Diagnostics

# Concurrent evaluation of cytokines improves the accuracy of antibodies against *Mycobacterium tuberculosis* antigens in the diagnosis of active tuberculosis

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## ARTICLE INFO

## ABSTRACT

*Background:* Antibodies against mycobacterial proteins are highly specific, but lack sensitivity, whereas cytokines have been shown to be sensitive but not very specific in the diagnosis of tuberculosis (TB). We assessed combinations between antibodies and cytokines for diagnosing TB.
*Methods:* Immuoglubulin (Ig) A and IgM antibody titres against selected mycobacterial antigens including Apa, NarL, Rv3019c, PstS1, LAM, "Kit 1" (MTP64 and Tpx)", and "Kit 2" (MPT64, Tpx and 19 kDa) were evaluated by ELISA in plasma samples obtained from individuals under clinical suspicion for TB. Combinations between the antibody titres and previously published cytokine responses in the same participants were assessed for diagnosing active TB.
*Results:* Antibody responses were more promising when used in combination (AUC of 0.80), when all seven antibodies were combined. When anti-"Kit 1"-IgA levels were combined with five host cytokine biomarkers, the AUC increased to 97% (92–100%) with a sensitivity of 95% (95% CI, 73–100%), and specificity of 88.5% (95% CI, 68.7–97%) achieved after leave-one-out cross validation.
*Conclusion:* When used in combination, IgA titres measured with ELISA against multiple *Mycobacterium tuberculosis* antigens may be useful in the diagnosis of TB. However, diagnostic accuracy may be improved if the antibodies are used in combination with cytokines.

## 1. Background

Tuberculosis (TB) remains one of the leading causes of death worldwide and was responsible for nearly 1.5 million deaths in 2019 [1]. Early case detection and proper treatment are highly important for the control of the disease. To reduce the burden of the disease, rapid and accurate tools for both the diagnosis and monitoring of TB treatment response are required. The diagnosis of TB still largely relies on initial clinical suspicion and subsequent microbiological confirmation by Ziehl-Neelsen staining, molecular diagnosis and culture [2]. However, these methods have several drawbacks: smear microscopy remains the most common and affordable method used especially in resource poor settings, but its sensitivity is compromised and in addition, this approach is unable to distinguish between *Mycobacterium tuberculosis (M.tb)* complex organisms and non-tuberculous mycobacteria, live and dead bacilli, amongst other limitations [3], making it impractical to be used as a tool to monitor TB treatment response [4,5]. Although culture as the gold standard test for TB is more sensitive, this method requires bacterial growth for up to 6 weeks before case detection and is not

**Abbreviations**

| | |
|---|---|
| PstS1 | Periplasmic phosphate-binding lipoprotein |
| Apa | alanine and proline rich secreted protein |
| NarL | nitrate/nitrite response transcriptional regulatory protein |
| LAM | lipoarabinomannan |
| kDa | kilo Dalton |
| "Kit 1" | MTP64+Tpx |
| "Kit 2" | MPT64+Tpx+19 kDa |
| WHO | World Health Organisation |

readily available in resource-limited settings. The automated gene amplification test GeneXpert (Cepheid Inc., Sunnyvale, USA) yields rapid results and is also able to detect resistance to rifampicin [6] but the use of the test is limited in resource-limited settings due to high operating costs and infrastructural needs [2]. Diagnostic tests that are based on sputum, including these microbiological and molecular methods, are not very useful in individuals with difficulty in providing sputum samples such as children. Immunodiagnostic assays may be useful for the diagnosis of TB since they have the potential to be easily adaptable into rapid, point-of-care tests, which would be suitable at primary health care centres in resource-limited settings.

Previous studies have shown that exposure to *M.tb* elicits antibody production to various antigens [7,8]. TB serological diagnostic assays have been widely investigated and have been improved over the years by using highly purified recombinant antigens, as previous generations of the assays were criticized for their low sensitivity in TB endemic regions [9,10]. Moreover, inflammatory biomarkers have been shown to have potential in the diagnosis of TB [11,12]. As these inflammatory biomarkers have sub-optimal specificity for TB, it is not known whether combining the cytokine biomarkers with their good sensitivity but poor specificity with antibodies with good specificity but poor sensitivity, will result in a better diagnostic biosignature for TB. A biosignature making use of both classes of biomarkers may therefore benefit from the strengths of each individual diagnostic approach.

In previous work, we evaluated antibodies against novel *M.tb* antigens for their ability to diagnose TB and as tools for monitoring of the response to TB treatment. These studies revealed that a combination of multiple antibody classes (IgA, IgG, IgM) against multiple *M.tb* antigens including LAM, Tpx and PPE proteins diagnosed TB with high accuracy [13]. Furthermore, combinations between other antibodies including anti-alanine dehydrogenase IgG, anti-Tpx IgG, anti-ESAT-6 IgG and anti-ESAT-6 IgA, measured prior to TB treatment initiation showed promise as markers for the prediction of early TB treatment response [14]. However, in other work done by Legesse et al., it was demonstrated that the discriminatory ability of *M.tb*-specific IgA antibodies was better than IgG antibodies for active TB and endemic controls in Africa [15]. This was also the case between healthy individuals with close contacts of TB patients and individuals without such contacts in another study [16]. Other studies also demonstrated a protective role for IgA in murine models of mycobacterial infection [17]. Contrary to our previous work, the present study focused on IgA antibodies. Furthermore, we also explored the utility of IgM antibodies against LAM as this kit was not available in previous panels evaluated in our high endemic TB setting.

The aim of the present study was therefore to explore the diagnostic potential of IgA antibodies against six *M.tb* specific antigens, and IgM antibodies against LAM in plasma samples from active TB patients and individuals with other respiratory diseases (ORD). We subsequently combined responses against the antibodies, with host inflammatory biomarker responses, to evaluate potential increased diagnostic performance resulting from the additional evaluation of host inflammatory

biomarkers together with the antibody responses.

## 2. Methods

### 2.1. Study participants

Participants enrolled into the present study were individuals who presented with signs and symptoms requiring investigation for TB at the Fisantekraal Community Clinic in the outskirts of Cape Town, South Africa. The study was a sub-study of a larger diagnostic biomarker project (the African European Tuberculosis Consortium; AE-TBC), that was conducted at field sites in six African countries between June 2010 and December 2013. As previously described [11,18,19], all study participants presented with signs and symptoms suggestive of active TB, including persistent cough lasting ≥2 weeks and at least one of either fever, malaise, recent weight loss, night sweats, knowledge of close contact with a TB patient, haemoptysis, chest pain or loss of appetite, and were recruited prior to clinical or laboratory assessment for TB. Participants were eligible for the study, if they were 18 years or older and willing to give written informed consent for participation in the study, including consent for HIV testing. Patients were excluded if they were pregnant, had not been residing in the study community for more than 3 months, were severely anaemic (haemoglobin <10 g/l), were on anti-TB treatment, had received anti-TB treatment in the previous 90 days or if they were on quinolone or aminoglycoside antibiotics during the past 60 days. The study was approved by the Health Research Ethics Committee of the Faculty of Medicine and Health Sciences of the University of Stellenbosch (Ethics reference number: N10/08/274).

### 2.2. Sample collection

As previously described [20], 6 ml of blood was collected into heparinized BD vacutainer tubes (BD Biosciences, Franklin Lakes, NJ, USA) at enrolment and transported to the laboratory at 4–8 °C for further processing. Upon receipt in the laboratory, tubes were centrifuged at 2000 rpm for 10 min after which plasma was harvested, aliquoted and stored at −80 °C until analysed. Sputum samples were collected from all study participants and cultured using the MGIT method (BD Biosciences). Positive MGIT cultures were examined for acid fast bacilli using the Ziehl-Neelsen technique (to check for contamination), followed by Capilia TB testing (TAUNS, Numazu, Japan), to confirm the isolation of organisms of the *M.tb* complex, before being designated as positive cultures.

### 2.3. Classification of study participants

As previously described [11,20], participants were classified as definite TB patients (sputum culture confirmed), probable TB (a combination of Chest X-ray or AFB smear positivity with confirmed clinical response to anti-TB treatment), other respiratory diseases (ORD) or questionable disease status using a combination of clinical, radiological, and laboratory findings. As also described previously [11], individuals with ORD had other diagnoses, including upper and lower respiratory tract infections and acute exacerbations of chronic obstructive pulmonary disease or asthma, but we did not attempt to identify these organisms due to funding limitations.

### 2.4. Antigen preparation

Cloned and purified recombinant proteins of *M.tb* were used for the production of the ELISA kits. All protein antigens were purified at LIONEX Diagnostics and Therapeutics, Braunschweig, Germany, using standard chromatographic methods (affinity chromatography, ion exchange chromatography, size exclusion chromatography) as previously described [13,14,21].

## 2.5. Enzyme -linked immunosorbent assay

ELISA experiments were performed on all study participants according to manufacturer's instructions (LIONEX Diagnostics and Therapeutics, Braunschweig, Germany), as previously described [22]. The investigators performing the experiments were blinded to the clinical groups. All antigens against which antibodies were evaluated are listed in Table 1.

## 2.6. Evaluation of host inflammatory biomarker responses

In a previous study that was conducted on the same subset of the study participants [20] the concentrations of 74 host inflammatory biomarkers were evaluated in plasma. These biomarkers were: alpha-2-macroglobulin (A2M), haptoglobin, C-reactive protein (CRP), serum amyloid P (SAP), procalcitonin (PCT), ferritin, tissue plasminogen activator (TPA), fibrinogen, serum amyloid A (SAA) (Bio-Rad Laboratories, Hercules, CA, USA), vitronectin, extracellular matrix protein 1 (ECM1), antithrombin III, vitamin D binding protein (VDBP), sFas, granzyme A, sFasL, sCD137, granzyme B, perforin, myoglobulin, ADAMTS13, P-selectin, lipocalin-2, growth differentiation factor (GDF) −15, thrombopoietin (TPO), stem cell factor (SCF), B-cell attracting chemokine (BCA)-1, epithelial neutrophil activating protein (ENA-78), thymic stromal lymphopoietin (TSLP), I-309(CCL-1), stromal cell derived factor-1 alpha (SDF-1α), IFN-γ, IFN-α2, interferon gamma inducible protein (IP)-10 (CXCL10), macrophage inflammatory protein (MIP)-1β, tumor necrosis factor (TNF)-α, TNF-β, vascular endothelial growth factor (VEGF), soluble CD40 ligand (sCD40L), apolipoprotein (Apo) A-1, Apo CIII, complement component 3 (CC3), transthyretin, complement factor H (CFH), total plasminogen activator inhibitor-1 (PAI-1), neural cell adhesion molecule (NCAM), brain-derived neurotrophic factor (BDNF), cathepsin D, myeloperoxidase (MPO), matrix metalloproteinase (MMP)-2, MMP-9, monokine induced by gamma interferon (MIG/CXCL9), granulocyte chemotactic protein-2 (GCP2), interferon inducible T-cell alpha chemoattractant (I-TAC/CXCL11), hemofiltrate CC chemokine-1 (HCC1), α1-antitrypsin, pigment epithelium derived factor (PEDF), macrophage inflammatory protein-4 (MIP-4/CCL18), complement C4, interleukin (IL)-17F, IL-17A, IL-22, IL-33, IL-21, IL-23, IL-25, IL- 31, IL-28A, IL-16, IL-1β, IL-12(p40), IL-13, IL-11 and IL-29 (Merck Millipore, Billerica, MA, USA), and were evaluated as described in detail in Ref. [20]. Only data from participants that were common between the two studies were evaluated in the current study.

## 2.7. Statistical analysis

Data were analysed using Statistica (Statsoft, TIBCO Software, Palo Alto, CA, USA) and Graphpad Prism version 5 (Graphpad Software Inc., CA, USA). Differences in antibody responses between TB patients and individuals with ORD were analysed using the Mann-Whitney *U* test. The diagnostic abilities of individual antibody responses were assessed

by receiver operator characteristics (ROC) curve analysis. The cut-off values for each antibody and associated sensitivity and specificity were determined by selecting the maximum values of Youden's index [23]. The predictive abilities of combinations between different antibodies and between antibodies and cytokines were investigated by general discriminant analysis (GDA), with leave-one-out cross validation.

## 3. Results

A total of 156 study participants, 22 of whom were definite and 4 that were probable TB patients [11] were included in this study (Fig. 1). The mean age of all study participants was 37 ± 11.2 years and 28 (n = 4 TB and n = 24 ORD; 18%) were HIV infected. The clinical and demographic characteristics of study participants are shown in Table 2.

### 3.1. Utility of individual anti-M.tb antibodies in the diagnosis of TB

Titres of Ig A antibodies against six antigens (Apa, NarL, Rv3019c, PstS1, "kit1"; a mixture of MTP64 and Tpx, and "Kit2"; a mixture of MPT64, Tpx and 19 kDa, and titres of IgM antibodies against LAM (Table 1) were evaluated in plasma samples from all study participants. When antibody levels detected in the TB patients (n = 26) were compared to the levels obtained in the 130 individuals with ORD with the Mann-Whitney *U* test, IgA antibodies against four *M.tb* antigens (Rv3019c, "Kit 1","Kit 2" and NarL) differentiated significantly between TB patients and individuals with ORD (Table 3, Fig. 2). When the diagnostic accuracies of the antibodies were evaluated by ROC curve analysis, anti-NarL IgA was the only individual antibody that showed promise, with an area under the ROC curve (AUC) of 0.74 (95% CI, 0.64–0.83), and sensitivity and specificity of 92% (95% CI, 75–99%) and 52% (95% CI, 34–60%) respectively, regardless of HIV infection status of the study participant. When HIV infected individuals were excluded, the most useful antibodies were Rv3019c Ig A (AUC of 0.70, 95%CI, 0.56–0.54) and NarL IgA (AUC of 0.75, 95% CI, 0.65–0.86) (Supplementary Table 1).

### 3.2. Utility of multi-antibody models in the diagnosis of TB

When all seven antibodies (anti-Rv3019c IgA + anti-PstS1 IgA + anti-"Kit 1" IgA + anti-"Kit 2" IgA + anti-Apa IgA + anti-NarL IgA + anti-LAM IgM) were used in combination, regardless of HIV status of the study participant, the antibodies discriminated between TB and ORD with an AUC of 0.80 (95% CI, 0.72–0.88), sensitivity of 65.4% (95% CI 44.4–82%) and specificity of 76.9% (95% CI, 68.6–83.7%) (Fig. 3). After leave-one-out cross validation, the sensitivity of the combination was 58% (95% CI, 37.2–76%) and specificity was 78% (95% CI, 66.9–82.3%), with positive and negative predictive values of 58% (95% CI, 37.2–76.0%) and 75.3% (95% CI, 66.9–82.3%) respectively.

When the GDA procedure was repeated after excluding the HIV infected individuals, the AUC for the seven-antibody combination was 0.79 (95% CI, 0.7–0.89), with a sensitivity of 55% (95% CI, 32.7–74.9%) and specificity of 80.2% (95% CI,71.1–87.1%). After leave-one-out-cross validation, the sensitivity was 50% (95% CI, 37.2–76%) and specificity was 76% (95% CI, 66.9–82.3%) (Fig. 3).

### 3.3. Diagnostic accuracy of combinations between antibodies and cytokines

Aliquots of plasma from a subset of study participants included in this study were previously used in a study that focused on host inflammatory biomarkers [20]. To ascertain whether the measurement of cytokines may improve the accuracy of the antibody responses, we assessed the diagnostic potential of combinations between the antibodies evaluated in the current study and host inflammatory biomarker responses that were previously measured in the common study
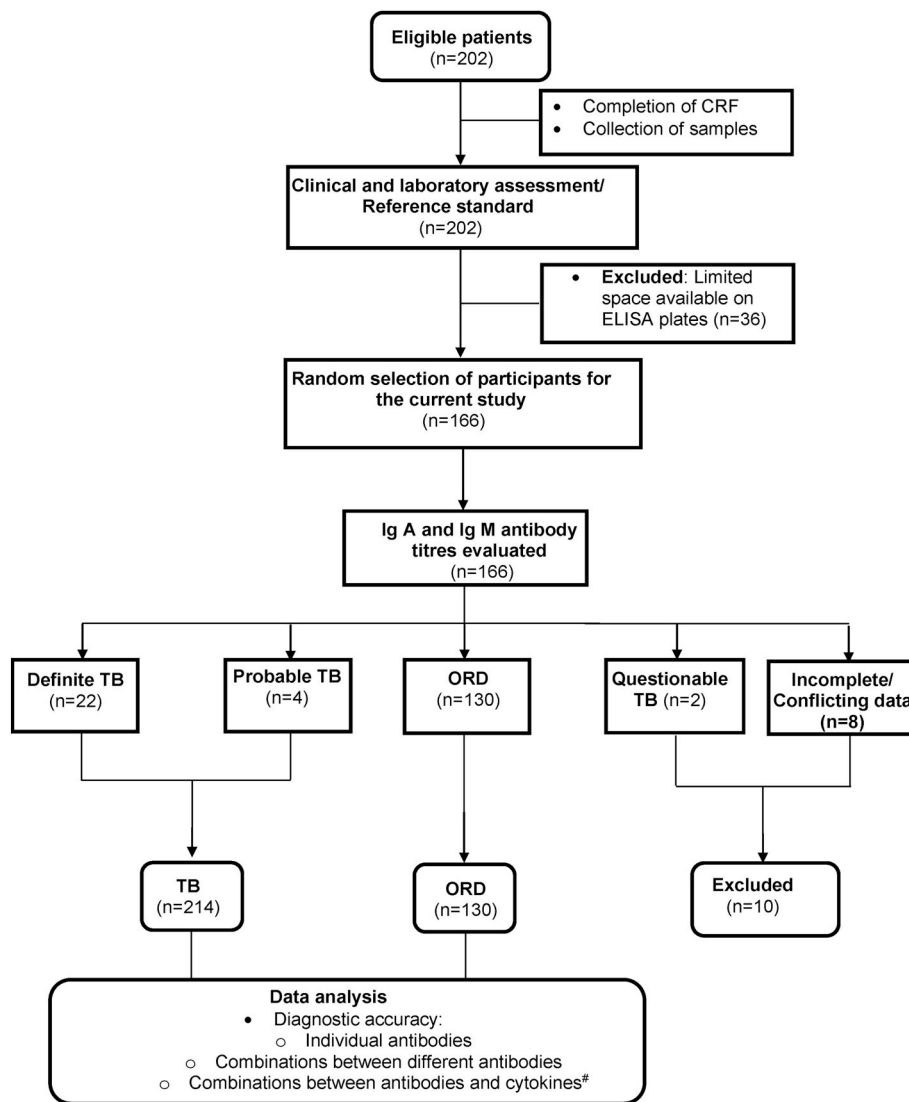
**Table 1**
Recombinant antigens of *M. tuberculosis* used in this study.

| *M. tuberculosis* antigens | Mol mass (kDa) | Rv number | Ig Class |
|---|---|---|---|
| EsxR (TB10.3) | 36 | Rv3019c | IgA |
| PstS1 | 37.37 | Rv0934 | IgA |
| "Kit 1" | - | - | IgA |
| "Kit 2" | - | - | IgA |
| Apa | 32.7 | Rv1860 | IgA |
| NarL | 23.9 | Rv0844c | IgA |
| LAM | - | - | IgM |

"Kit 1" = MTP64+Tpx, "Kit 2" = MPT64+Tpx+19 kDa.
PstS1 = Periplasmic phosphate-binding lipoprotein, Apa = alanine and proline rich secreted protein, NarL = nitrate/nitrite response transcriptional regulatory protein, LAM = lipoarabinomannan, kDa = kilo Dalton.

**Fig. 1.** Flow diagram showing the study design and classification of study participants. CRF, case report form; ELISA, Enzyme linked immunosorbent assay; TB, Pulmonary tuberculosis; ORD, Individuals presenting with symptoms and investigated for pulmonary TB but in whom TB disease was ruled out. #Combinations between antibodies and cytokines were evaluated in a subset of study participants (n = 46; n = 20 culture confirmed TB, and n = 26 ORD) who were included into a previous study [20].

participants (n = 46; n = 20 culture confirmed TB and n = 26 ORD). When the host inflammatory biomarker- and antibody data for each participant were fitted into GDA models, regardless of HIV status, a biosignature comprising of one antibody kit (anti-"Kit 1" IgA) and 5 host markers namely; neural cell adhesion molecule (NCAM)-1, vitronectin, complement factor H, ferritin and α-2 macroglobulin (A2M) (model 1) diagnosed TB with a sensitivity of 95% (95% CI, 73–100%) and specificity of 88.5% (95% CI, 68.7–97.0%). The accuracy of this biosignature did not change after leave-one-out cross validation (Fig. 4). The positive and negative predictive values of this biosignature were 86.4% (95% CI, 64–96.4%) and 95.8% (95% CI, 76.9–99.8%), respectively. An alternative biosignature (model 2) comprising three host inflammatory biomarkers (NCAM-1, vitronectin and sFas) and two antibodies (Apa IgA and NarL IgA) diagnosed TB with an AUC of 0.91 (95% CI, 0.82–1.00), sensitivity of 100% (95% CI, 79.9–100%) and specificity of 80.8% (95% CI, 60.0–92.6%) after leave-one-out cross validation.

When the diagnostic accuracies of the antibodies and host inflammatory biomarkers that were included in each of the two biosignatures (models 1 and 2 respectively) were evaluated separately (to assess the contribution of each biomarker type to the models), the host inflammatory biomarker – only signatures were more accurate as might be expected. However, the addition of the antibodies to the models increased both the sensitivity (by 10%) and specificity (especially in the case of model 2 which included more antibodies) by 11.6% (Table 4).

## 4. Discussion

Serological diagnostic methods for TB have extensively been studied with variable success [9]. Although the WHO advised against the use of commercial serological tests for the diagnosis of TB due to their suboptimal performance in a policy document [24], it encouraged further investigations on strategies to improve the accuracies of these tests. In the present study, we evaluated the serodiagnostic potentials of antibodies against seven *M.tb* specific antigens (Apa, NarL, Rv3019c, PstS1, LAM, "kit1"; a mixture of MTP64 and Tpx, and "Kit2"; a mixture of MPT64, Tpx and 19 kDa), for the immunological diagnosis of TB. IgA titres against NarL, Rv3019c, "Kit 1" and "Kit 2" were significantly different between TB patients and individuals with ORD, with the most promising single antibody being anti-NarL IgA. The diagnostic performance of antibodies improved when combined with host inflammatory biomarkers.

NarL has been described as a putative nitrate response regulator and is part of the membrane fraction of *M.tb* [25,26]. IgA antibodies against NarL in serum samples have previously been investigated for the diagnosis of active TB and discriminated between active TB patients and healthy controls (in contrast to ORD controls used in the present study) with 78.6% sensitivity and 100% specificity [21]. Our findings that IgA antibodies against NarL may be useful in the diagnosis of TB are therefore in keeping with these previous observations.

**Table 2**
Clinical and demographic characteristics of study participants.

| Number of participants | All Participants | Definite TB | Probable TB | All TB | ORD |
|---|---|---|---|---|---|
| | (n = 156) | (n = 22) | (n = 4) | (n = 26) | (n = 130) |
| Males, n (%) | 65 (42) | 6 (27) | 0 (0) | 6 (23) | 59 (45) |
| Mean age, (Years)±SD | 37 ± 11.2 | 39 ± 9.9 | 40 ± 11.4 | 39 ± 9.9 | 37 ± 11.5 |
| HIV Infected, n (%) | 28 (18) | 4 (18) | 0 (0) | 4 (15) | 24 (18) |
| Quantiferon results | | | | | |
| Positive, n (%) | 104 (70) | 16 (80) | 3 (75) | 19 (79) | 85 (69) |
| Negative, n (%) | 43 (29) | 3 (15) | 1 (25) | 4 (17) | 39 (31) |
| Indeterminate, n (%) | 1 (1) | 1 (5) | 0 (0) | 1 (4) | 0 (0) |
| **Symptoms at enrolment** | | | | | |
| Cough, n (%) | 156 (100) | 22 (100) | 4 (100) | 26 (100) | 130 (100) |
| Weight loss, n (%) | 103 (66) | 16 (23) | 4 (100) | 20 (77) | 83 (64) |
| Sputum production, n (%) | 155 (99) | 22 (100) | 4 (100) | 26 (100) | 129 (99) |
| Night sweats, n (%) | 110 (71) | 19 (86) | 4 (100) | 23 (88) | 87 (67) |
| Malaise, n (%) | 87 (56) | 15 (68) | 3 (75) | 18 (69) | 69 (53) |
| Fever, n (%) | 44 (28) | 10 (45) | 2 (50) | 12 (46) | 32 (25) |
| Anorexia, n (%) | 80 (51) | 13 (59) | 4 (100) | 17 (65) | 63 (48) |
| Haemoptysis, n (%) | 23 (15) | 4 (18) | 1 (25) | 5 (19) | 18 (14) |
| Chest pain, n (%) | 115 (74) | 19 (86) | 3 (75) | 22 (85) | 93 (72) |
| Short Breath, n (%) | 129 (83) | 18 (82) | 3 (75) | 21 (81) | 108 (83) |

Abbreviations: TB = pulmonary tuberculosis, SD = standard deviation.

When the TB-specific antibodies were used in combination with host inflammatory protein biomarkers, namely IgA antibodies against 'kit 1' (a mixture of MTP64 and Tpx) plus 5 host markers (NCAM-1, vitronectin, CFH, ferritin and α-2-macroglobulin), the antibody-host inflammatory biomarker signature diagnosed TB with accuracy that was superior to what was obtained with antibodies alone (sensitivity of 95% and specificity of 88.5% after leave-one-out cross validation). In another of such signatures comprising more antibodies (model 2), combinations between NCAM-1, vitronectin, sFas and two antibodies (Apa IgA and NarL IgA) also showed strong potential, with the inclusion of the host inflammatory biomarkers increasing the sensitivity and specificity of the two antibodies (Apa + NarL) by values of 35% and 11.6% respectively. A

5-marker biosignature (NCAM-1+SAP + ferritin + CFH + ECM-1) diagnosed TB with a sensitivity of 95.2% and specificity of 89.3% after leave-one-out cross validation in the initial host inflammatory biomarker study (19), with other previous studies also demonstrating the promising nature of host inflammatory biomarker-based signatures in the diagnosis of active TB [11,27,28]. Despite these previous studies showing the undeniable value of host inflammatory biosignatures in the diagnosis of TB, we showed in this study that the accuracy of these signatures may also improve by the concurrent testing of antibodies, as the addition of antibody (Apa + NarL) responses from the same participants increased both the sensitivity and specificity of the inflammatory marker combination (NCAM-1 + vitronectin + sFas) by 10% and 15.4% respectively. These data show that the combination of inflammatory biomarker responses with antibody levels may be a useful approach for the diagnosis of TB and requires evaluation in independent studies.

Apa is an actively secreted antigen of *M.tb* and IgA responses against this antigen diagnosed TB disease in serum samples with promising accuracy [13,29]. Although IgA antibodies against Apa did not perform well as an individual biomarker in the current study, it's inclusion in the alternative antibody + cytokine signature (model 2) and the fact that it was the most frequently occurring antigen in the multi-antigen biosignatures that were generated shows that it may indeed be a useful diagnostic candidate and requires further evaluation. This antigen was also included in an antigenic cocktail (Apa, CFP-10, ESAT-6, PstS-1 and Ag85) that was generated in a Cuban population for the diagnosis of active TB, and diagnosed TB with a sensitivity of 87.1% and specificity of 97.1% [30].

Despite several studies demonstrating the potential usefulness of host inflammatory biomarkers in the diagnosis of TB [11,12,28,31], it is well-established that these markers are not particularly disease specific, as their levels are raised in multiple infections and non-infectious inflammatory conditions. Combining host inflammatory biomarkers with antibodies may be a useful approach, which will benefit from the strengths of either type of biomarker as demonstrated in the current study. NCAM-1 has been found to be important in cell-cell or cell-matrix interactions [32] and its role in lung tumor progression has been described [33]. NCAM-1 was found to be the most frequently occurring biomarker in the TB biosignatures identified in our previous study [20]. The other prominent members of the antibody + cytokine signatures (ferritin and A2M) are acute phase proteins that have previously been shown to have potential as biomarkers for the diagnosis of TB [34,35]. A further prominent member of this combination (complement factor H) is a soluble complement regulator that is important in the alternative pathway [36], and which also showed potential in previous TB diagnostic studies [11]. Our observations regarding the potential usefulness of these biomarkers when evaluated alongside antibodies as TB diagnostic candidates require validation in future larger studies.

Although the sample size for this study (n = 156) was relatively small, it was considerably larger than sample sizes employed in most
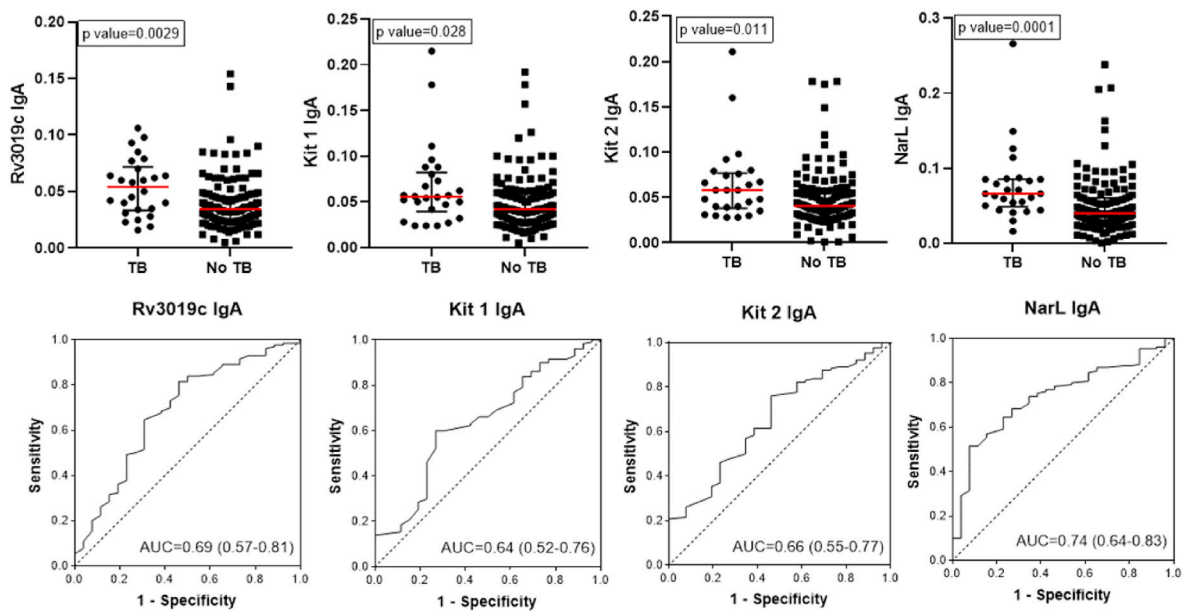
**Table 3**
**Median optical density values (and inter-quartile ranges in parenthesis) and diagnostic accuracies of individual antibodies against *M. tuberculosis* antigens in plasma samples to distinguish between TB (n = 26) and individuals with ORD (n = 130). Cut-off values were selected based on the Youden's index.**

| *M. tuberculosis* antigens | Ig class | TB | ORD | P-value | AUC (95% CI) | Sensitivity (95% CI) | Specificity (95% CI) | Cut-off Value |
|---|---|---|---|---|---|---|---|---|
| Rv3019c (TB10.3) | IgA | 0.054 (0.03–0.07) | 0.35 (0.02–0.05) | 0.0029 | 0.69 (0.57–0.81) | 54 (33–73) | 82 (74–88) | >0.0495 |
| PstS1 | IgA | 0.044 (0.03–0.07) | 0.034 (0.02–0.05) | 0.07 | 0.61 (0.49–0.73) | 77 (56–91) | 48 (39–57) | >0.0325 |
| Kit 1 | IgA | 0.056 (0.04–0.08) | 0.042 (0.03–0.06) | 0.028 | 0.64 (0.52–0.76) | 73 (52–88) | 60 (51–68) | >0.0465 |
| Kit 2 | IgA | 0.058 (0.038–0.078) | 0.041 (0.029–0.057) | 0.011 | 0.66 (0.55–0.77) | 54 (33–73) | 76 (68–83) | >0.0575 |
| Apa | IgA | 0.024 (0–0.037) | 0.025 (0.006–0.044) | 0.42 | 0.55 (0.43–0.67) | 92 (75–99) | 26 (18–35) | <0.0410 |
| NarL | IgA | 0.066 (0.05–0.09) | 0.040 (0.03–0.06) | 0.0001 | 0.74 (0.64–0.83) | 92 (75–99) | 51 (34–60) | >0.0405 |
| LAM | IgM | 0.073 (0.04–0.1) | 0.071 (0.05–0.1) | 0.98 | 0.50 (0.37–0.62) | 73 (52–88) | 35 (26–43) | <0.0865 |

"Kit 1" = MTP64+Tpx, "Kit 2" = MPT64+Tpx+19 kDa, PstS1 = Periplasmic phosphate-binding lipoprotein, Apa = alanine and proline rich secreted protein, NarL = nitrate/nitrite response transcriptional regulatory protein, LAM = lipoarabinomannan, kDa = kilo Dalton.

**Fig. 2.** Scatter plots showing the median optical density (450 nm) for anti-Rv3019c IgA, anti-"Kit 1" IgA, anti-"Kit 2" IgA and anti-NarL IgA in plasma samples from TB patients (n = 26) and individuals with ORD (n = 130) and ROC curves showing the accuracies of these antibodies in the diagnosis of TB. Error bars in the scatter plots represent the median with interquartile range. "Kit 1" = MTP64+Tpx, "Kit 2" = MPT64+Tpx+19 kDa, NarL = nitrate/nitrite response transcriptional regulatory protein, No TB = Individuals with other respiratory diseases (ORD).
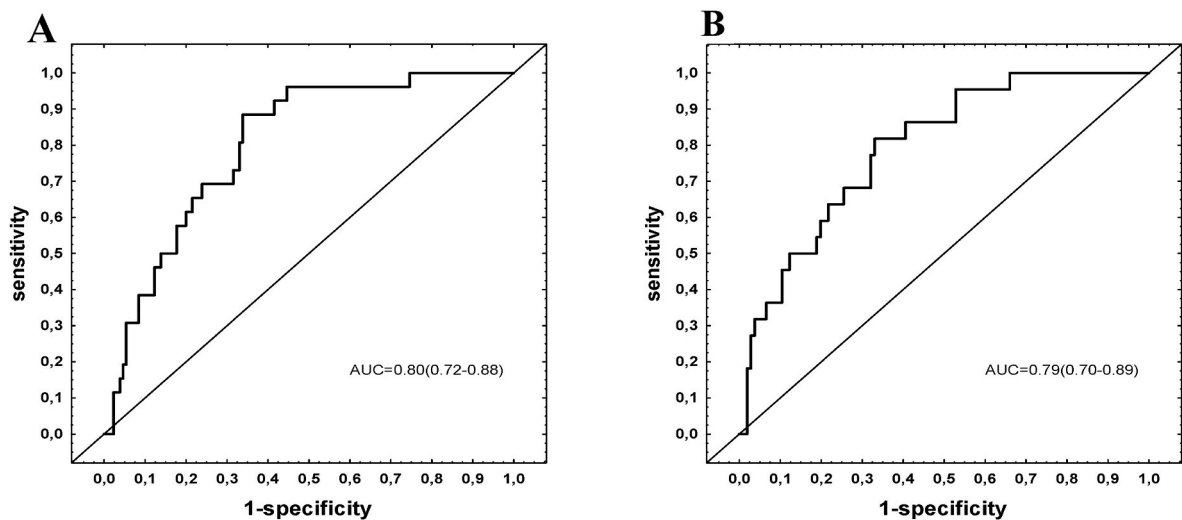


**Fig. 3. Accuracy of multi-antibody models in the serodiagnosis of TB.** Receiver operator characteristics (ROC) curve showing the accuracy of a seven-marker biosignature (anti-Apa IgA, anti- "Kit 1" IgA, anti-"Kit 2" IgA, anti- NarL IgA, anti-Rv3019c IgA, anti- PstS1 IgA and anti-LAM IgM) in all study participants, regardless of HIV infection (A), ROC curve showing the diagnostic accuracy of the seven-marker biosignature (anti-Apa IgA, anti- "Kit 1" IgA, anti-"Kit 2" IgA, anti- NarL IgA, anti-Rv3019c IgA, anti- PstS1 IgA and anti-LAM IgM) in HIV uninfected individuals (B). "Kit 1" = MTP64+Tpx, "Kit 2" = MPT64+Tpx+19 kDa, PstS1 = Periplasmic phosphate-binding lipoprotein, Apa = alanine and proline rich secreted protein, NarL = nitrate/nitrite response transcriptional regulatory protein, LAM = lip-oarabinomannan, kDa = kilo Dalton.

similarly published studies. The use of specimens that were collected from individuals that presented at a primary health care centre in a high burden setting with symptoms requiring investigation for TB, prior to the establishment of a clinical diagnosis, is a strength of our study. Although our findings may be relevant and have implications for the design of future point-of-care tests based on cytokines and antibodies, more validation studies, including sufficiently powered studies conducted in other geographical regions are required to confirm our observations. Such future studies should include more HIV-infected individuals and patients presenting with symptoms for other diseases that are often confused with TB including bacterial or viral upper or lower respiratory tract infections, acute exacerbations of chronic

obstructive airway disease end malignancy, to evaluate the specificity of the biosignatures.

In conclusion, our study is proof-of-concept that the combination of antibodies against *M.tb* antigens and host inflammatory biomarkers may be a useful approach for diagnosis of active TB. Further studies, also including other highly promising antibody-based biomarkers such as anti-LAM-IgG [13], are required to confirm and further improve the usefulness of this approach.
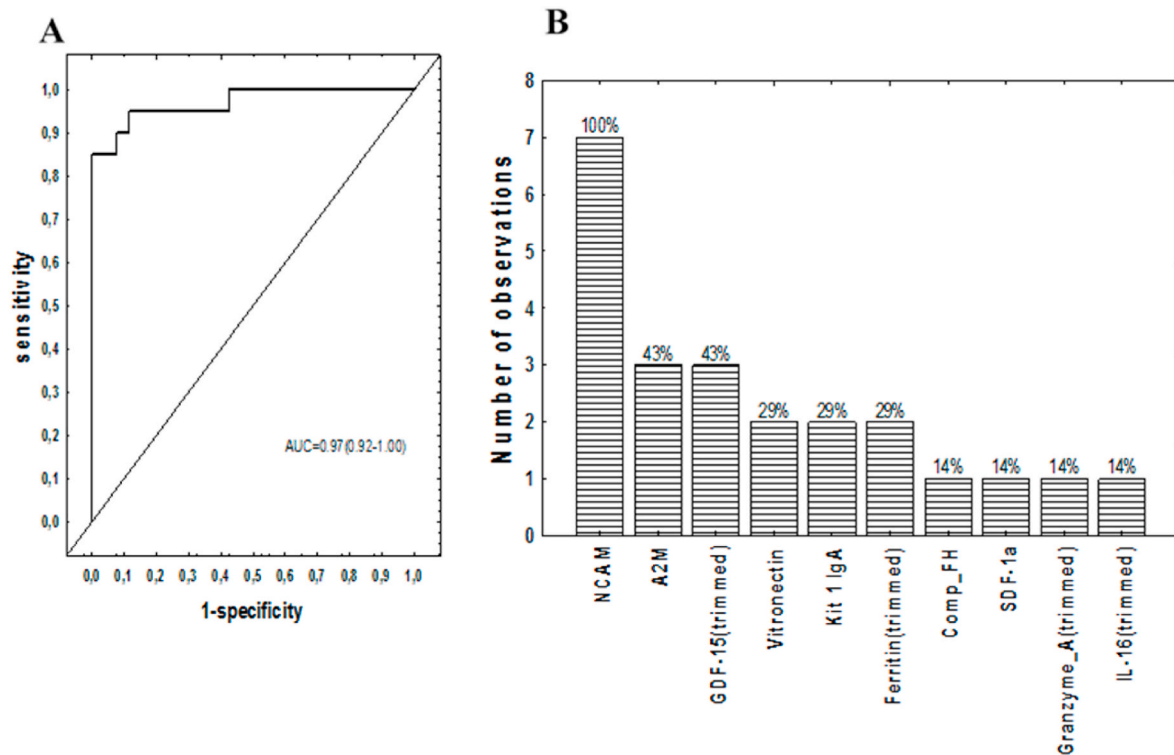
**Fig. 4. Accuracy of an antibody plus host inflammatory marker biosignature in the diagnosis of TB disease**. Receiver operator characteristic curve showing the diagnostic accuracy of the six-marker biosignature (anti-"Kit 1" IgA, NCAM-1, vitronectin, Complement factor H, ferritin and A2M) in all participants, regardless of HIV infection (A). Bar graph showing the frequency of analytes (antibodies and host inflammatory biomarkers) in the top 7 general discriminant analysis (GDA) models that most accurately classified study participants as TB or ORD irrespective of HIV status (B). "Kit 1" = MTP64+Tpx.

**Table 4**
Percentage increase in sensitivity and specificity obtained through the concurrent evaluation of host inflammatory biomarkers and antibodies in the same study participants.

| Biosignature | Biosignature type (and model from this study) | AUC (95% CI) | Sensitivity, % (95% CI), after LOOCV | Specificity, % (95% CI), after LOOCV | % Increase in Sensitivity* | % Increase in specificity* |
|---|---|---|---|---|---|---|
| "Kit 1" Ig A | Antibody only (model 1) | 0.64 (0.52–0.76) | 73 (52–88) | 60 (51–68) | NA | NA |
| NCAM-1, vitronectin, CFH, ferritin, A2M | Inflammatory markers only (Model 1) | 0.97 (0.93–1.00) | 95.0 (75.1–99.9) | 84.6 (65.1–95.6) | NA | NA |
| "Kit 1" Ig A + NCAM-1, vitronectin, CFH, ferritin, A2M | Antibodies + Inflammatory markers (Model 1) | 0.97 (0.92–1.00) | 95.0 (73–100) | 88.5 (68.7–97) | 0 | 3.9% |
| Apa IgA + NarL IgA | Antibodies only (model 2) | 0.78 (0.65–0.92) | 65.0 (40.8–84.6) | 69.2 (48.2–85.7) | NA | NA |
| NCAM-1, vitronectin, sFas | Inflammatory markers only (Model 2) | 0.89 (0.78–0.99) | 90.0 (68.3–98.8) | 65.4 (44.3–82.8) | NA | NA |
| Apa IgA, NarL IgA + NCAM-1, vitronectin, sFas | Antibodies + Inflammatory markers (Model 2) | 0.91 (0.82–1.00) | 100 (79.9–100%) | 80.8 (60.0–92.6%) | 10 | 11.6 |

"Kit 1" = MTP64+Tpx, Apa = alanine and proline rich secreted protein, NarL = nitrate/nitrite response transcriptional regulatory protein, CFH = complement factor H, A2M = alpha-2-macroglobulin; 95% CI = 95% Confidence Interval, AUC = Area under the receiver operator characteristics curve, LOOCV = Leave-one-out cross validation. *% increase values shown were obtained by subtracting the sensitivity and specificity of the respective antibody only or inflammatory biomarker only models (whichever was highest) from the combined antibody + host inflammatory biomarker model values.

**Data availability statement**

All the important data relevant to this study was reported in the manuscript. Any additional data will be made available upon request from the corresponding author.

**Author contributions**

RJ performed the laboratory experiments, analysed and interpreted data and wrote the first draft of the manuscript. DOA assisted with

laboratory experiments, interpretation of results and revised the manuscript for important intellectual content. RB contributed to the design of the study, interpretation of results and revised the manuscript critically. STM, SM recruited and characterised study participants and critically revised the manuscript. KS designed the database, collated, and interpreted data, SK and MS provided reagents and contributed to writing the draft and revision of the manuscript. GW contributed to the design of the study, provision of resources, coordination of the project and revision of the manuscript for important intellectual content. NC contributed to the design of the study, data analysis and interpretation and revision of the manuscript for important intellectual content. All authors read and approved the final version of the manuscript.

## Declaration of competing interest

None.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2022.102169.

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