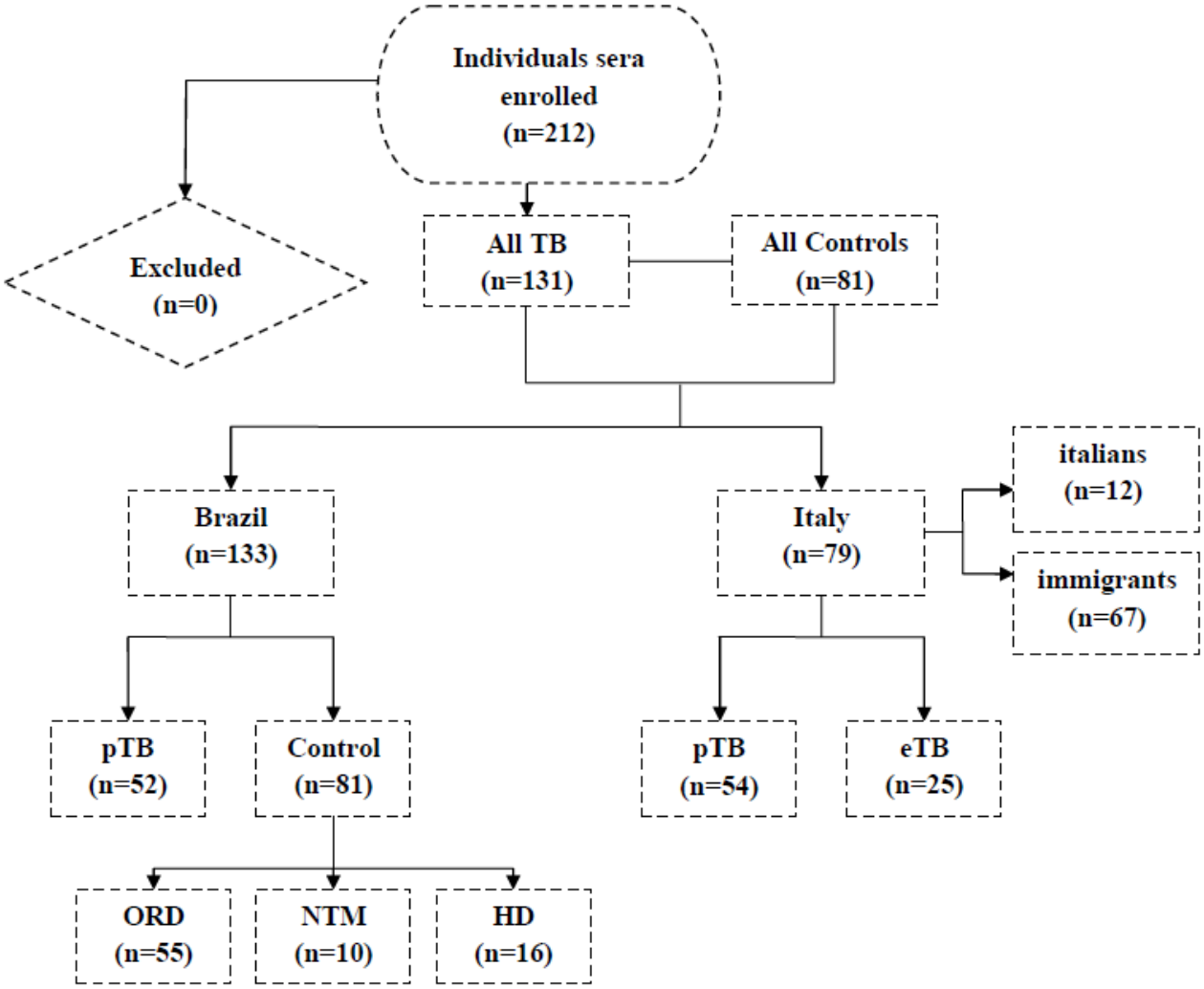


Figure 1

Flowchart of patients groups enrolled in the present study.

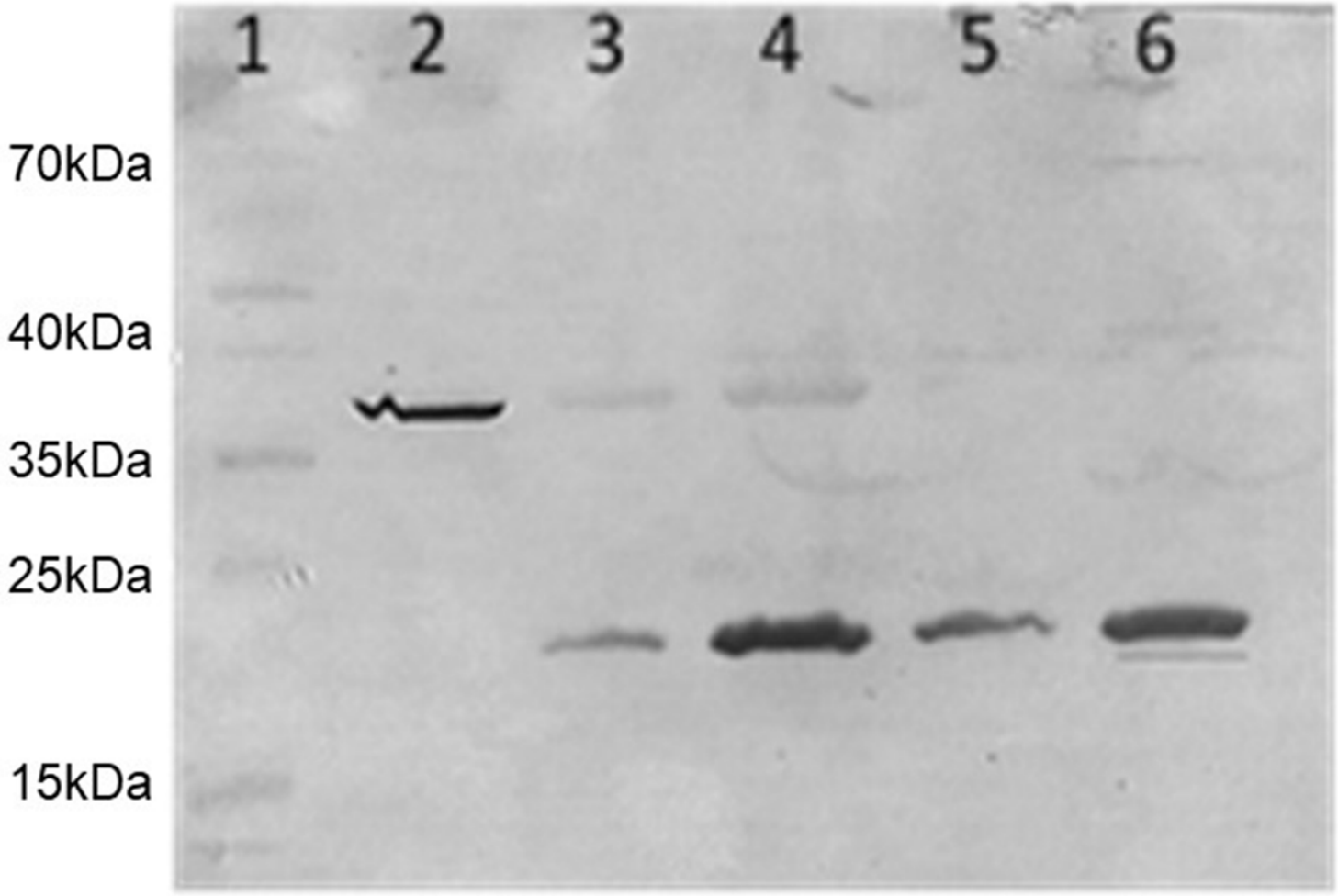


Figure 2

Western blot of recombinant PP59 protein.

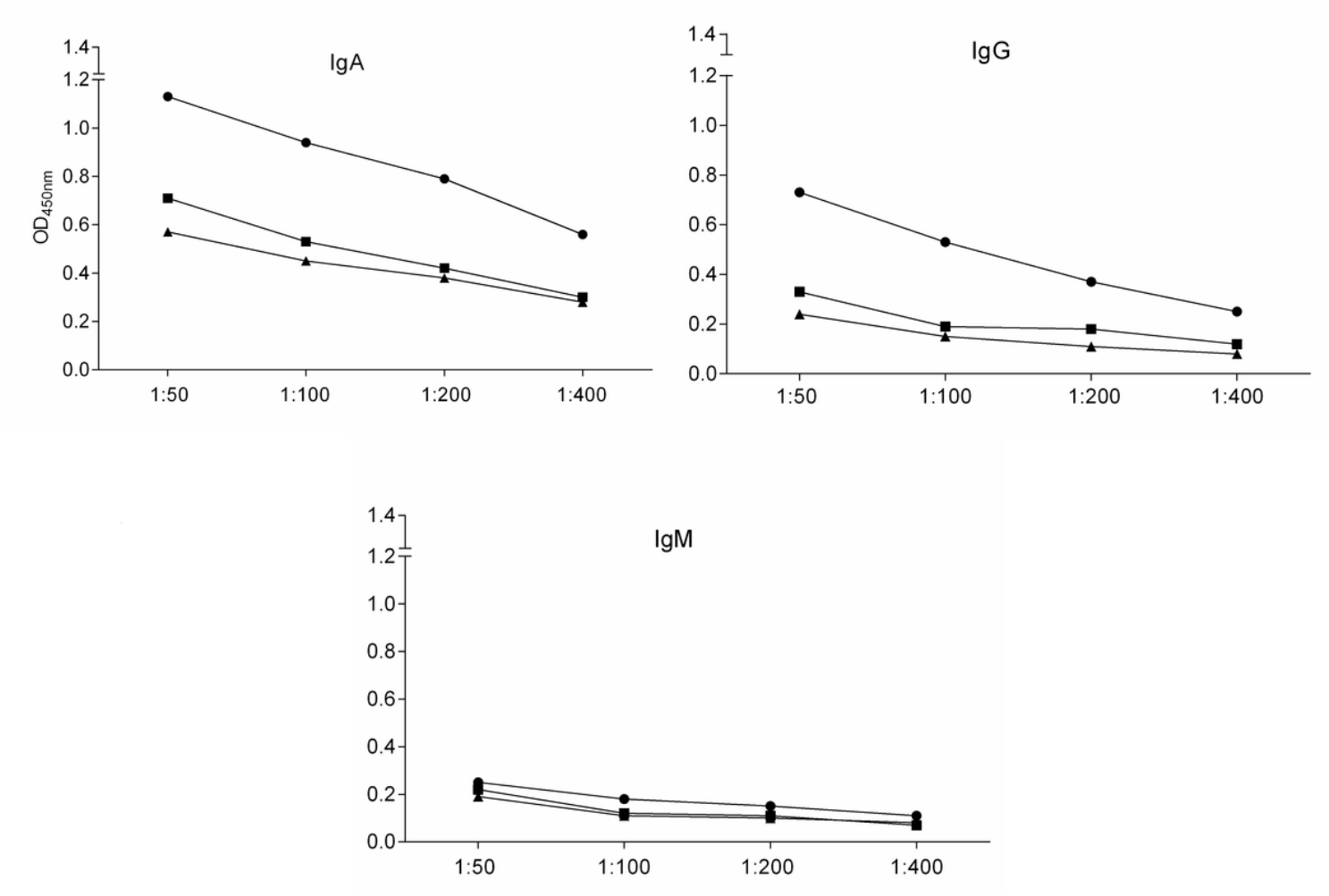
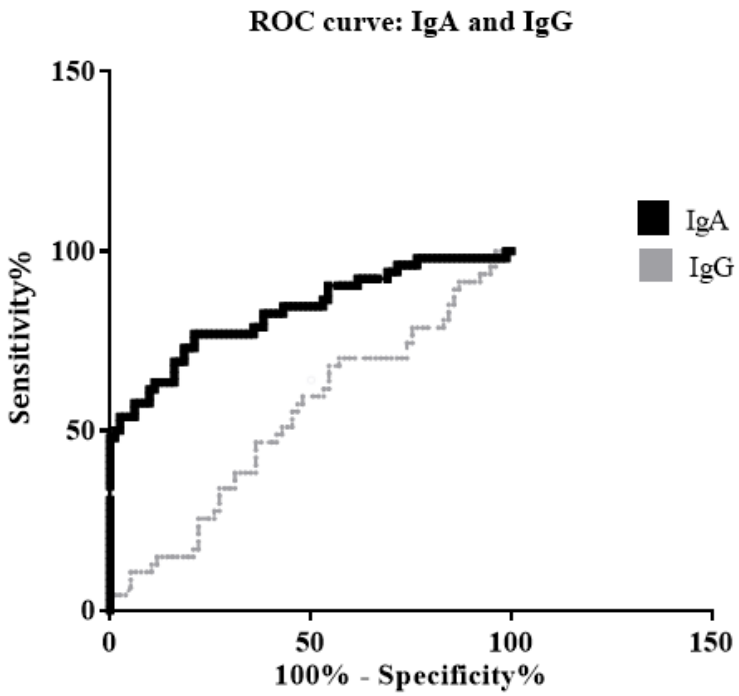


Figure 3

The in house IgA, IgG and IgM-ELISA standardization with the recombinant PPE 59.



Antibodies test	AUC (95% CI)	P valor	cut off	Likehood ratio (LR)	A	PPV	NPV
PPE59-IgA	0.832 (0.758 to 0.902)	<0.0001	1.030	21.81	61.8	96.5	47.9
PPE59-IgG	0.467 (0.361 to 0.572)	0.537	0.732	1.043	36.9	56	34.1
IgA + IgG-PPE59	-	-	-	-	58.9	83.3	45.3

Figure 4

Evaluation of AUC of the Receiver Operating Characteristic (ROC) PPE59-IgA/IgG. Area under the curve (AUC) was calculated with 52 TB patients and 81 controls. ROC (95% CI). PPV: positive predictive value; NPV: negative predictive value; A: accuracy.

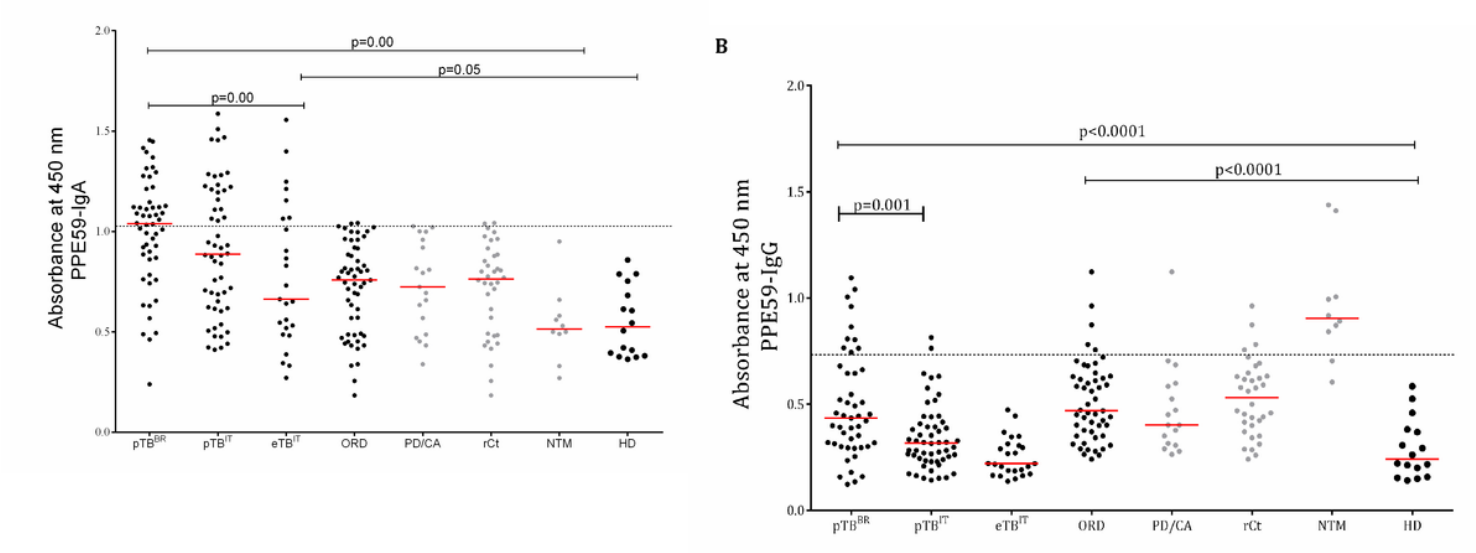


Figure 5

Distribution of IgA/IgG response to PPE59 in sera of Brazilians (BR) and Italians/immigrants (IT). Dotted line: Cut-off point 1.030 for PPE59-IgA and 0.732 for PPE59-IgG. Light grey: Stratified ORD by asthma, bronchitis, sinusitis, pneumonia, Wegener's granulomatosis, cancer and chronic obstructive pulmonary disease (PD/CA), recent contact of active TB patient (rCt) and NTM: nontuberculous mycobacteria pulmonary infection. 5A and 5B: ELISA PPE59-IgA and IgG dot plot, respectively.