ERJ Express. **Published on April 22, 2009 as doi: 10.1183/09031936.00005309**

Utility of quantitative T cell responses versus unstimulated IFN-γ for the diagnosis of pleural tuberculosis

Dheda K[+*], van Zyl-Smit RN[+], Sechi LA[†], Badri M[+], Meldau R[+], Meldau S[+], Symons G[+], Semple L[+], Maredza A[+], Dawson R[+], Wainright H^{+}, Whitelaw A[#], Vallie Y[+], Raubenheimer P[+], Bateman ED[+], Zumla A*

[+]Lung Infection and Immunity Unit & CTBRI, UCT Lung Institute & Division of Pulmonology, Department of Medicine, University of Cape Town, South Africa

[*]Centre for Infectious Diseases and International Health, Royal Free and University College Medical School, London;

[†]Department of Biomedical Sciences, University of Sassari, Sardinia, Italy.

National Health Laboratory Service, South Africa and Division of Medical Microbiology, Department of Clinical laboratory Sciences, University of Cape Town, Cape Town, South Africa

Short descriptive title: Diagnosis of TB pleural effusion

Word count: 3018

Corresponding author: Keertan Dheda, Lung Infection and Immunity Unit, Division of Pulmonology, Department of Medicine, University of Cape Town.

Current address: Department of Medicine. J flr. Old Main Bldg. Groote Schuur Hospital. Observatory, Cape Town, South Africa, 7925.

Tel: +27 21 4066509 , Fax: +27 21 4486815, email: keertan.dheda@uct.ac.za

Copyright 2009 by the European Respiratory Society.

Key words: diagnosis, diagnostic accuracy, human, IFN-γ, pleural effusion,

tuberculosis

ABSTRACT:

Background: The clinical utility of antigen-specific IFN-γ-release-assays (IGRAs), using pleural mononuclear cells, for the diagnosis of TB requires clarification.

Objective/methods: We compared the diagnostic utility of unstimulated pleural IFN-γ levels to several pleural antigen-specific T-cell IGRAs [ESAT-6 and CFP-10 (T-SPOT.®TB, QuantiFERON®-TB Gold-In-Tube), PPD and HBHA] in 78 South African tuberculosis suspects. Test results were compared against a clinical score and a reference standard.

Results: Of 74 evaluable subjects 48, 7 and 19 had definite, probable and no TB, respectively. 11/74 (15%) of pleural samples [9/ 48 (19%) of the definite TB cases] had total cell counts that were inadequate for T-cell processing. In the remaining 63 samples the [sensitivity; specificity; PPV; NPV %] of different diagnostic methods were: maximal bio-clinical score [54; 89; 92; 43], T-SPOT.®TB [86; 60; 84; 64], QuantiFERON®-TB Gold-In-Tube [57; 80; 87; 44], HBHA-specific IGRA [59; 31; 64; 27], PPD-specific IGRA [81; 40; 76; 46] and pleural fluid unstimulated IFN-γ [97; 100; 100; 94].

Conclusion: Unstimulated IFN-γ was the most accurate test to distinguish TB from non-TB effusions in a high burden setting. The antigen-specific T-cell IFN-γ release assays were limited by sub-optimal accuracy and the inability to isolate sufficient mononuclear cells to perform the assay.

Pleural effusions are common in clinical practice in developing countries and have various aetiologies including tuberculosis (TB). Annually, over half a million cases of tuberculosis-related pleural effusions (TB-PE) occur worldwide [1]. Tuberculosis is out of control in Africa and accounts for a significant proportion of pleural effusions in this setting [2]; thus making an accurate diagnosis becomes essential. Smear and culture of the pleural fluid is helpful in a minority (~30%) of cases [3]. Currently, testing for adenosine-deaminase (ADA), a biomarker for TB, is used in several laboratories but this facility is not widely available in high burden developing countries and it is not specific for TB [3, 4]. Closed pleural biopsy has a good yield (approximately 80 %) but it is less sensitive, invasive, relatively expensive, and may be associated with complications [3]. Consequently, empiric anti-TB treatment is often administered. There is a need for alternative, rapid and less invasive, methods to diagnose TB-PEs, especially in developing countries. Recently, candidate biomarkers IP-10 (IFN-γ-inducible-10kDa protein, a Th-1 associated chemokine) [5], and unstimulated IFN-γ levels [6] have shown promise. The latter has shown good discriminatory value in intermediate and low burden settings [6]. However, data about the utility and performance outcome of pleural fluid unstimulated IFN-γ levels in a high TB prevalence setting is limited.

More recently peripheral blood quantitative RD-1 antigen-specific (ESAT-6 and CFP-10) T lymphocyte responses have become established as sensitive, specific and rapid immunodiagnostic tests for TB infection [7-9]. However, they cannot

distinguish latent infection from active disease [8]. By contrast, at the site of active pleural [10] or pulmonary [11, 12] disease the frequency of clonally expanded antigen-specific T-cells is over 10-fold higher than peripheral blood and should theoretically not be present in non-TB pleural effusions. We therefore hypothesized that TB antigen-specific responses could distinguish active pleural TB from alternative diagnoses and should have better discriminatory value than unstimulated IFN-γ, a non-specific biomarker of inflammation. A recent case-control study from Europe showed a high diagnostic accuracy with the T-SPOT.®TB (Oxford Immunotec, Abingdon, UK) assay [13] but overall data on the utility of two known Interferon-gamma response assays (IGRAs), T-SPOT.®TB and QuantiFERON® TB-Gold-In-Tube (Cellestis, Carnegie, Victoria, Australia), (QFT-TB-GIT), including a head-to-head comparison, for the diagnosis of TB-PE are limited [13-16]. There are several other unresolved questions including the optimal cell number and pleural fluid volume required, optimal cut-point and utility in high HIV-prevalence settings [17]. Other novel antigens like heparin-binding-haemagglutinin (HBHA), shown to be discriminatory in peripheral blood [18, 19], have hitherto not been evaluated using pleural fluid lymphocytes.

In this study the main aim was to evaluate the diagnostic utility, in clinical practice, of several different IGRAs using pleural fluid T cells in a high burden TB and HIV setting. We prospectively studied the comparative performance outcomes of unstimulated pleural IFN-γ levels against four antigen-specific IGRAs, including a head-to-head comparison between the T-SPOT.®TB and

QFT-TB-GIT, using pleural fluid mononuclear cells . Comparative peripheral blood responses were also evaluated.

Methods

Patient recruitment, characterization and routine laboratory tests

Study approval was obtained from the University of Cape Town Health Sciences Faculty research ethics committee. After obtaining informed consent, and counselling, seventy-eight consecutive patients with clinically suspected TB pleural effusion were prospectively recruited at the Groote Schuur, Somerset and Victoria hospitals in Cape Town, South Africa over a 12 month period (ending 30 April 2008). All patients had a history taken and physical examination performed, routine investigations including testing for HIV infection, chest x-ray, microbiological sputum examination whenever possible, and aspiration of approximately 20 ml of pleural fluid (or closest obtainable volume) for biochemical, cytological, and detailed microbiological evaluation. Four patients were excluded from further analysis (see figure 1 for details including summary of recruitment), and thus 74 patients contributed evaluable samples. For accurate characterization of disease multiple closed pleural biopsies were undertaken. Although offered, in 16 patients biopsies were not performed either because of patient refusal, a contra-indication or a positive culture of fluid, or histology, from another site prior to attempted pleural biopsy.

The reference standard used for diagnosis of TB was culture positivity for *M. tuberculosis* (using pleural fluid or tissue) and/ or histology suggestive of tuberculosis (caseous necrosis with acid fast bacilli with or without granuloma formation). Patients were thus characterized as (i) definite TB (meeting the reference standard), (ii) non-TB (no microbiological or histological evidence for TB, alternative diagnosis made, not treated for TB and did not develop TB over 6 month follow up) and (iii) probable TB (empiric anti-TB treatment but not meeting the criteria for definite TB). The definite and non-TB groups were used for sensitivity and specificity calculations. All assays were performed by an experienced laboratory technician who was blinded to patient and clinical details.

Antigen-specific interferon-γ release assays

Four different IGRAs were evaluated. (a) IFN-γ ELISPOT responses to ESAT-6/ CFP-10 peptide pools were performed according to manufacturer's instructions (T-SPOT.®TB); using peripheral blood (250 000 cells/well) and pleural mononuclear cells (200 000 cells/well) and enumerated using an ELISPOT reader. Data from 9 patients showed that 2×10^5 cells per well produced optimal IFN-γ responses compared to 1×10^5 cells per well (149 vs. 39 SFCs; p= 0.002 for ESAT-6 and 108 vs. 36 SFCs for CFP-10; p= 0.01). Using 4×10^5 cells per well did not significantly increase the spot counts. (b) IFN-γ responses using the QFT-TB-GIT assay were performed using 1 ml of blood and 1×10^6 pleural mononuclear cells re-suspended in 1ml of serum free medium in each tube. Data from 6 patients showed that antigen-specific responses were significantly

higher when using at least 1 x 10^6 vs. 1 x 10^5 pleural mononuclear cells per tube (2.53 vs. 0.026 IU/ml; p= 0.008); suspension of pleural cells in smaller volumes (0.25 and 0.5ml) had no effect on measured IFN-γ concentrations. (c) IFN-γ ELISPOT responses to HBHA were performed using IFN-γ pre-coated ELISPOT plates (Mabtech, Sweden). Dose response experiments revealed that optimum IFN-γ secretion occurred at a HBHA dose of 100μg/ ml using peripheral mononuclear cells. Methylated HBHA antigen was produced in cultures of *Mycobacterium smegmatis* 3.38 as previously described [20]. (d) IFN-γ ELISPOT responses to PPD (100ug/ml; Staten Serum Institute, Denmark) were performed using IFN-γ pre-coated ELISPOT plates. All assays were incubated for 16 to 20 hours.

Measurement of unstimulated IFN-γ

IFN-γ concentrations were measured in the unstimulated pleural fluid supernatant using the QFT ELISA kit.

Statistical analysis

Categorical variables and continuous variables were compared using the appropriate parametric and non-parametric tests. McNemar χ^2 test was used compare sensitivities and specificities of the different diagnostic tests [21]. To ascertain the relative value of newer tests in a high burden setting, a regression model was used to develop a bio-clinical score [22] appropriate for a resource poor setting. Thus, to ascertain the relative value of newer tests in a high burden

setting, regression models were fitted to identify variables independently associated with risk of tuberculosis, taking into account findings from the history, physical examination and pleural fluid biochemical data (the variables considered in the analysis included age, race, smoking and HIV status, history of previous TB, cell counts and pleural fluid protein level). Multi-colinearity was assessed, and variables contributing to the best fit of the final model, or most cost-effective and widely available in our setting, were retained in the final model. The final bio-clinical scoring rule was developed by assigning a relative score or points to each of the variables included in the final multivariate model. Reporting of the study was done using the Standards for Reporting of Diagnostic Accuracy (STARD) template [23].

Results

Clinical, demographic data and biochemical data

There were 48, 19 and 7 patients with definite, non-TB and probable TB, respectively. Smear, pleural fluid culture, and biopsy (tissue culture and histology) were positive in 1, 27 and 41 of the 48 definite TB cases, respectively, and by definition, in none of the non-TB cases. None of the probable TB cases were culture or biopsy positive but all were treated empirically for TB based on clinical suspicion. Effusions in the non-TB group were due to several causes (2 lymphoma, 2 myeloproliferative disorders, 9 adeno or small cell carcinoma, 3 parapneumonic, and 3 due to other causes). Twenty one percent (16/74) of patients did not have a pleural biopsy (refused by 4 patients, contra-indicated in 2

patients, 1 in whom a liver biopsy was done, and 9 in whom the culture result was positive prior to a biopsy being done [6 sputum culture positive and 3 pleural fluid culture positive]). Of all subjects in the study cohort tested for HIV, 46.4% (26/56) were HIV positive. Clinical, demographic and biochemical data are summarized in table 1 (n= 67 with definite or non-TB groups from which sensitivities and specificities were calculated).

In the final multivariate logistic regression model, only age (< 42 years), [odds ratio (OR) = 3.89, 95% CI 1.01-14.90, p=0.04] and pleural fluid protein levels (> 53 g/L) [OR = 3.59, 95% CI 1.02-12.56, p=0.04) were independently associated with the risk of tuberculosis. These variables, when incorporated into a bio-clinical score, had a maximal sensitivity and specificity of 54 and 89%, respectively (table 2).

The median (25th; 75th percentiles) pleural fluid cell count was 1.75×10^6 cells/ml (1.03; 5.45×10^6 cells/ml) and the median volume of fluid obtained was 20 ml (10; 25 ml). The pleural fluid ADA had a sensitivity and specificity of 96 and 69%, respectively, for the diagnosis of TB and further details are published elsewhere [24].

Performance outcomes of pleural fluid unstimulated IFN-γ

The median values for unstimulated IFN-γ were significantly higher in TB vs. non-TB effusions (10.95 vs. 0.105 IU/ml; p<0.001). At a receiver operating curve

(ROC) derived cut-point of 0.31 IU/ml the sensitivity, specificity, PPV and NPV (95% CI) of unstimulated IFN-γ was 97% (85-99); 100% (81-100); 100% (90-100) and 94% (73- 99), respectively. Accuracy was 98% (90-100) and area under the ROC was 0.99. A scatter-plot and area under the ROC of unstimulated IFN-γ levels in pleural fluid are shown in figure 2. When probable and definite TB cases were grouped together the sensitivity of the assay was 98%.

Performance outcome of pleural vs. peripheral blood T-SPOT.®TB and QFT-TB-GIT

Pleural T-cell assays could only be performed in 63 of the 74 (85%) patients because inadequate cell numbers were isolated from 11 patients during the pleural fluid processing stage (median cell count in this group was 1.3 x 10^4 cells/ml and the median volume of pleural fluid received was 14 ml). Of these 11 patients 9 had definite TB and thus 9/ 48 (19%) of the definite TB cases were unsuitable for T cell processing. This left 63 evaluable patients where a T cell assay had been performed on the pleural fluid.

The performance outcomes of both commercial IGRA assays (T-SPOT.®TB and QFT-TB- GIT) for pleural fluid (using mononuclear cells for both assays) and whole blood are shown in table 3. Of the 7 probable TB cases (data not shown), 2 and 3 had a positive pleural T-SPOT.®TB and QFT-TB- GIT results, respectively. The scatter-plot for the pleural T-SPOT.®TB and pleural

mononuclear cell-derived QFT-TB-GIT assays, including area under the ROC curve, are shown in figure 3A and B. In summary, the pleural T-SPOT.®TB assay, compared to the pleural mononuclear cell-derived QFT-TB-GIT assay, had a better sensitivity (80% vs. 51%; p= 0.002, McNemar Chi squared test) but poorer specificity (65% vs. 94%) and PPV (83% vs. 95%).

In an attempt to improve the specificity of the assay, we corrected pleural antigen-specific T-cell responses for peripheral antigen-specific T-cell counts (pleural RD1 spot count/ peripheral spot count ratio). This ratio, although higher in TB vs. non-TB patients (6.56 vs. 2.37) was not significant (p= 0.11). Based on the ROC (area under the ROC curve = 0.68) the optimal ratio cut-point was 3.9 for ESAT-6-specific responses which produced a sensitivity of 64% and specificity of 75%, and was thus not discriminatory.

Sixty three participants had a least one pleural T-cell assay and their outcome categorization by disease status is shown in table 4A. There was no difference in the number of indeterminate results using the QFT-TB-GIT pleural mononuclear cell-derived assay compared to the pleural T-SPOT.®TB assay (4/60 vs. 6/61; p= 0.40, Fisher exact). A head-to-head comparison of 53 paired pleural T-SPOT.®TB and QFT-TB GIT assays, in TB and non-TB subjects, is shown in table 4B. There was modest agreement between the pleural RD-1 assays (56 and 53% in TB vs. non-TB patients, respectively). Inter-assay concordance was poor (table 4B; kappa=0.21). When probable and definite TB cases were grouped together

the sensitivity of assays remained unchanged (85 and 81% vs. 53 and 50%, respectively, for the two T-SPOT.®TB and QFT-TB GIT cut-points specified in table 3).

Pleural HBHA and PPD ELISPOT responses

The performance outcomes of HBHA and PPD-driven ELISPOT responses, using pleural mononuclear calls, is in shown in table 3, whilst a scatter-plot of the data together with area under the ROC is shown in figure 3C. In summary both antigens had poor discriminatory value when using pleural mononuclear cells.

Pleural fluid interferon gamma responses did not differ significantly in HIV-infected and HIV un-infected patients, respectively [unstimulated IFN-γ median (range) 10.87 (0.7-20.1) vs. 12.4 (0.1-20.1) IU/ml; QFT-GIT 1.64 (0.0-13.2) vs. 0.74 (0.0-14.0) IU/ml; ESAT-6 292 (0-1250) vs. 420 (0-1250) SFC/million cells; CFP-10 182 (0-1250) vs. 397 (0-1250) SFC/million cells].

Discussion

In this study we prospectively evaluated the clinical diagnostic utility of four different IGRAs (RD-1 ELISPOT, RD-1 ELISA, HBHA and PPD ELISPOT) using pleural fluid mononuclear cells, which were compared against pleural fluid unstimulated IFN-γ levels, in South African patients suspected to have TB. A comparative study of the different RD-1 IGRAs, and evaluation of HBHA responses, has not hitherto been undertaken using pleural fluid lymphocytes. The

IGRAs, which are technically more demanding and expensive, performed sub-optimally and, at best, missed 15% of TB cases and incorrectly diagnosed a further 20%. By contrast, unstimulated IFN-γ levels in pleural fluid distinguished, with a high level of accuracy, between tuberculosis and non-TB effusions. The excellent outcomes with IFN-outcomes are borne out by several other studies from geographical areas outside Africa (Asia, Europe and South America), which has been summarized in a recent meta-analysis, where the pooled sensitivity and specificity of IFN-γ in unstimulated pleural fluid was 89% and 97%, respectively (individual studies and their outcomes are outlined in the meta-analysis [6]). We confirm this finding in African HIV co-infected subjects. Collectively, these data suggest that a point of care test incorporating IFN-γ testing holds promise for the diagnosis of TB-PE in a high burden setting. This approach however does not allow determination of drug-susceptibility status and hence microbiological investigation in appropriate patients may still be required. Thus, IFN-γ levels cannot necessarily replace culture of *M. tuberculosis*, but could be used as an adjunctive test to make rapid diagnosis, and possibly reduce the need for further investigations to exclude other causes of the pleural effusion.

Two recent preliminary studies from low burden countries (Europe and Japan) suggested that RD-1-based commercial IGRA assays, using pleural mononuclear cells, appear promising as diagnostic tools for TB-PE [13, 14]. In the last several months two case-control studies from high burden countries (South Africa), and using QFT-GIT, have been published [15, 16]. Both showed that the QFT-TB-GIT

assay had sub-optimal sensitivity (27% and 57%, respectively). However, the T-SPOT.®TB test, thought to be more sensitive [7], was not evaluated in these studies. Furthermore, details about optimization and rationale of laboratory protocols are unclear, the total cell number in the QFT-TB-GIT assay was not controlled for, and pleural biopsy was not undertaken or limited at the discretion of the operator, thus limiting the accuracy of patient categorization. In the current study preliminary experiments were performed to determine optimal cell number and antigen concentration, pleural biopsy was performed unless refused or contra-indicated, and both RD-1 commercial platforms were evaluated.

Overall, the T-SPOT.®TB assay was more sensitive than the QFT-TB-GIT assay in keeping with similar observations in peripheral blood [7], presumably because ELISPOT is generally regarded a more sensitive technique [9]. Although the QFT-TB-GIT had a high PPV it missed almost half the TB cases. By contrast, using pleural fluid unstimulated IFN-γ levels, a non-specific Th1 marker of inflammation, achieved a higher PPV. Thus, the specificity of IGRAs was sub-optimal despite the use of TB-specific antigens. How do we explain these results? One possibility is the translocation of blood RD-1-specific T cells, through an inflamed or 'leaky' pleura, in subjects with non-TB pleuritis (no active TB) who had a concomitant high frequency of peripheral antigen-specific T cells and hence LTBI (6/7 non-TB patients who had a higher than expected frequency of antigen-specific T cells in the pleural space, also had a high frequency of peripheral antigen-specific T cells, and hence LTBI). We hypothesise that the

ELISPOT was more prone to this effect because it is a more sensitive technique. Alternative explanations for the poor specificity include transient exposure to *M. tuberculosis*, organisms in a state of non-replicating persistence in the pleural space, or dual pathology (active TB and malignancy), though on follow-up (6 to 12 months), we detected no clinical evidence of TB in those with alternative diagnoses. Compensating for the frequency of peripheral antigen-specific T cells or the total pleural mononuclear cells (pleural RD1 spot count/ peripheral spot count ratio) did not significantly improve the specificity of the assay (thus, we reasoned that in cases of active pleural TB there would be a greater proportion of antigens-specific cells and hence a high ratio, and in cases of LTBI there would be fewer such cells and hence the ratio would be low). Adjustment of the cut-point improved the specificity but compromised on the sensitivity.

By contrast the unstimulated pleural fluid IFN-γ was highly sensitive and specific for pleural TB. This potent *M. tb*-driven compartment-specific Th1 response is not characteristic of non-mycobacterial or tumour antigens. Thus, we believe, based on the data presented here, and that of other investigators [6], that unstimulated IFN-γ should be used as a discriminatory diagnostic tool in clinical practice. Further research is now required to develop user-friendly formats to measure unstimulated IFN-γ levels in pleural fluid.

The optimal cell number and pleural fluid volume required to perform pleural T cell assays is unclear [17]. Hence, an additional aim of our study was to clarify

this aspect. We show that obtaining adequate cell numbers to perform the IGRA was an important practical limitation of the assay. In most cases less than less than 10ml of pleural fluid was more than adequate for the required experiments. However, in some cases, where effusions were fibrinous or loculated, a limited volume of pleural fluid was obtainable (less than 20 ml), or the fluid was pauci-cellular, thus limiting the number of cells that were isolated. Additional factors, likely more frequent in high burden settings, that may have modulated the number of viable cells obtained include: unequal distribution of cells in the pleural fluid, cell loss during processing, lymphocyte-poor and neutrophil predominant effusions, excessive debris and degenerating cells in complicated effusions, and pauci-cellular pleural inflammation in HIV+ subjects (six of the eight patients with inadequate cell numbers, who were tested, had advanced HIV disease). Thus, for successful T cell assays to be performed it is crucial that adequate volumes of pleural fluid are obtained. In contrast to previous reports [15], to enable precise quantification of the number of cells being used in the assay we washed pleural mononuclear cells and re-incubated them in serum free media. This had the additional advantage of minimizing indeterminate results due to high readouts in the negative control well/ tube. There was only modest inter-assay agreement/ concordance and this may reflect differences in the technique (ELISA vs. ELISPOT), cell numbers and antigen cocktails used (QFT-TB-GIT has an additional peptide antigen, TB 7.7). For this study all samples were processed in a category 3 containment laboratory. However, this may not always be possible

in a resource-poor setting where safety precautions may be in line with local policy and regulations.

To meaningfully evaluate the relative clinical value of newer and established tests, we compared their utility to a simple bio-clinical score, generated through regression analysis, and relevant to a resource-poor setting [25, 26]. We also investigated the effect of HIV status on test performance outcomes. All tests performed equally well in HIV+ and negative patients.

We also tested in the ELISPOT assay, a novel antigen, HBHA, a virulence factor that mediates binding of *M. tuberculosis* to alveolar epithelial cells, and whose antigenicity is dependent on methylation of the C-terminal domain, which is absent from recombinant forms of HBHA [19]. HBHA-responses were recently found to be a sensitive marker of TB infection [18, 27]. We therefore used *M. smegmatis* electroporated with a plasmid vector to produce methylated *M.tb* HBHA [20, 28, 29]. Human tuberculous pleural fluid was recently shown to contain high titres of anti-HBHA antibody [30] but the utility of antigen-specific HBHA responses for the diagnosis of TB-PE is unknown. We found generally poor responses to methylated HBHA compared to the RD-1 antigens. The reasons for this are unclear but may be related to the altered pleural fluid regulatory T-cell profile [31] in TB-PE, which is known to attenuate HBHA responses [32], and may underlie the proposed ability of this antigen to

distinguish latent from active TB [18]. The differential effect of *M. smegmatis* vs. *M. tuberculosis* methylated HBHA on IFN-γ responses, if any, is unclear.

Investigator bias in our study was minimized through several steps that ensured our study's validity, including consecutive recruitment with universally applied and pre-specified inclusion criteria, an experienced operator blinded to clinical details, invasive procedures to ensure accurate classification of patient and control sub-groups, and use of a pre-specified reference standard. However, our results are probably generalisable only to high TB/HIV burden settings. Thus, further and larger studies are required to evaluate whether outcomes are different in low burden settings using the cut-points identified here, and the effect of HIV infection on IFN-γ-related pleural assays. The high background rate of LTBI and possible transient infection are likely to have impacted heavily on the T cell assay results in the non-TB group.

In conclusion, unstimulated IFN-γ was the most accurate test to distinguish TB from non-TB effusions in a high burden setting. In this well-categorized cohort of unselected patients from a high burden setting the antigen-specific T-cell IGRAs were limited by sub-optimal accuracy and the inability to isolate sufficient mononuclear cells to perform the assay.

Acknowledgements:

K Dheda is supported by the South African National Research Foundation (SARChI), the South African Medical Research Council, EU FP7, and the UCL-UCT Collaboration Initiative. A Zumla is in receipt of funding from the EC Framework 7 programme, EDCTP and from EuropeAID, Brussells, Belgium. We thank Drs P Govender, H Khalfey, A Aboo, I Carr, Prof R van Zyl-Smit, and registered nurses C Cooper and M Isaacs for facilitating patient recruitment. We are also grateful to Professors Zanetti and Delogu for the gift of the pMV 3-38.

References

1. Gopi A, Madhavan SM, Sharma SK, Sahn SA. Diagnosis and treatment of tuberculous pleural effusion in 2006. *Chest* 2007: 131(3): 880-889.

2. Sinzobahamvya N, Bhakta HP. Pleural exudate in a tropical hospital. *Eur Respir J* 1989: 2(2): 145-148.

3. Trajman A, Pai M, Dheda K, van Zyl Smit R, Zwerling AA, Joshi R, Kalantri S, Daley P, Menzies D. Novel tests for diagnosing tuberculous pleural effusion: what works and what does not? *Eur Respir J* 2008: 31(5): 1098-1106.

4. Greco S, Girardi E, Masciangelo R, Capoccetta GB, Saltini C. Adenosine deaminase and interferon gamma measurements for the diagnosis of tuberculous pleurisy: a meta-analysis. *Int J Tuberc Lung Dis* 2003: 7(8): 777-786.

5. Pokkali S, Das SD, R L. Expression of CXC and CC type of chemokines and its receptors in tuberculous and non-tuberculous effusions. *Cytokine* 2008: 41(3): 307-314.

6. Jiang J, Shi HZ, Liang QL, Qin SM, Qin XJ. Diagnostic value of interferon-gamma in tuberculous pleurisy: a metaanalysis. *Chest* 2007: 131(4): 1133-1141.

7. Menzies D, Pai M, Comstock G. Meta-analysis: new tests for the diagnosis of latent tuberculosis infection: areas of uncertainty and recommendations for research. *Ann Intern Med* 2007: 146(5): 340-354.

8. Pai M, Dheda K, Cunningham J, Scano F, O'Brien R. T-cell assays for the diagnosis of latent tuberculosis infection: moving the research agenda forward. *Lancet Infect Dis* 2007: 7(6): 428-438.

9. Pai M, Zwerling A, Menzies D. Systematic review: T-cell-based assays for the diagnosis of latent tuberculosis infection: an update. *Ann Intern Med* 2008: 149(3): 177-184.

10. Wilkinson KA, Wilkinson RJ, Pathan A, Ewer K, Prakash M, Klenerman P, Maskell N, Davies R, Pasvol G, Lalvani A. Ex vivo characterization of early secretory antigenic target 6-specific T cells at sites of active disease in pleural tuberculosis. *Clin Infect Dis* 2005: 40(1): 184-187.

11. Jafari C, Ernst M, Strassburg A, Greinert U, Kalsdorf B, Kirsten D, Lange C. Local immunodiagnosis of pulmonary tuberculosis by enzyme-linked immunospot. *Eur Respir J* 2008: 31(2): 261-265.

12. Jafari C, Ernst M, Kalsdorf B, Greinert U, Diel R, Kirsten D, Marienfeld K, Lalvani A, Lange C. Rapid diagnosis of smear-negative tuberculosis by bronchoalveolar lavage enzyme-linked immunospot. *Am J Respir Crit Care Med* 2006: 174(9): 1048-1054.

13. Losi M, Bossink A, Codecasa L, Jafari C, Ernst M, Thijsen S, Cirillo D, Ferrarese M, Greinert U, Fabbri LM, Richeldi L, Lange C. Use of a T-cell interferon-gamma release assay for the diagnosis of tuberculous pleurisy. *Eur Respir J* 2007: 30(6): 1173-1179.

14. Ariga H, Kawabe Y, Nagai H, Kurashima A, Masuda K, Matsui H, Tamura A, Nagayama N, Akagawa S, Machida K, Hebisawa A, Nakajima Y, Yotsumoto H, Mori T. Diagnosis of active tuberculous serositis by antigen-specific interferon-gamma response of cavity fluid cells. *Clin Infect Dis* 2007: 45(12): 1559-1567.

15. Baba K, Sornes S, Hoosen AA, Lekabe JM, Mpe MJ, Langeland N, Dyrhol-Riise AM. Evaluation of immune responses in HIV infected patients with pleural tuberculosis by the QuantiFERON TB-Gold interferon-gamma assay. *BMC Infect Dis* 2008: 8: 35.

16. Chegou NN, Walzl G, Bolliger CT, Diacon AH, van den Heuvel MM. Evaluation of Adapted Whole-Blood Interferon-gamma Release Assays for the Diagnosis of Pleural Tuberculosis. *Respiration* 2008.

17. Joshi R, Pai M. Can pleural tuberculosis be diagnosed using interferon-gamma release assays? *Respiration* 2008: 76(2): 128-130.

18. Hougardy JM, Schepers K, Place S, Drowart A, Lechevin V, Verscheure V, Debrie AS, Doherty TM, Van Vooren JP, Locht C, Mascart F. Heparin-binding-hemagglutinin-induced IFN-gamma release as a diagnostic tool for latent tuberculosis. *PLoS ONE* 2007: 2(10): e926.

19. Locht C, Hougardy JM, Rouanet C, Place S, Mascart F. Heparin-binding hemagglutinin, from an extrapulmonary dissemination factor to a powerful diagnostic and protective antigen against tuberculosis. *Tuberculosis (Edinb)* 2006: 86(3-4): 303-309.

20. Delogu G, Bua A, Pusceddu C, Parra M, Fadda G, Brennan MJ, Zanetti S. Expression and purification of recombinant methylated HBHA in Mycobacterium smegmatis. *FEMS Microbiol Lett* 2004: 239(1): 33-39.

21. Trajman A, Luiz RR. McNemar chi2 test revisited: comparing sensitivity and specificity of diagnostic examinations. *Scand J Clin Lab Invest* 2008: 68(1): 77-80.

22. Moons KG, Harrell FE, Steyerberg EW. Should scoring rules be based on odds ratios or regression coefficients? *J Clin Epidemiol* 2002: 55(10): 1054-1055.

23. Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig LM, Lijmer JG, Moher D, Rennie D, de Vet HC. Towards complete and accurate reporting of studies of diagnostic accuracy: the STARD initiative. Standards for Reporting of Diagnostic Accuracy. *Clin Chem* 2003: 49(1): 1-6.

24. Dheda K, Van-Zyl Smit RN, Sechi LA, Badri M, Meldau R, Symons G, Khalfey H, Carr I, Maredza A, Dawson R, Wainright H, Whitelaw A, Bateman ED, Zumla A. Clinical diagnostic utility of IP-10 and LAM antigen levels for the diagnosis of tuberculous pleural effusions in a high burden setting. *PLoS ONE* 2009: 4(3): e4689.

25. Neves DD, Dias RM, Cunha AJ. Predictive model for the diagnosis of tuberculous pleural effusion. *Braz J Infect Dis* 2007: 11(1): 83-88.

26. Porcel JM, Vives M. Differentiating tuberculous from malignant pleural effusions: a scoring model. *Med Sci Monit* 2003: 9(5): CR175-180.

27. Temmerman S, Pethe K, Parra M, Alonso S, Rouanet C, Pickett T, Drowart A, Debrie AS, Delogu G, Menozzi FD, Sergheraert C, Brennan MJ, Mascart F, Locht C. Methylation-dependent T cell immunity to Mycobacterium tuberculosis heparin-binding hemagglutinin. *Nat Med* 2004: 10(9): 935-941.

28. Delogu G, Sanguinetti M, Posteraro B, Rocca S, Zanetti S, Fadda G. The hbhA gene of Mycobacterium tuberculosis is specifically upregulated in the lungs but not in the spleens of aerogenically infected mice. *Infect Immun* 2006: 74(5): 3006-3011.

29. Zanetti S, Bua A, Delogu G, Pusceddu C, Mura M, Saba F, Pirina P, Garzelli C, Vertuccio C, Sechi LA, Fadda G. Patients with pulmonary tuberculosis develop a strong humoral response against methylated heparin-binding hemagglutinin. *Clin Diagn Lab Immunol* 2005: 12(9): 1135-1138.

30. Yang CS, Lee JS, Lee HM, Shim TS, Son JW, Jung SS, Kim JS, Song CH, Kim HJ, Jo EK. Differential cytokine levels and immunoreactivities against Mycobacterium tuberculosis antigens between tuberculous and malignant effusions. *Respir Med* 2008: 102(2): 280-286.

31. Chen X, Zhou B, Li M, Deng Q, Wu X, Le X, Wu C, Larmonier N, Zhang W, Zhang H, Wang H, Katsanis E. CD4(+)CD25(+)FoxP3(+) regulatory T cells suppress Mycobacterium tuberculosis immunity in patients with active disease. *Clin Immunol* 2007: 123(1): 50-59.

32. Hougardy JM, Place S, Hildebrand M, Drowart A, Debrie AS, Locht C, Mascart F. Regulatory T cells depress immune responses to protective antigens in active tuberculosis. *Am J Respir Crit Care Med* 2007: 176(4): 409-416.

Table 1. Baseline characteristics of the definite (n= 48) and non- tuberculosis (n= 19) patients. P-value: * t-test, †Fisher exact test, ‡ χ^2 test.

Characteristic	Definite TB n(%)	Non-TB n(%)	P-value
Age, Mean(SD)	37(14.4)	55.3(15.7)	**<0.0001** *
Sex			**0.99**‡
Male	23(47.9)	9(47.4)	
Female	25(52.1)	10(52.6)	
Race:			**0.03**†
Black African	25(52.1)	4(21.1)	
Mixed-race	23(47.9)	15(78.9)	
HIV status			**0.07**†
HIV +	22(45.8)	3(15.8))	
HIV negative	16(33.3)	10(52.6)	
Refused testing	10(20.8)	6(31.6)	
History of previous TB			**0.01**†
Yes	3(6.2)	6(31.6)	
No	45(93.8)	13(68.3)	
BCG status			**0.44**†
Positive	17(35.4)	8(42.1)	
Negative	19(37.6)	9(47.4)	
Unknown	12(25.0)	2(10.5)	
Current smoker			**0.02**‡
Yes	11(22.9)	10(52.6)	
No	37(77.1)	9(47.4)	
Employment status			**0.84**
Employed	29(60.4)	12(63.2)	
Unemployed	19(39.6)	7(36.8)	
Pleural Fluid, Mean(SD)			**0.003***
Protein (g/l)	58.9(15.7)	43.4(18.5)	

Table 2. Performance outcomes of a bio-clinical score for use in a high burden setting. Age less than 42 years or pleural fluid protein level > 53 g/l scored 4 points each; thus a score of 0, 4 or 8 was possible. Sens= sensitivity %; Spec= specificity %; PPV= positive predictive value; NPV= negative predictive value; [95% confidence interval].

Score	Sens	Spec	PPV	NPV	accuracy
0	19 [10; 31]	58 [36; 76]	53 [31; 73]	22 12; 35]	29 [20; 42]
4	81 [68; 89]	58 [32; 76]	82 [69; 91]	55 34; 78]	74 [63; 83]
8	54 [40; 67]	89 [68; 97]	92 [77; 98]	43 [29; 59]	64 [52; 74]

Table 3. Performance outcomes (95% CI) of IFN-γ release assays (T-SPOT®.TB, QFT-TB GIT, and HBHA and PPD-specific responses) at different cut-points, using pleural fluid mononuclear cells (3A) and peripheral blood (3B), in pleural TB suspects. Sixty-one and 60 T-SPOT®.TB, QFT-TB GIT assays were performed, respectively. * = manufacturer-derived cut-point for whole blood [≥ 24 spot forming cells (SFCs) per million mononuclear cells, either in the ESAT-6 or CFP-10 containing wells for T-SPOT®.TB, and ≥ 0.35 IU/ml for QFT-GIT]; ** = AUC-derived cut-point. Sens= sensitivity; Spec= specificity. n/a = not applicable (as this is a dichotomized variable the AUC result cannot be calculated). Comparative values for unstimulated IFN-γ levels are shown in figure 2 and in the results section.

3A

RD1 antigen-specific IGRAs using pleural fluid							
	Cut-point	Sens	Spec	PPV	NPV	Accuracy	AUC
T-SPOT.®TB (combined RD-1 antigen)	≥24SFC*	86% (71; 94)	60% (36; 80)	84% (69; 92)	64% (39;84)	78% (65; 88)	n/a
	> 100 SFC**	80% (65; 90)	65% (41; 83)	83% (67; 92)	61% (39;80)	76% (62; 85)	
T-SPOT.®TB ESAT-6	≥> 24 SFC*	86% (71;94)	67% (42;84)	86% (71;94)	67% (42;85)	80% (68;89)	0.88
	> 137 SFC**	72% (56;84)	93% (70;98)	96% (82;100)	58% (38;76)	78% (65;87)	
T-SPOT.®TB CFP-10	≥ 24 SFC*	78% (62;88)	60% (36;80)	82% (66;92)	53% (31;73)	73% (59;83)	0.84
	> 225 SFC**	56% (40;71)	93% (70;99)	95% (77;99)	47% (30;64)	67% (53;78)	
QFT TB GIT	≥ 0.35 IU/ml*	57% (41;72)	80% (55;93)	87% (68;96)	44% (28-63)	64% (50-76)	0.80
	> 0.73 IU/ml**	51% (36;67)	94% (72;94)	95% (75;99)	47% (31;64)	65% (51;76)	
HBHA and PPD-specific IGRAs using pleural fluid							
HBHA stimulation	>17 SFC**	59% (41;75)	31% (13;58)	64% (45;80)	27% (11;52)	59% (40;75)	0.51
	>158 SFC**	22% (11;41)	69% (31;83)	60% (31;83)	30% (17;48)	38% (24;53)	
PPD stimulation	> 12 SFC**	81% (65;90)	40% (20;64)	76% (61;87)	46% (23;71)	69% (55;80)	0.61
	> 362 SFC**	24% (14;38)	87% (61;96)	85% (58;96)	27% (16;41)	39% (28;52)	

3B

RD1 antigen-specific IGRAs using peripheral whole blood							
	Cut-point	Sens	Spec	PPV	NPV	Accuracy	AUC
T-SPOT.®TB	≥ 24 SFC*	83% (68;92)	56% (33;77)	81% (66;91)	60% (36;80)	75% (62;85)	n/a
QFT TB GIT	≥ 0.35 IU/ml*	87% (70;95)	69% (42;87)	87% (70-95)	69% (42;87)	81% (67;90)	0.76

Table 4A. Performance outcomes, including indeterminate results, of T-SPOT.®TB (n= 61) and QFT TB GIT (n= 60), by disease status, using pleural fluid mononuclear cells.

	TB	Non-TB	Probable TB	Total
T-SPOT.®TB positive QFT GIT positive	31 20	6 3	3 2	40 25
T-SPOT.®TB negative QFT GIT negative	5 15	9 12	1 4	15 31
T-SPOT.®TB indeterminate QFT GIT indeterminate	3 1	2 3	1 0	6 4

Table 4B. Head-to-head comparison of paired pleural fluid T-SPOT.®TB and QFT-TB GIT results, when available, in 36 definite TB patients and 17 non-TB patients.

Definite TB				
	T-SPOT.®TB positive	T-SPOT.®TB negative	T-SPOT.®TB indeterminate	Total
QFT GIT positive	17	0	3	20
QFT GIT negative	12	3	0	15
QFT indeterminate	0	1	0	1
Total	29	4	3	36
Kappa= 0.21 95% CI -0.02 – 0.41, p= 0.03; agreement = 56%				
Non-TB				
	T-SPOT.®TB positive	T-SPOT.®TB negative	T-SPOT.®TB indeterminate	Total
QFT GIT positive	2	1	0	3
QFT GIT negative	4	6	1	11
QFT indeterminate	0	2	1	3
Total	6	9	2	17
Kappa= 0.20 95%CI -0.27 – 0.67, p= 0.20; agreement = 53%				

Figure legends

Figure 1. Summary and flow chart of recruited patients. In addition, blood samples were taken for the RD-1 ELISPOT and RD-1 ELISA IFN-γ release assays.

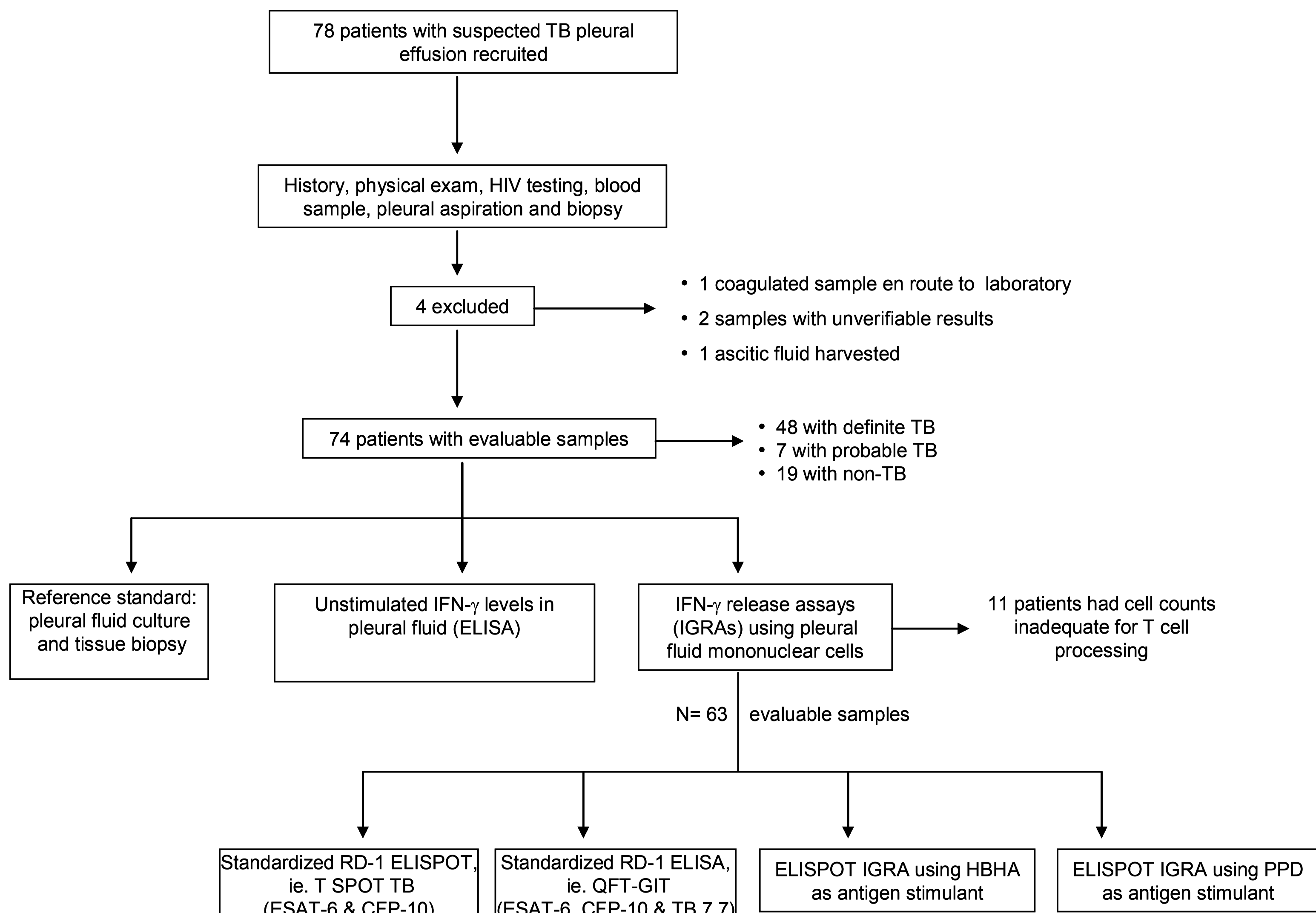


Figure 2. Scatter-plot of unstimulated IFN-γ using pleural fluid from patients with tuberculous (TB), non-tuberculous (non-TB) and probable tuberculosis pleural effusions (left panel). The right panel shows the receiver operating curve (ROC) for TB vs. non-TB pleural effusion. Area under the ROC was 0.99.

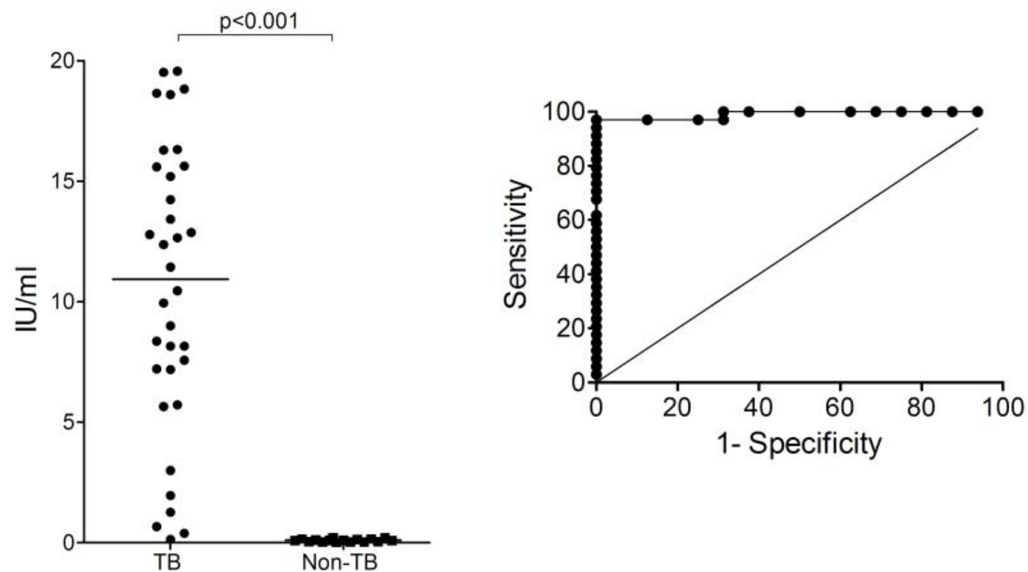
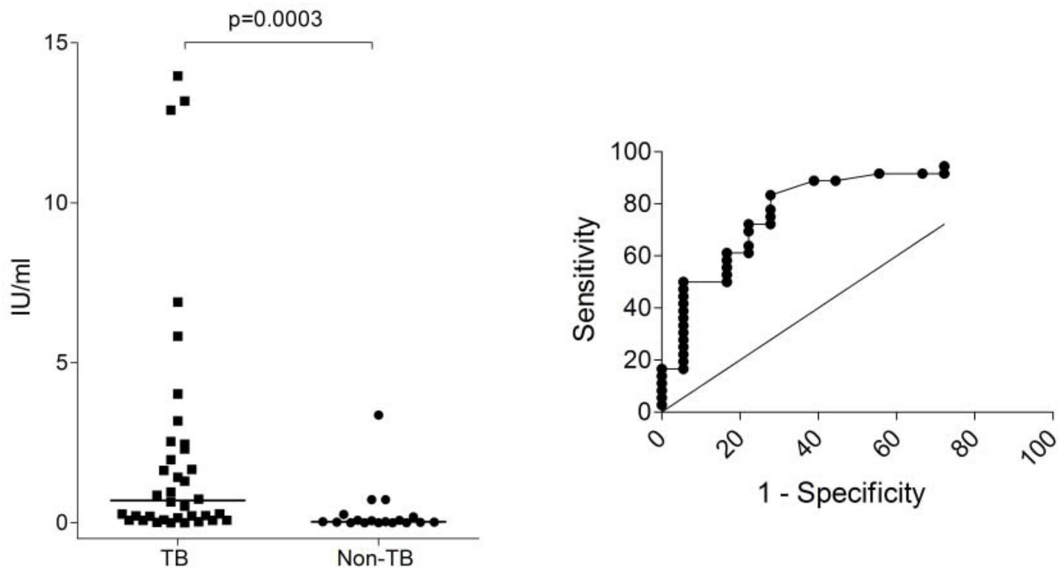
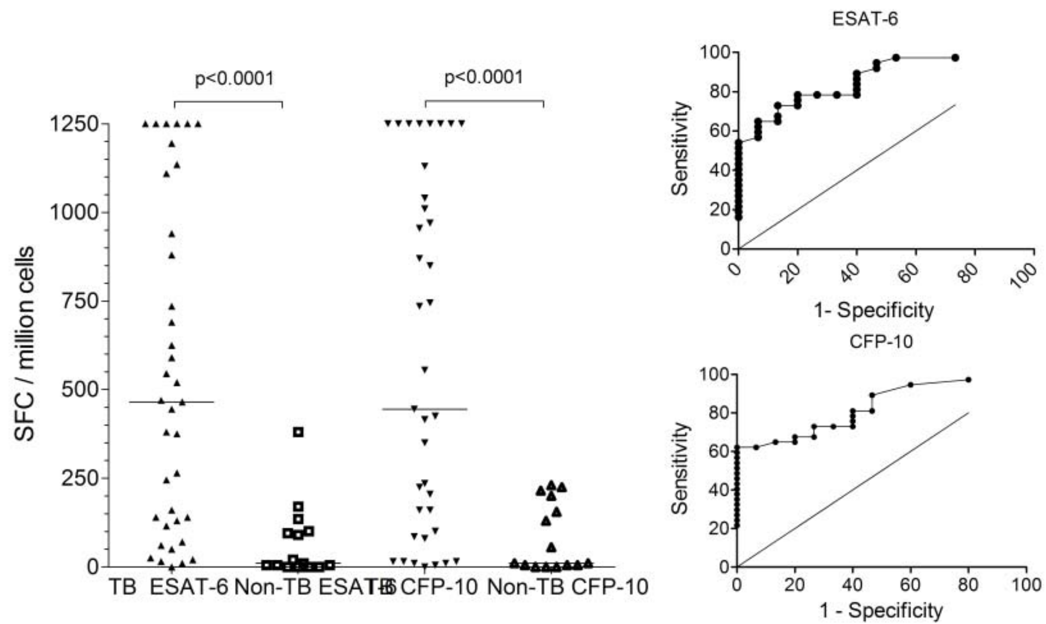


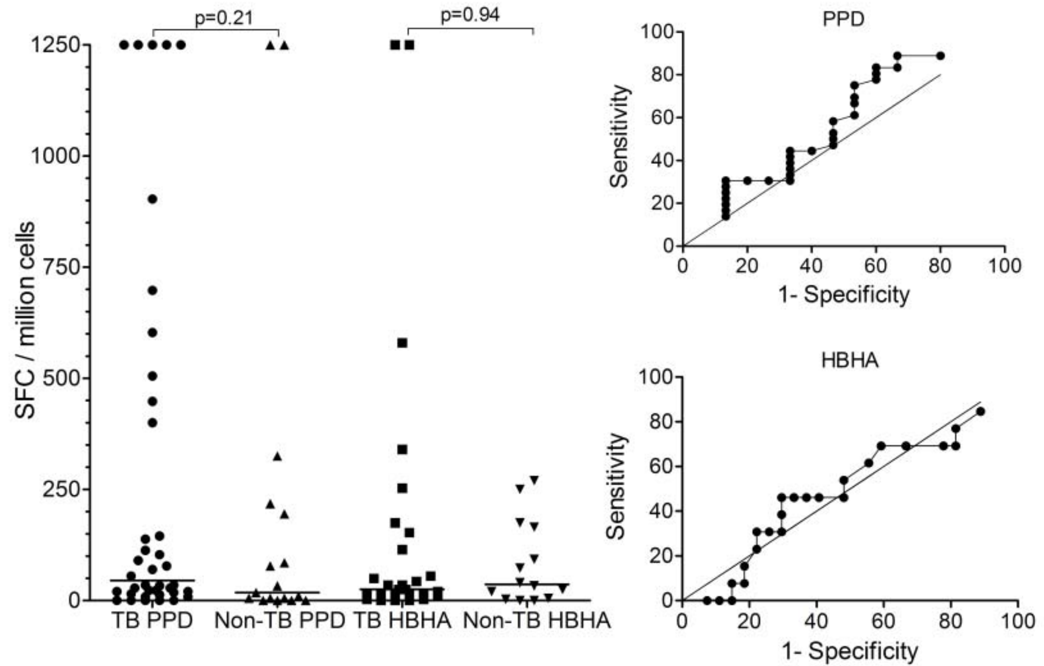
Figure 3. Scatter-plot of the frequency (spot forming cells per 10^6 pleural mononuclear cells) of the antigen-specific IFN-γ responses using the QFT-TB-GIT (figure 3A), T-SPOT.®TB assay (figure 3B), and of HBHA and PPD-specific IFN-γ responses (3C), in patients with tuberculosis (TB), non-tuberculosis (non-TB) and probable tuberculosis pleural effusions.

The right panel of each figure shows the respective area under the ROC curve analyses for TB vs. non-TB (actual values shown in table 3).





p<0.0001
p<0.0001
SFC / million cells
1250
1000
750
500
250
0
TB ESAT-6
Non-TB ESAT-6
TB CFP-10
Non-TB CFP-10
ESAT-6
Sensitivity
1- Specificity
CFP-10
Sensitivity
1 - Specificity



SFC / million cells
p=0.21
p=0.94
TB PPD
Non-TB PPD
TB HBHA
Non-TB HBHA
PPD
HBHA
Sensitivity
1- Specificity