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Host urine immunological biomarkers as potential candidates for the diagnosis of tuberculosis

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ABSTRACT

Objective: To investigate the potential of host urinary biomarkers as diagnostic candidates for tuberculosis (TB).
Methods: Adults self-presenting with symptoms requiring further investigation for TB were enrolled in Cape Town, South Africa. Participants were later classified as having TB or other respiratory diseases (ORD) using results from TB confirmatory tests. The concentrations of 29 analytes were evaluated in urine samples from participants using the Luminex platform, and their diagnostic potential was assessed using standard statistical approaches.
Results: Of the 151 study participants, 34 (22.5%) were diagnosed with TB and 26 (17.2%) were HIV-positive. Seven biomarkers showed potential as TB diagnostic candidates, with accuracy improving (in HIV-positives) when stratified according to HIV status (area under the receiver operating characteristics curve; AUC $\geq$0.80). In HIV-positive participants, a four-marker biosignature (sIL6R, MMP-9, IL-2Ra, IFN-γ) diagnosed TB with AUC of 0.96, sensitivity of 85.7% (95% confidence interval (CI) 42.1–99.6%), and specificity of 94.7% (95% CI 74.0–99.9%). In HIV-negatives, the most promising was a two-marker biosignature (sIL6R and sIL-2Ra), which diagnosed TB with AUC of 0.76, sensitivity of 53.9% (95% CI 33.4–73.4%), and specificity of 79.6% (95% CI 70.3–87.1%).
Conclusions: Urinary host inflammatory biomarkers possess TB diagnostic potential but may be influenced by HIV infection. The results of this study require validation in larger studies.

Introduction

Annual tuberculosis (TB) illness stands at 10 million, with roughly 1.7 billion people infected with *Mycobacterium tuberculosis* (Mtb) worldwide (WHO, 2019). The World Health Organization End TB Strategy (2016–2035), proposes to accomplish a 95% decrease in TB mortality and a 90% drop in the rate of occurrence of TB by 2035 (WHO, 2019). To achieve these targets, improved and reliable diagnostics, in addition to new and more effective drugs and vaccines, are urgently needed.

Current diagnostic approaches for TB are limited by challenges that either do not guarantee the accuracy of results or render their extensive use impossible, especially in resource-constrained settings (Yong et al., 2019). For example, the reference standard, sputum culture, provides results after a significant delay, in addition to the challenge of cost, contamination, and requirement for high-tech laboratory infrastructure (Chegou et al., 2011; Chegou et al., 2018; Luo et al., 2019; WHO, 2019; Yong et al., 2019). Conversely, sputum-smear microscopy is, to a great extent, accessible but lacks sensitivity, missing diagnosis in more than one-third of patients requiring care (Davies and Pai, 2008; Goletti et al., 2016). The GeneXpert MTB/RIF or ULTRA, a nucleic acid-based test, has a high sensitivity, rapid turnaround time, and identifies rifampicin resistance. However, the problem of cost limits its extensive deployment in resource-limited settings where the burden of TB disease is highest (Albert et al., 2016; Pantoja et al., 2013). Furthermore, these available methods rely on sputum specimens and are not very useful in patients who have difficulty providing quality sputum, such as children and individuals with

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extrapulmonary TB (Yong et al., 2019). For these reasons, there is a need for new and more efficient tests for TB that could assist in identifying or ruling out active TB in individuals suspected of having TB disease.

Immunodiagnostic techniques may be valuable for TB diagnosis, mainly if they utilize samples that are easily collected, namely, blood, saliva, or urine. Interferon gamma-release assays (IGRAs) and the tuberculin skin test (TST), the existing and widely used immunological tests for detecting people infected with Mtb, do not discriminate between an ongoing TB disease and latent infection (Cho et al., 2020; Goletti et al., 2018; Pai et al., 2014; Wawrocki et al., 2019; WHO, 2011). This limitation has led to an intensified search for alternative host biomarkers that could accurately diagnose active TB disease, in addition to distinguishing TB from other diseases of the respiratory tract with related symptoms. Aside from the potential of host biomarkers to diagnose TB with high accuracy, there is the prospect for their incorporation into a rapid, affordable, and user-friendly test that can be widely deployed to points of primary care, especially in resource-constrained settings.

We have previously measured and identified several host biomarkers in whole blood culture supernatants, serum, plasma, saliva, and cerebrospinal fluid. Some of these individual proteins or biosignatures have shown good prospects in the diagnosis of various forms of TB disease (Chegou et al., 2009; Chegou et al., 2016; Chegou et al., 2018; Jacobs et al., 2016a; Manngo et al., 2019; Manyelo et al., 2019). Urine is easily collected and commonly used for diagnostic tests. Immunological biomarkers in urine have shown applications in conditions such as cancers and sepsis (Kustán et al., 2017; Li et al., 2016; Su et al., 2011). However, urine has not been widely investigated to identify host immunological biomarkers for TB diagnosis. To date, lipoarabinomannan (LAM), a mycobacterial antigen, is the only approved biomarker in urine for TB diagnosis, and LAM test kits are commercially available (Bulterys et al., 2020; Peter et al., 2012; Songkhla et al., 2019; WHO, 2015). Nevertheless, LAM tests have low sensitivity and are only useful in HIV-positive TB cases with low CD4 cell counts (Abbasi, 2018; Songkhla et al., 2019). Elevated levels of inducible protein (IP)-10 in the urine of individuals with active TB have been reported in previous studies (Petrone et al., 2019; Petrone et al., 2016). Another recent study explored the use of a urine metabolomic biosignature in the diagnosis of TB disease (Isa et al., 2018). Altogether, these studies give credence, and further show that urine could be an alternative candidate specimen for the discovery of biomarkers for TB diagnosis.

Therefore, in the present study, the aim was to measure and identify potential urine-based immunological biomarkers that may be useful in the diagnosis of pulmonary TB in adults recruited from a TB-endemic setting.

Methods

Study participants

A total of 151 adults (18 years or older), who reported to a primary health clinic in Cape Town, South Africa, between November 2010 and November 2012 with symptoms suggestive of TB, were recruited. The participants were enrolled before the confirmation of a clinical diagnosis, and formed part of a larger cohort of the African European Tuberculosis Consortium for TB Diagnostic Biomarkers project (Awoniyi et al., 2016; Chegou et al., 2016; MacLean et al., 2019). We included participants who

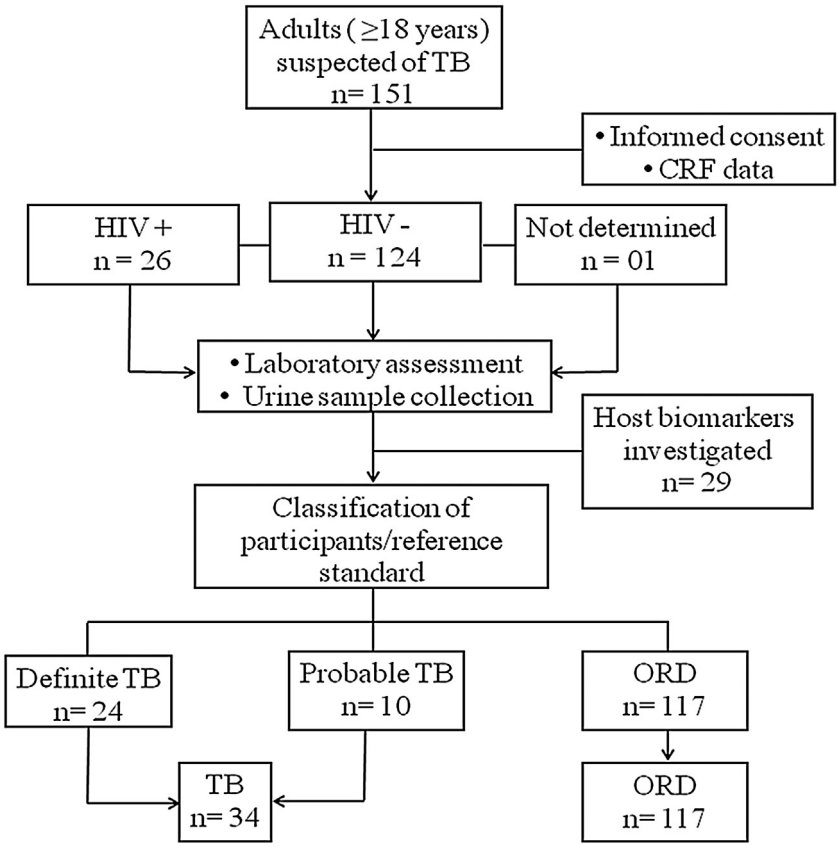

Figure 1. Flow diagram showing the classification of participants and the study design. TB = tuberculosis; ORDs = other respiratory diseases (these were individuals with negative smear, culture, and chest X-rays); CRF = case report file.

self-presented with a cough that had been persisting for a minimum of 2 weeks, in addition to presenting with one of either chest pain, loss of appetite, fever, knowledge of close contact with a confirmed TB case, weight loss, sweating at night, or haemoptysis. Furthermore, only participants who consented in writing to participate in the study and to undergo HIV testing were enrolled. Participants who were severely anaemic (haemoglobin <10 g/l), pregnant, receiving treatment for TB, had received anti-TB drugs in the past 90 days, had resided in the neighbourhood for less than 3 months, or were on aminoglycoside or quinolone antibiotics during the past 60 days were excluded from the study. The bacille Calmette–Guérin (BCG) vaccine is routinely administered at birth in the study community. The study was approved by the Health Research Ethics Committee of the Faculty of Medicine and Health Sciences of the University of Stellenbosch (reference number N10/08/274).

Sample collection and processing

Midstream urine samples were collected into 15-ml tubes and transported to the laboratory at 4–8 °C. The samples were centrifuged at 1000×*g* for 2 min, and the supernatants were aliquoted into multiple 2-ml cryovials and stored at −80 °C until the assays were performed. To microbiologically confirm TB, sputum specimens were collected from the participants, and these were cultured using the mycobacteria growth indicator tube (MGIT) technique (BD Biosciences). Positive cultures for Mtb were confirmed by performing Ziehl–Neelsen acid-fast bacillus staining, followed by Capilia TB assay (TAUNS, Numazu, Japan), as reported previously by Chegou et al. (2016) and Jacobs et al. (2016a).

Reference standard for the classification of study participants

The participants were classified using a pre-defined combination of radiological, clinical, and laboratory results as definite/probable TB, questionable, or other respiratory diseases (ORDs), as reported in previous studies (Chegou et al., 2016; Jacobs et al., 2016a). As mentioned in these previous reports (Chegou et al., 2016; Jacobs et al., 2016a), individuals with a questionable status were those who could not be classified as TB or ORD due to various reasons, including contamination of cultures, coupled with a lack of data from other diagnostic tests. Participants classified as ORD had negative results for all TB tests and had never been initiated on TB treatment by the national TB control program. These individuals had a range of other lower and upper respiratory tract conditions, including asthma and acute exacerbation of chronic pulmonary disease, as reported previously (Chegou et al., 2016; Jacobs et al., 2016a). However, they were not investigated further with cultures for bacterial or viral pathogens. Only individuals who were diagnosed with definite/probable TB or ORD were included in the present study (Figure 1).

Immunoassay

The concentrations of 29 host biomarkers were investigated in urine samples obtained from the participants using the Luminex platform. These biomarkers were selected from the literature, based on the potential shown in previous studies conducted on serum or plasma samples as candidate biomarkers for the diagnosis of TB (Chegou et al., 2016; Jacobs et al., 2016b) or monitoring of the response to TB treatment (Ronacher et al., 2019). The biomarkers measured include ferritin, matrix metalloproteinase 9 (MMP-9), macrophage-derived chemokine (MDC/CCL22), interleukin (IL)-2 receptor alpha (IL-2Ra), interferon-gamma (IFN-γ), procalcitonin (PCT), vascular endothelial growth factor receptor 3 (VEGF-R3), macrophage inflammatory protein-1 beta (MIP-1β/CCL4), inducible protein 10 (IP-10/CXCL10), IL-6Ra, VEGF-A (R&D Systems Inc., Minneapolis, MN, USA), C-reactive protein (CRP), fibrinogen, serum amyloid protein P (SAP), alpha-2-macroglobulin (A2M), haptoglobin, soluble IL6R (sIL6R), sIL4R, sIL2Ra, sVEGF-R3, soluble cluster of differentiation 30 (sCD30), soluble glycoprotein 130 (sgp130), sIL1RI, sIL1RII, soluble receptor for advanced glycation end products (sRAGE), soluble tumour necrosis factor receptor 1 (sTNFR I), sTNFR II, sVEGF-R1, and sVEGF-R2 (Merck Millipore, Massachusetts, USA). The analysis of samples was done on the Bio-Plex platform (Bio-Rad Laboratories, Hercules, CA, USA), and the amount of the measured biomarkers in the reagents used for quality control were within the expected ranges. In cases where a kit had not been validated for use on urine, the procedure for analysis of serum samples was followed. Experiments were performed in a blinded manner. Bio-Plex Manager Software version 6.1 was used for bead acquisition and analysis.

Statistical analysis

Data were analysed using Graph Pad Prism version 7.00 (GraphPad Software, San Diego, CA, USA) and Statistica (TIBCO Software Inc., Palo Alto, CA, USA). The Mann–Whitney *U*-test was used to determine statistical differences between the TB and ORD groups. The accuracy of individual biomarkers to diagnose TB was investigated using receiver operating characteristics (ROC) curve analysis. Optimal cut-off values and associated sensitivity and specificity were determined using the Youden's index (Fluss et al., 2005). The predictive ability of combined host biomarkers was assessed using general discriminant analysis (GDA), followed by leave-one-out cross-validation.

Results

Of the 151 individuals with a definite TB, probable TB, or ORD diagnosis (Figure 1), 26 (17%) were confirmed positive for HIV, whereas 93 (62%) of the participants were QuantiFERON-TB Gold (QFT)-positive (Table 1). For analysis purposes, the definite TB and

Table 1
Demographic and clinical characteristics of the study participants.

	All	TB group	ORD group
Number of patients	151	34	117
Sex; male *n* (%)/female *n* (%)	63 (42)/88 (58)	9 (26.5)/25 (73.5)	54 (46)/63 (54)
Age (years), mean ± SD	38.1 ± 11.5	39.6 ± 10.3	37.7 ± 11.9
HIV-positive, *n* (%)	26 (17)	7 (21)	19 (16)
QFT			
Positive, *n* (%)	93 (62)	22 (65)	71 (61)
Negative, *n* (%)	47 (31)	6 (18)	40 (34)
Indeterminate, *n* (%)	0 (0)	0 (0)	0 (0)

TB, tuberculosis; ORD, other respiratory diseases; SD, standard deviation; QFT, QuantiFERON-TB Gold.

probable TB patients were combined (TB group) for comparison with individuals with ORD, as has been done in previous studies (Awoniyi et al., 2016; Chegou et al., 2016; Jacobs et al., 2016a,c). The characteristics of the study participants are shown in Table 1.

Potential of individual host urine biomarkers in the diagnosis of TB regardless of HIV infection status

The potential of individual host urine biomarkers to diagnose TB was assessed by comparing differences in the concentrations of the analytes between the TB group (definite TB + probable TB) and individuals with ORDs using the Mann–Whitney *U*-test. Out of the 29 host biomarkers evaluated, the median levels of 10 markers were significantly higher in TB patients than in those with ORDs (Table 2). Following the investigation of the diagnostic potential of the 10 biomarkers using ROC curve analysis, seven performed in the diagnosis of TB regardless of HIV infection status with AUCs ≥0.65: sIL2Ra, sCD30, sIL1RI, haptoglobin, sTNFR II, ferritin, and IL-2Ra. sIL2Ra showed the highest accuracy (AUC = 0.70) (Figure 2a) and diagnosed TB with a sensitivity and specificity of 59% (95% CI 41–75%) and 81% (95% CI 72–88%), respectively (Table 2).

Performance of individual urine biomarkers stratified according to HIV infection status of the study participants

After stratification of the study participants according to HIV status, ferritin, sTNFR II, haptoglobin, sIL2Ra, and sIL1RI were significantly elevated in the HIV-negative participants diagnosed with TB compared to those with ORDs, and performed in the diagnosis of TB with AUCs ≥0.63 (see Supplementary Material Table S1). Haptoglobin was the most accurate biomarker (AUC = 0.70) (Figure 2b) in HIV-negative participants, with a sensitivity of 65% (95% CI 46–81%) and specificity of 72% (95% CI 63–80%) (see Supplementary Material Table S1). Both sCD30 and IL-2Ra showed potential in HIV-positive and negative participants. However, the accuracies of both markers (sCD30, AUC = 0.82; IL-2Ra, AUC = 0.80) were higher in the HIV-positive patients (Figure 3). MMP-9 was significantly elevated in TB cases compared to those with ORDs, and this observation was unique to the HIV-positive participants. MMP-9 showed potential in the diagnosis of TB in the HIV-positive participants, with an AUC of 0.83 (Figure 2c) and a sensitivity and specificity of 86% (95% CI 49–99%) and 68% (95% CI 0.41–81%), respectively (see Supplementary Material Table S2).

Potential of combinations of host urine biomarkers in the diagnosis of TB

GDA was applied to evaluate the potential usefulness of combinations of urine biomarkers in the diagnosis of TB. Upon fitting the data from all host biomarkers into GDA models, a three-marker biosignature consisting of IL2-Ra, sIL2Ra, and MDC (CCL22) was the most accurate biosignature for the diagnosis of TB, regardless of the HIV status of the participants. The three-marker biosignature performed with a sensitivity of 51.5% (95% CI 33.5–69.2%) and specificity of 84.2% (95% CI 76.2–90.4%) after leave-one-out cross-validation. The positive predictive value (PPV) and negative predictive value (NPV) of the three-marker biosignature was 48.6% (95% CI 35.5–61.8%) and 85.7% (95% CI 80.7–89.6%), respectively (Table 3). The frequency of the analytes in the top 20 most accurate GDA models is shown in Figure 4a. When only the definite TB patients were considered, irrespective of HIV status, a two-marker biosignature comprising sIL2Ra and MDC diagnosed TB with a sensitivity of 62.5% (95% CI 40.6–81.2%) and specificity of 86.0% (95% CI 78.2–91.8%) after leave-one-out cross-validation (see Supplementary Material Table S3).

When the participants were stratified according to HIV infection status, a two-marker urine biosignature made up of sIL6R and sIL2Ra diagnosed TB in the HIV-negative participants with an AUC of 0.76 (95% CI 0.64–0.78) (Figure 4b), sensitivity of 53.9% (95% CI 33.4–73.4%), and specificity of 79.6% (95% CI 70.3–87.1%). The biosignature had a PPV of 41.2% (95% CI 29.2–54.3%) and NPV of 86.7% (95% CI 80.9%-90.9%) (Table 3). When only the definite TB patients were considered in the TB group, a three-marker biosignature consisting of sIL6R, sTNFR II, and sIL2Ra diagnosed TB in the HIV-negative participants with an AUC of 0.83 (95% CI 0.72–0.93) (see Supplementary Material Figure S1a), sensitivity of 63.2% (95% CI 38.4–83.7%), and specificity of 87.8% (95% CI 79.6–93.5%) (see Supplementary Material Table S3). In the HIV-positive participants, a four-marker urine biosignature, which comprised sIL6R, MMP-9, IL-2Ra, and IFN-γ, diagnosed TB with an AUC of 0.96 (95% CI 0.89–1.00) (Figure 4c), sensitivity and specificity of 85.7% (95% CI 42.1–99.6%) and 94.7% (95% CI 74–99.9%), respectively, after leave-one-out cross-validation. The biosignature had a PPV of 85.7% (46.5–97.6%) and NPV of 94.7% (95% CI 74.5–99.1%) (Table 3). When only definite TB patients were considered in the TB group, a two-marker biosignature made up of MMP-9 and IL-2Ra diagnosed TB in the HIV-positive participants with an AUC of 0.92 (95% CI 0.78–1.00) (see Supplementary Material Figure S1c). The sensitivity and specificity of the biosignature after leave-one-out cross-validation was 80.0% (95%

Table 2
Median concentrations (with interquartile range) of individual host urine biomarkers in participants diagnosed with TB ($n = 34$) compared to those in patients with ORDs ($n = 117$), regardless of HIV infection status, and their diagnostic performance.

Marker	Concentration, median (IQR)		*p*-Value	AUC (95% CI)	Cut-off	Sensitivity % (95% CI)	Specificity % (95% CI)
	ORD	TB					
sIL2Ra	2364 (1789–3342)	4267 (2273–6255)	0.0003	0.70 (0.59–0.81)	3537.83	59 (41–75)	81 (72–88)
sCD30	58 (0.0–81.24)	100 (0.0–187.4)	0.0015	0.67 (0.56–0.79)	86.24	56 (38–73)	79 (71–86)
sIL1RI	12 (8.02–19.98)	18 (12.85–27.36)	0.0017	0.67 (0.56–0.78)	14.64	71 (55–85)	62 (52–71)
Haptoglobin	13 (3.605–26.99)	26 (13.31–35.47)	0.0021	0.67 (0.58–0.77)	19.065	68 (49–83)	67 (57–75)
sTNFR II	6726 (4514–151 000)	151 000 (6398–151 000)	0.0046	0.66 (0.55–0.76)	150624	56 (38–73)	71 (62–79)
Ferritin	3114 (1035–6033)	4438 (2358–13 866)	0.0064	0.65 (0.56–0.75)	3336.76	71 (52–85)	54 (44–63)
IL-2Ra	722 (403.2–1014)	1322 (460.7–2371)	0.0068	0.65 (0.53–0.78)	1319.765	52 (34–69)	85 (77–91)
MMP-9	2692 (777.2–14 323)	5831 (2671–20 840)	0.0334	0.62 (0.51–0.72)	2891.46	76 (59–89)	51 (42–61)
sTNFR I	2215 (1362–3595)	2955 (1912–25 000)	0.0370	0.62 (0.51–0.73)	2916.53	53 (38–73)	66 (56–74)
sVEGFR3	2352 (1531–17 159)	3884 (1531–17 159)	0.0406	0.61 (0.50–0.73)	2490.13	71 (53–85)	52 (43–61)

TB, tuberculosis; ORDs, other respiratory diseases; IQR, interquartile range; AUC, area under the receiver operating characteristics curve; CI, confidence interval. The table only displays biomarkers that differed significantly between the TB and ORD groups using the Mann–Whitney *U*-test. Youden's index was used to estimate the best cut-off values and the related sensitivity and specificity. Except for haptoglobin, which is in units of ng/ml, the levels of all analytes in urine were measured in pg/ml.

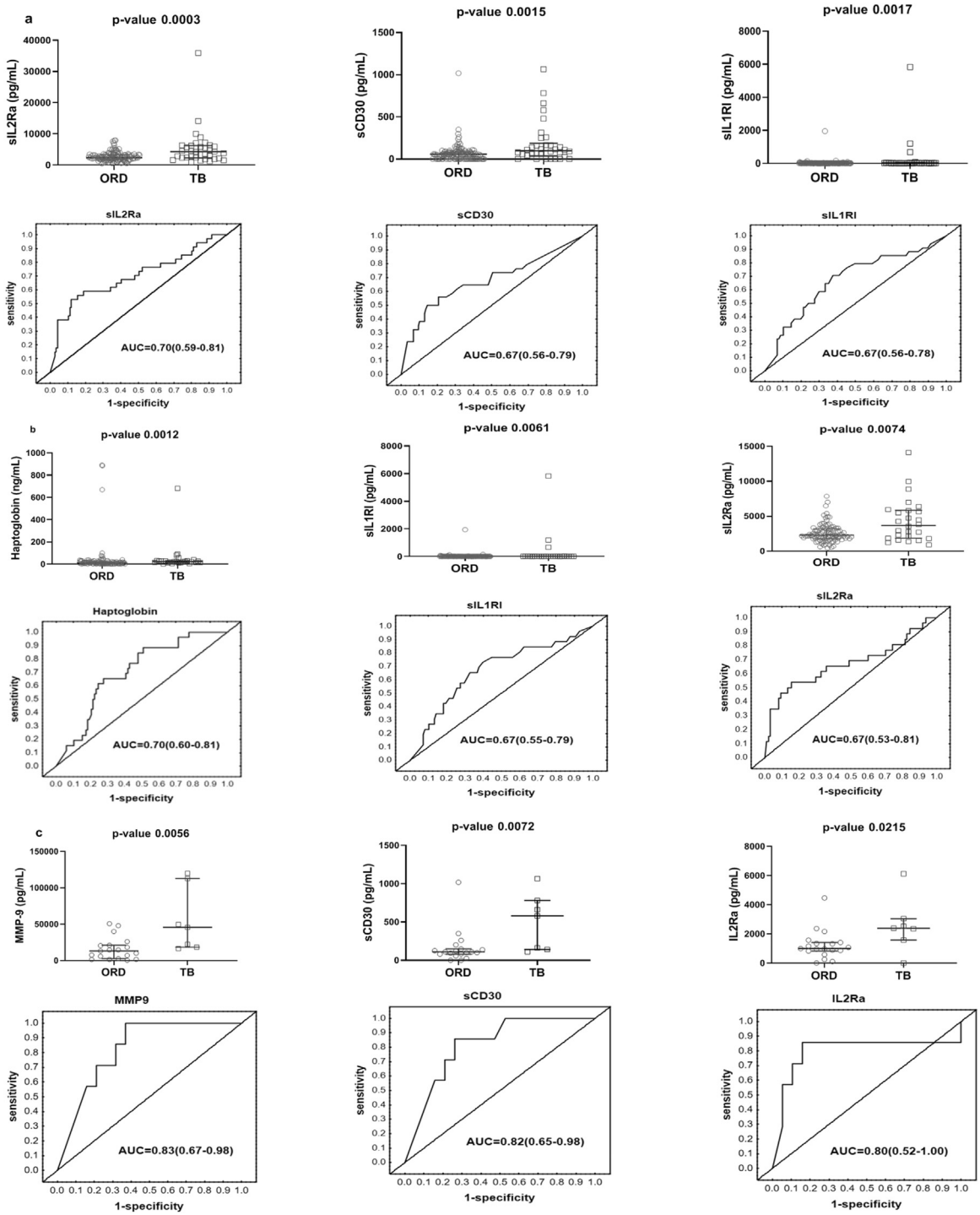

Figure 2. Scatter plots displaying the levels of host urine biomarkers measured in (a) participants diagnosed with TB ($n = 34$) and participants who had ORDs ($n = 117$) regardless of HIV infection status; (b) HIV-negative participants diagnosed with TB ($n = 26$) and participants who had ORDs ($n = 98$); (c) HIV-positive participants diagnosed with TB ($n = 7$) and participants who had ORDs ($n = 19$). The error bars in the scatter plots depict the median with the interquartile range. The ROC curves for three representative biomarkers (sIL2Ra, sCD30, and sIL1RI), (haptoglobin, sIL1RI and sIL2Ra), and (MMP-9, sCD30 and IL-2Ra), respectively, for the different groups, are also presented (TB, tuberculosis; ORDs, other respiratory diseases; ROC, receiver operating characteristics).

CI 28.4–99.5%) and 89.5% (95% CI 66.9–98.7%), respectively (see Supplementary Material Table S3).

Discussion

The accurate and rapid diagnosis of TB, especially at the point-of-care, is crucial for curbing the spread of the disease, and remains challenging. There have been numerous studies, including studies conducted in our laboratory, which have aimed to identify biomarkers for the diagnosis of TB. Most of the previous studies involved the analysis of serum, plasma, or other body fluids, with only a few studies assessing the potential of urinary host inflammatory protein biomarkers as TB diagnostic candidates.

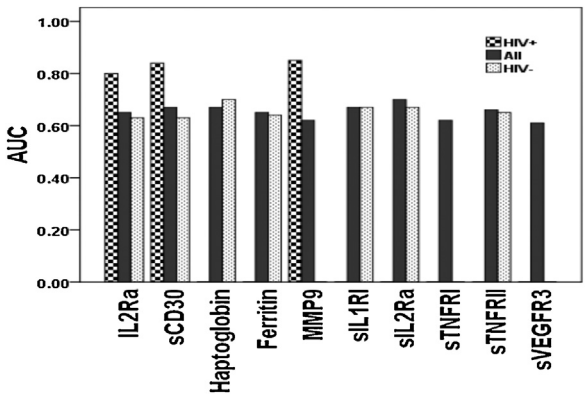

Figure 3. Areas under the receiver operating characteristics curve (AUCs) for the individual host urine biomarkers after the participants diagnosed with tuberculosis or other respiratory diseases were stratified according to HIV infection status.

In addition to being able to discriminate between active TB and latent TB infection, a potential TB diagnostic biomarker should be able to separate patients with TB from those with other respiratory diseases (ORDs) with related clinical symptoms. Furthermore, ideal TB diagnostic biomarkers should preferably be non-sputum-based (WHO, 2014). In this study, we investigated the potential of 29 host urine biomarkers to diagnose active TB disease while distinguishing TB cases from ORDs. The results revealed that seven individual host proteins diagnosed TB regardless of HIV infection and in the participants who were HIV-negative with AUCs $\geq$0.63, whereas three biomarkers performed in the diagnosis of TB in the HIV-positive participants with AUCs $\geq$0.80. Considering the wide variation in social and demographic characteristics, the heterogeneity in immune responses against Mtb, and the influence of comorbidities such as HIV on the accuracy of diagnostic tests, it may be unlikely that a single biomarker would fulfil all of the requirements for a reliable diagnostic test for TB. The present study is the first to investigate a large number of host inflammatory protein biomarkers that have previously shown potential as TB diagnostic candidates (up to 29 biomarkers that have previously been investigated in serum or plasma), in urine samples collected from individuals who were suspected of having active TB in a high-burden setting.

Primary in this study was the identification of three biosignatures that diagnosed TB regardless of HIV infection, and in the HIV-negative and positive participants, respectively, with promising accuracies: (1) IL-2Ra, sIL2Ra, and MDC, (2) sIL6R and sIL2Ra, and (3) sIL6R, MMP-9, IL-2Ra, and IFN-γ. IL-2Ra, included in the biosignatures, is a transmembrane protein expressed constitutively on regulatory T-cells as part of the heterotrimeric IL-2 receptor (Goudy et al., 2013). It is also expressed on activated T- and B-cells, macrophages, and dendritic cells (Vanmaris and Rijkers, 2017). IL-2Ra is involved in both T-cell expansion and tolerance regulation (Goudy et al., 2013). In previous TB studies, higher levels of IL-2Ra were reported in plasma of active TB patients compared to latently infected and healthy individuals (La Manna et al., 2018). sIL2Ra is an inflammatory mediator released from membrane-bound IL-2Ra. Serum levels of sIL2Ra are used as a biomarker to assess disease severity in some inflammatory conditions such as sarcoidosis (Vanmaris and Rijkers, 2017). In previous TB studies, differential levels of sIL2Ra in plasma, serum, and bronchoalveolar lavage fluid between TB patients and individuals with latent TB infection were reported and used in the grading of pulmonary TB (Tsao et al., 2002; Yao et al., 2017). MDC, also included in the biosignatures, is a chemokine mainly produced by macrophages and dendritic cells. It is upregulated by Th2 cytokines, such as IL-4 and IL-5, and plays a vital role in the regulation of Th2 responses (Yamashita and Kuroda, 2002). Serum levels of MDC have been investigated as a potential biomarker for predicting the risk of developing lung cancer (Shiels et al., 2013; Zhang et al., 2016). MMPs play a vital role in inflammation and wound healing. MMP-9, which is among the biosignatures, is secreted by endothelial cells, macrophages, cardiomyocytes, neutrophils, and fibroblasts and has been investigated as a potential biomarker in cardiovascular (Medeiros et al., 2019; Mirhafez et al., 2017) and TB diseases (Kathamuthu et al., 2020). Monocytes, endothelial cells, and hepatocytes secrete sIL6R, and it is detected in various body fluids (Nilsson et al., 2005; Wang et al., 2013). Elevated concentrations of circulating sIL6R have been investigated as a potential biomarker for myocardial infarction (Velásquez et al., 2015), the severity of asthma (Hawkins et al., 2010), and in predicting treatment outcomes in TB (Ronacher et al., 2019) and in patients receiving chemoradiotherapy for some cancers (Makuuchi et al., 2013).

There are only a few published studies on the potential use of host urine biomarkers or biosignatures in the diagnosis of TB. Urine IP-10 was previously reported as a potential diagnostic and treatment response monitoring biomarker for TB (Cannas et al., 2010; Kim et al., 2018; Petrone et al., 2019; Petrone et al., 2016). Another previous study by Isa et al. (2018) reported a host urine metabolomic biosignature comprising sialic acid, neopterin, diacetylspermine, and *N*-acetylhexosamine with potential in distinguishing TB patients from non-tuberculous pulmonary cases and healthy individuals (Isa et al., 2018). It is known that the production of paucibacillary sputum samples and the frequent extrapulmonary presentation of TB in HIV-positive patients limits the usefulness of GeneXpert MTB/RIF and sputum-smear microscopy, resulting in high mortality. LAM, a mycobacterial antigen, which is currently the only approved urine biomarker test, has an estimated sensitivity and specificity of 46% and 89%, respectively. The use of the LAM assay in combination with other available diagnostic tools has been reported to improve the proportion of immunocompromised HIV-positive patients diagnosed with TB who would otherwise have been missed by 38% (Huerga et al., 2019) and to result in a 17% reduction in all-cause deaths (Peter et al., 2016). In the present study, IL-2Ra performed with a sensitivity of 86% and specificity of 84%. The four-marker urine biosignature diagnosed TB in HIV-positive individuals with high accuracy (AUC = 0.96) and a sensitivity of 85.7% and specificity of 94.7%. A urine host biomarker or biosignature-based point-of-care

Table 3
Performance of the urine biosignatures in the diagnosis of TB classified according to HIV infection status.

	Leave-one-out cross-validation			
	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
Optimal biosignature regardless of HIV infection IL-2Ra + sIL2Ra + MDC	51.5 (33.5–69.2)	84.2 (76.2–90.4)	48.6 (35.5–61.8)	85.71 (80.7–89.6)
Optimal biosignature in HIV-negative participants sIL6R + sIL2Ra	53.9 (33.4–73.4)	79.6 (70.3–87.1)	41.2 (29.2–54.3)	86.7 (80.9–90.9)
Optimal biosignature in HIV-positive participants sIL6R + MMP-9 + IL-2Ra + IFN-γ	85.7 (42.1–99.6)	94.7 (74–99.9)	85.7 (46.5–97.6)	94.7 (74.5–99.1)

TB, tuberculosis; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

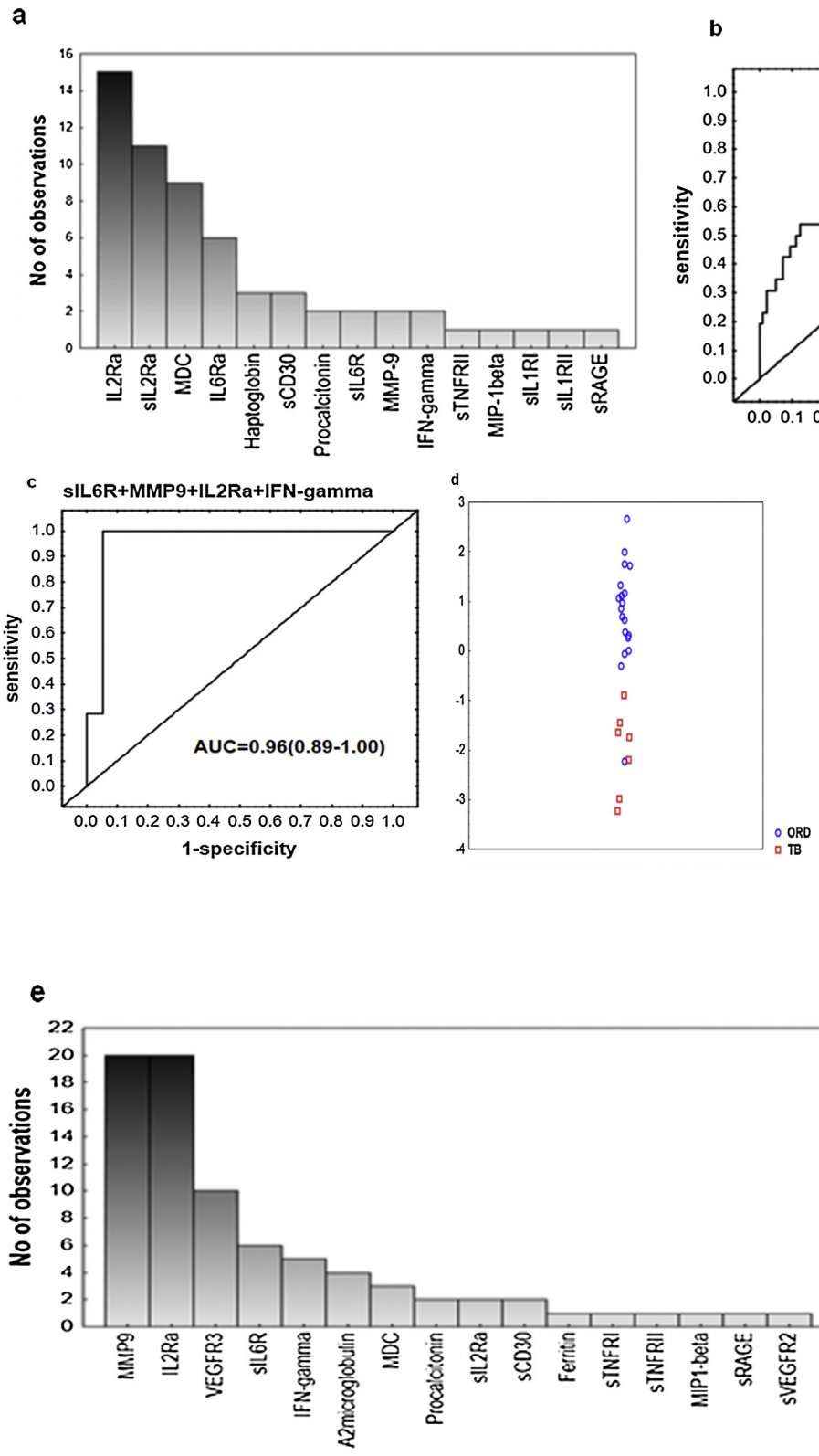

Figure 4. (a) Frequency of analytes in the top 20 most accurate GDA models that discriminated between TB and ORDs regardless of HIV infection status. (b) ROC curve showing the accuracy of the two-marker urine biosignature that diagnosed TB in the HIV-negative participants. (c) ROC curve showing the accuracy of the four-marker biosignature that diagnosed TB in the HIV-positive participants. (d) Red squares: HIV-positive participant diagnosed with TB; blue circles: HIV-positive participants with ORDs. (e) Frequency of analytes in the top 20 most accurate GDA models that discriminated between TB and ORD HIV-positive participants. (GDA, general discriminant analysis; TB, tuberculosis; ORD, other respiratory disease; ROC, receiver operating characteristics curve).

test with improved diagnostic performance may result in even more significant improvements in TB diagnosis and reductions in deaths in HIV-positive people, especially in resource-constrained settings.

We acknowledge that the performance of the urine biosignatures regardless of HIV and in the HIV-negative participants are below the optimal levels and especially when put at par with other serum-based biosignatures from previous studies conducted in our laboratory (Chegou et al., 2016). However, as the biosignatures had a considerably high specificity and NPV, they may be useful in ruling out TB, especially when combined with, for example, sputum smear results and other clinical parameters. Besides, urine is a waste product of metabolism and is not subject to homeostatic mechanisms compared to blood (An and Gao, 2015). Therefore, one may argue that urine may as well be a source of useful biomarkers for TB diagnosis compared to blood. Furthermore, as an advantage, urine collection is non-invasive, poses fewer biohazard dangers, and requires minimal sample processing. Urine-based host biomarkers may also be useful in the diagnosis of extrapulmonary TB and also TB in children, who have difficulty in producing quality sputum; these questions require further investigation.

We acknowledge as limitations of the study, the relatively small number of participants and the small number of HIV-positive TB cases. Furthermore, this study did not consider the severity of HIV infection in the participants with HIV-positive status. Also, the observed performances of the biosignatures may likely be over-estimated, as they were based on leave-one-out cross-validation and not on a separate test set. However, these preliminary data may be used as the basis for the design of larger validation studies. It is acknowledged that biomarker discovery technologies such as the Luminex platform are expensive and may not be available in many resource-constrained environments. However, following the discovery and validation of biomarkers using such expensive technologies, the incorporation of these biomarkers into lateral flow or dip-stick-like tests will make them cheaper and easily implementable as point-of-care tests in these settings. Such an approach, i.e. TB biomarker discovery and validation using expensive technologies, followed by conversion into simpler lateral flow-based tests, was done previously in the Africa-wide study, which contributed specimens for the current study (Corstjens et al., 2016). Of note, a multi-biomarker fingerprick-based version of the test is currently undergoing field evaluation in multicentre studies, including the TriageTB project (https://www.triagetb.com/).

In conclusion, we report new urine-based host proteins and biosignatures that show promise for the diagnosis of pulmonary TB in adults. While searching for other urine-based inflammatory markers, there is a need to evaluate the performance of these individual urine biomarkers and biosignatures in reiterated studies, including larger cohorts of participants and possibly optimized for high sensitivity. Future studies should also include grading of HIV infection states of participants based on CD4 counts and viral load, as these are known to influence the accuracy of biomarker-based diagnostic tests.

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Conflict of interest

All authors declare no conflicts of interest.

Authors' contribution

N.C. and G.W. conceived and designed the study; S.M., S.M., M.L., and O.E recruited the study participants and/or performed laboratory experiments; M.L., O.E., K.S., G.S., G.W., and N.C. helped with analysis and interpretation of the data; M.L. and O.E. drafted the article; all authors reviewed and approved the final version of the manuscript. All authors were part of the AE-TBC Consortium.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at https://doi.org/10.1016/j.ijid.2020.08.019.

References

Abbasi J. Urine test for tuberculosis in development. JAMA 2018;319(6):539–40.
Albert H, Nathavitharana RR, Isaacs C, Pai M, Denkinger CM, Boehme CC. Development, roll-out and impact of Xpert MTB/RIF for tuberculosis: what lessons have we learnt and how can we do better?. Eur Respir J 2016;48(2):516–25.
An M, Gao Y. Urinary biomarkers of brain diseases. Genomics Proteomics Bioinform 2015;13(6):345–54.
Awoniyi DO, Teuchert A, Sutherland JS, Mayanja-Kizza H, Howe R, Mihret A, et al. Evaluation of cytokine responses against novel Mtb antigens as diagnostic markers for TB disease. J Infect 2016;73(3):219–30.
Bulterys MA, Wagner B, Redard-Jacot M, Suresh A, Pollock NR, Moreau E, et al. Point-of-care urine LAM tests for tuberculosis diagnosis: a status update. J Clin Med 2020;9(1):111.
Cannas A, Calvo L, Chiacchio T, Cuzzi G, Vanini V, Lauria FN, et al. IP-10 detection in urine is associated with lung diseases. BMC Infect Dis 2010;10(1):333.
Chegou NN, Black GF, Kidd M, Van Helden PD, Walzl G. Host markers in QuantiFERON supernatants differentiate active TB from latent TB infection: preliminary report. BMC Pulm Med 2009;9(1):21.
Chegou NN, Hoek KG, Kriel M, Warren RM, Victor TC, Walzl G. Tuberculosis assays: past, present and future. Expert Rev Anti-infect Ther 2011;9(4):457–69.
Chegou NN, Sutherland JS, Malherbe S, Crampin AC, Corstjens PL, Geluk A, et al. Diagnostic performance of a seven-marker serum protein biosignature for the diagnosis of active TB disease in African primary healthcare clinic attendees with signs and symptoms suggestive of TB. Thorax 2016;71(9):785–94.
Chegou NN, Sutherland JS, Namuganga A-R, Corstjens PL, Geluk A, Gebremichael G, et al. Africa-wide evaluation of host biomarkers in QuantiFERON supernatants for the diagnosis of pulmonary tuberculosis. Sci Rep 2018;8(1):1–12.
Cho Y, Park Y, Sim B, Kim J, Lee H, Cho S-N, et al. Identification of serum biomarkers for active pulmonary tuberculosis using a targeted metabolomics approach. Sci Rep 2020;10(1):1–11.
Corstjens PL, Fat EMTK, Claudia J, van der Ploeg-van JJ, Franken KL, Chegou NN, et al. Multi-center evaluation of a user-friendly lateral flow assay to determine IP-10 and CCL4 levels in blood of TB and non-TB cases in Africa. Clin Biochem 2016;49(1–2):22–31.
Davies P, Pai M. The diagnosis and misdiagnosis of tuberculosis [State of the art series, Tuberculosis. Edited by ID Rusen. Number 1 in the series]. Int J Tubercul Lung Dis 2008;12(11):1226–34.
Fluss R, Faraggi D, Reiser B. Estimation of the Youden Index and its associated cutoff point. Biometrical J 2005;47(4):458–72.
Goletti D, Lee MR, Wang JY, Walter N, Ottenhoff TH. Update on tuberculosis biomarkers: from correlates of risk to correlates of active disease and of cure from disease. Respirology 2018;23(5):455–66.
Goletti D, Petruccioli E, Joosten SA, Ottenhoff TH. Tuberculosis biomarkers: from diagnosis to protection. Infect Dis Rep 2016;8(2).
Goudy K, Aydin D, Barzaghi F, Gambineri E, Vignoli M, Mannurita SC, et al. Human IL2RA null mutation mediates immunodeficiency with lymphoproliferation and autoimmunity. Clin Immunol 2013;146(3):248–61.
Hawkins G, Robinson M, Moore W, Hastie A, Peters S, Meyers D, et al. Serum levels of soluble IL6R are genetically regulated and correlate with lung function in

asthmatics: Severe Asthma Research Program (SARP). J Allergy Clin Immunol 2010;125(2):AB357.
Huerga H, Rucker SCM, Cossa L, Bastard M, Amoros I, Manhica I, et al. Diagnostic value of the urine lipoarabinomannan assay in HIV-positive ambulatory patients with CD4 below 200 cells/μl in 2 low-resource settings: a prospective observational study. PLoS Med 2019;16(4).
Isa F, Collins S, Lee MH, Decome D, Dorvil N, Joseph P, et al. Mass spectrometric identification of urinary biomarkers of pulmonary tuberculosis. EBioMedicine 2018;31:157–65.
Jacobs R, Maasdorp E, Malherbe S, Loxton AG, Stanley K, Van Der Spuy G, et al. Diagnostic potential of novel salivary host biomarkers as candidates for the immunological diagnosis of tuberculosis disease and monitoring of tuberculosis treatment response. PLoS One 2016a;11(8).
Jacobs R, Malherbe S, Loxton AG, Stanley K, Van Der Spuy G, Walzl G, et al. Identification of novel host biomarkers in plasma as candidates for the immunodiagnosis of tuberculosis disease and monitoring of tuberculosis treatment response. Oncotarget 2016b;7(36):57581.
Jacobs R, Tshehla E, Malherbe S, Kriel M, Loxton AG, Stanley K, et al. Host biomarkers detected in saliva show promise as markers for the diagnosis of pulmonary tuberculosis disease and monitoring of the response to tuberculosis treatment. Cytokine 2016c;81:50–6.
Kathamuthu GR, Kumar NP, Moideen K, Nair D, Banurekha VV, Sridhar R, et al. Matrix metalloproteinases and tissue inhibitors of metalloproteinases are potential biomarkers of pulmonary and extra-pulmonary tuberculosis. Front Immunol 2020;11:419.
Kim SY, Kim J, Kim DR, Kang YA, Bong S, Lee J, et al. Urine IP-10 as a biomarker of therapeutic response in patients with active pulmonary tuberculosis. BMC Infect Dis 2018;18(1):240.
Kustán P, Szirmay B, Horváth-Szalai Z, Ludány A, Kovacs GL, Miseta A, et al. Urinary orosomucoid: a novel, early biomarker of sepsis with promising diagnostic performance. Clin Chem Lab Med 2017;55(2):299–307.
La Manna MP, Orlando V, Donni PL, Sireci G, Di Carlo P, Cascio A, et al. Identification of plasma biomarkers for discrimination between tuberculosis infection/disease and pulmonary non tuberculosis disease. PLoS One 2018;13(3).
Li F, Yu Z, Chen P, Lin G, Li T, Hou L, et al. The increased excretion of urinary orosomucoid 1 as a useful biomarker for bladder cancer. Am J Cancer Res 2016;6(2):331.
Luo J, Zhang M, Yan B, Li F, Guan S, Chang K, et al. Diagnostic performance of plasma cytokine biosignature combination and MCP-1 as individual biomarkers for differentiating stages *Mycobacterium tuberculosis* infection. J Infect 2019;78(4):281–91.
MacLean E, Broger T, Yerlikaya S, Fernandez-Carballo BL, Pai M, Denkinger CM. A systematic review of biomarkers to detect active tuberculosis. Nat Microbiol 2019;4(5):748–58.
Makuuchi Y, Honda K, Osaka Y, Kato K, Kojima T, Daiko H, et al. Soluble interleukin-6 receptor is a serum biomarker for the response of esophageal carcinoma to neoadjuvant chemoradiotherapy. Cancer Sci 2013;104(8):1045–51.
Manngo PM, Gutschmidt A, Snyders CI, Mutavhatsindi H, Manyelo CM, Makhoba NS, et al. Prospective evaluation of host biomarkers other than interferon gamma in QuantiFERON Plus supernatants as candidates for the diagnosis of tuberculosis in symptomatic individuals. J Infect 2019;79(3):228–35.
Manyelo CM, Solomons RS, Snyders CI, Manngo PM, Mutavhatsindi H, Kriel B, et al. Application of cerebrospinal fluid host protein biosignatures in the diagnosis of tuberculous meningitis in children from a high burden setting. Mediat Inflamm 2019;2019.
Medeiros NI, Gomes JA, Fiuza JA, Sousa GR, Almeida EF, Novaes RO, et al. MMP-2 and MMP-9 plasma levels are potential biomarkers for indeterminate and cardiac clinical forms progression in chronic Chagas disease. Sci Rep 2019;9(1):1–9.
Mirhafez SR, Avan A, Tajfard M, Mohammadi S, Moohebati M, Fallah A, et al. Relationship between serum cytokines receptors and matrix metalloproteinase 9 levels and coronary artery disease. J Clin Lab Anal 2017;31(5):e22100.
Nilsson MB, Langley RR, Fidler IJ. Interleukin-6 secreted by human ovarian carcinoma cells, is a potent proangiogenic cytokine. Cancer Res 2005;65(23):10794–800.
Pai M, Denkinger CM, Kik SV, Rangaka MX, Zwerling A, Oxlade O, et al. Gamma interferon release assays for detection of *Mycobacterium tuberculosis* infection. Clin Microbiol Rev 2014;27(1):3–20.
Pantoja A, Fitzpatrick C, Vassall A, Weyer K, Floyd K. Xpert MTB/RIF for diagnosis of tuberculosis and drug-resistant tuberculosis: a cost and affordability analysis. Eur Respir J 2013;42(3):708–20.
Peter JG, Theron G, van Zyl-Smit R, Haripersad A, Mottay L, Kraus S, et al. Diagnostic accuracy of a urine lipoarabinomannan strip-test for TB detection in HIV-infected hospitalised patients. Eur Respir J 2012;40(5):1211–20.
Peter JG, Zijenah LS, Chanda D, Clowes P, Lesosky M, Gina P, et al. Effect on mortality of point-of-care, urine-based lipoarabinomannan testing to guide tuberculosis treatment initiation in HIV-positive hospital inpatients: a pragmatic parallel-group multicountry open-label randomised controlled trial. Lancet 2016;387(10024):1187–97.
Petrone L, Bondet V, Vanini V, Cuzzi G, Palmieri F, Palucci I, et al. First description of agonist and antagonist IP-10 in urine of patients with active TB. Int J Infect Dis 2019;78:15–21.
Petrone L, Cannas A, Vanini V, Cuzzi G, Aloi F, Nsubuga M, et al. Blood and urine inducible protein 10 as potential markers of disease activity. Int J Tubercul Lung Dis 2016;20(11):1554–61.
Ronacher K, Chegou NN, Kleynhans L, Siawaya JFD, du Plessis N, Loxton AG, et al. Distinct serum biosignatures are associated with different tuberculosis treatment outcomes. Tuberculosis 2019;118:101859.
Shiels MS, Pfeiffer RM, Hildesheim A, Engels EA, Kemp TJ, Park J-H, et al. Circulating inflammation markers and prospective risk for lung cancer. J Natl Cancer Inst 2013;105(24):1871–80.
Songkhla MN, Tantipong H, Tongsai S, Angkasekwinai N. Lateral flow urine lipoarabinomannan assay for diagnosis of active tuberculosis in adults with human immunodeficiency virus infection: a prospective cohort study. Open forum infectious diseases. US: Oxford University Press US; 2019. p. ofz132.
Su L-X, Feng L, Zhang J, Xiao Y.-J, Jia Y.-H, Yan P, et al. Diagnostic value of urine sTREM-1 for sepsis and relevant acute kidney injuries: a prospective study. Crit Care 2011;15(5):R250.
Tsao T, Huang C, Chiou W, Yang P, Hsieh M, Tsao K. Levels of interferon-γ and interleukin-2 receptor-α for bronchoalveolar lavage fluid and serum were correlated with clinical grade and treatment of pulmonary tuberculosis. Int J Tubercul Lung Dis 2002;6(8):720–7.
Vanmaris RM, Rijkers GT. Biological role of the soluble interleukin-2 receptor in sarcoidosis. Sarcoidosis Vasculitis Diffuse Lung Dis 2017;34(2):122–9.
Velásquez IM, Golabkesh Z, Källberg H, Leander K, de Faire U, Gigante B. Circulating levels of interleukin 6 soluble receptor and its natural antagonist sgp130 and the risk of myocardial infarction. Atherosclerosis 2015;240(2):477–81.
Wang Q, Chen X, Feng J, Cao Y, Song Y, Wang H, et al. Soluble interleukin-6 receptor-mediated innate immune response to DNA and RNA viruses. J Virol 2013;87(20):11244–54.
Wawrocki S, Seweryn M, Kielnierowski G, Rudnicka W, Wlodarczyk M, Druszczynska M. IL-18/IL-37/IP-10 signalling complex as a potential biomarker for discriminating active and latent TB 14(12). PLoS One; 2019.
WHO. Use of tuberculosis interferon-gamma release assays (IGRAs) in low-and middle-income countries: policy statement. 2011.
WHO. High priority target product profiles for new tuberculosis diagnostics: report of a consensus meeting, 28–29 April 2014. Geneva, Switzerland: World Health Organization; 2014.
WHO. The use of lateral flow urine lipoarabinomannan assay (LF-LAM) for the diagnosis and screening of active tuberculosis in people living with HIV: policy guidance. World Health Organization; 2015.
WHO. Global tuberculosis report 2019. Geneva, Switzerland: World Health Organization; 2019.
Yamashita U, Kuroda E. Regulation of macrophage-derived chemokine (MDC/CCL22) production. Crit Rev Immunol 2002;22(2).
Yao X, Liu Y, Liu Y, Liu W, Ye Z, Zheng C, et al. Multiplex analysis of plasma cytokines/chemokines showing different immune responses in active TB patients latent TB infection and healthy participants. Tuberculosis 2017;107:88–94.
Yong YK, Tan HY, Saeidi A, Wong WF, Vignesh R, Velu V, et al. Immune biomarkers for diagnosis and treatment monitoring of tuberculosis: current developments and future prospects. Front Microbiol 2019;10.
Zhang Y, Yu K, Hu S, Lou Y, Liu C, Xu J, et al. MDC and BLC are independently associated with the significant risk of early stage lung adenocarcinoma. Oncotarget 2016;7(50):83051.