Biomarkers for Tuberculosis Based on Secreted, Species-Specific, Bacterial Small Molecules

Shih-Jung Pan,1,2 Asa Tapley,1,3,4 John Adamson,1 Tessa Little,5 Michael Urbanowski,2 Keira Cohen,1,4 Alexander Pym,1 Deepak Almeida,2 Alton Dorasamy,1 Emilie Layre,2 David C. Young,7 Ravesh Singh,8 Vinod B. Patel,9 Kristina Wallengren,10 Thumbi Ndung’u,1,4,11,12 Douglas Wilson,13 D. Branch Moody,7 and William Bishai1,4

1KwaZulu-Natal Research Institute for Tuberculosis and HIV (K-RITH), Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa; 2Center for Tuberculosis Research, Johns Hopkins University School of Medicine, Baltimore, Maryland; 3San Francisco School of Medicine, University of California, Oakland; 4Howard Hughes Medical Institute, Chevy Chase, Maryland; 5School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Durban, South Africa; 6Division of Pulmonary and Critical Care Medicine, Department of Medicine, and 7Division of Rheumatology, Immunology and Allergy, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts; 8HIV Pathogenesis Programme, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, 9Department of Neurology, University of KwaZulu Natal, and 10Tuberculosis & HIV Investigative Network in KwaZulu-Natal (THINK), Durban, South Africa; 11Max Planck Institute for Infection Biology, Berlin, Germany; 12Ragon Institute of Massachusetts General Hospital, Massachusetts Institute of Technology and Harvard University, Cambridge, and 13Department of Medicine, Edendale Hospital, University of KwaZulu-Natal, Pietermaritzburg, South Africa

Improved biomarkers are needed for tuberculosis. To develop tests based on products secreted by tubercle bacilli that are strictly associated with viability, we evaluated 3 bacterial-derived, species-specific, small molecules as biomarkers: 2 mycobactin siderophores and tuberculosinyladenosine. Using liquid chromatography–tandem mass spectrometry, we demonstrated the presence of 1 or both mycobactins and/or tuberculosinyladenosine in serum and whole lung tissues from infected mice and sputum, cerebrospinal fluid (CSF), or lymph nodes from infected patients but not uninfected controls. Detection of the target molecules distinguished host infection status in 100% of mice with both serum and lung as the target sample. In human subjects, we evaluated detection of the bacterial small molecules (BSMs) in multiple body compartments in 3 patient cohorts corresponding to different forms of tuberculosis. We detected at least 1 of the 3 molecules in 90%, 71%, and 40% of tuberculosis patients’ sputum, CSF, and lymph node samples, respectively. In paucibacillary forms of human tuberculosis, which are difficult to diagnose even with culture, detection of 1 or more BSM was rapid and compared favorably to polymerase chain reaction–based detection. Secreted BSMs, detectable in serum, warrant further investigation as a means for diagnosis and therapeutic monitoring in patients with tuberculosis.

Keywords. biomarker; diagnostic; mycobactin; small molecule; tuberculosinyladenosine; tuberculosis.

Tuberculosis remains a leading cause of mortality and morbidity worldwide, resulting in 1.3 million deaths annually and infecting approximately one-third of the global human population. The recent emergence of drug-resistant forms of tuberculosis underscores the inadequacies of current public health-control efforts. Major factors in the poor state of global tuberculosis control are the lack of rapid and specific point-of-care diagnostics and new drugs to treat the newly resistant forms of tuberculosis. Many experts regard the lack of validated markers as a major obstacle to conducting the clinical trials required to bring a new drug to market in a cost-effective manner [1]. Consequently, there is an urgent need for effective new tuberculosis biomarkers both for diagnosing disease and monitoring its response to treatment.

While there has been relative success in developing blood-based diagnostics for latent tuberculosis based on host immune responses with interferon-γ release assays, the majority of current diagnostics for active tuberculosis are sputum-based. This limits their utility both in children, who do not reliably produce sputum [2], and as treatment response markers because coughing and sputum production resolve many months before cure in most active tuberculosis patients. Moreover, since human immunodeficiency virus (HIV) coinfection and other forms of immunocompromise are common risk factors for tuberculosis, diagnostics targeting host antibody or T-cell responses may have limitations in this significant sector of at-risk individuals [3]. For these reasons, we focused our studies on secreted, bacterial-derived small molecules, which may enter compartments outside of the lung such as blood or urine.

After phagocytosis by alveolar macrophages, Mycobacterium tuberculosis depends on a number of strategies for surviving intracellularly; these include mechanisms for harvesting host iron stores and for preventing phagosome maturation. M. tuberculosis acquires iron through the secretion and subsequent reinternalization of siderophores mycobactin T (MBT) and carboxymycobactin (cMBT) [4]. Species-specific differences
in mycobactins T, S, and J, from *M. tuberculosis*, *M. smegmatis*, and *M. paratuberculosis*, respectively, have been identified [5]. *M. avium*, *M. tuberculosis*, *M. smegmatis*, and *M. bovis* all secrete cMBT. Disruption of siderophore production or secretion significantly attenuates mycobacterial growth and virulence [6]. Additionally, *M. tuberculosis* produces and sheds the terpene...
The nucleoside 1-tuberculosinyladenosine (1-TbAd), which likely regulates macrophage phagosomal maturation [7, 8]. The genes encoding its biosynthetic enzymes (Rv3377c and Rv3378c) are only found within the \textit{M. tuberculosis} complex. However, the locus contains loss-of-function mutations in \textit{M. bovis} and \textit{M. bovis} bacillus Calmette-Guérin (BCG), and it appears that \textit{M. tuberculosis} may be the only complex member that produces 1-TbAd [8, 9]. These molecules—MBT, cMBT, and TbAd—have shared properties that are attractive as diagnostic targets, including their abundant biosynthesis, export to the surface for shedding, and their promotion of \textit{M. tuberculosis} virulence, which suggests continuous expression in vivo. Also, their similar polarity and mass profiles facilitated the development of a single liquid chromatography–tandem mass spectrometry (LC-MS/MS)–based method for their detection.

RESULTS AND DISCUSSION

MBT and cMBT were purified from sterilized lipid extracts of \textit{M. tuberculosis} H37Rv cultured in iron-deficient media to
induce siderophore production. We confirmed detection of the molecules using a collision-induced mass spectrometry (CID-MS) protocol. For both MBT and cMBT, the method revealed 3 major molecular variants (Figure 1A and 1B). 1-TbAd and an isomer recently identified as N6-TbAd were separately purified (see Supplementary Methods), and their CID-MS profiles characterized (Figure 1C) [9]. The isomer fragments similarly to 1-TbAd, but with 1 additional major fragment ion at m/z 148 (Figure 1C). The structure of 1-TbAd used in this study was confirmed by nuclear magnetic resonance (Figure 1D). Beyond these studies of purified, naturally derived mycobacterial lipids, we confirmed that the method successfully detected them in uninfected mouse lung tissue spiked with these small molecules. For positivity of a given molecule, we required detection of at least 1 parent ion and associated transition pair with a signal-to-noise (S/N) ratio ≥3:1. For the purposes of analysis, we
combined the 2 TbAd isomers into 1 category and did not differentiate between individual molecular variants of MBT or cMBT.

To evaluate target molecule detection in samples from M. tuberculosis–infected hosts, we infected BALB/c mice with a high dose of the virulent M. tuberculosis H37Rv. Mice were sacrificed at postinfection day 1 for determination of the initial bacterial implantation, and at day 21 for evaluation of the final bacterial burden and presence of the 3 targeted bacterial small molecules (BSMs). The implantation and final bacterial burdens were found to be typical for a high-dose aerosol infection (Supplementary Figure 1A), and we observed discrete pulmonary lesions in the lungs of mice 21 days postinfection (Supplementary Figure 1B).

LC-MS/MS evaluation revealed MBT in lung tissue and serum of 100% (7/7) M. tuberculosis–infected mice (Table 1a and Figure 1A–C). TbAd was detected in lung tissue of 100% (7/7) of infected mice, but in serum samples of only 43% (3/7). By contrast, we detected cMBT in the serum of only 1 infected mouse and in no lung samples. The sensitivity for any BSMs being positive in either mouse serum or lung tissue was 100%. Lung and serum samples from 100% (6/6) of the uninfected mice were negative for all 3 molecules of interest, thus the specificity for any BSMs being positive in mouse serum or lung tissue was also 100%. Because TbAd is not produced by M. bovis BCG [7], we tested lungs of BCG-infected mice and showed that TbAd was not detected despite the presence of $1.1 \times 10^4$ colony forming units of BCG in the animal lungs at 3 weeks postinfection.

To test the utility of these BSMs as markers of tuberculosis disease in humans, we analyzed sputum samples from pulmonary tuberculosis patients and culture-negative, healthy controls. All tuberculosis patients were positive for tuberculosis by acid-fast bacilli (AFB) sputum smear, culture, and GeneXpert testing. As shown in Table 1b, we detected MBT in 90% (9/10) of sputum samples from infected individuals and TbAd in 60% (6/10), and in none of 10 uninfected control sputum samples (90% sensitivity, 100% specificity for any BSMs being positive). The 1 sputum sample negative for MBT had a low bacterial burden as indicated by GeneXpert and AFB smear. The S/N ratio of cMBT was below the detection threshold in this and subsequent analyses.

To determine whether MBT and TbAd levels may correlate with bacterial loads, we compared signal intensities of these small molecules with other quantitative markers of M. tuberculosis abundance in the above sputum study. We correlated the ion counts per second of the molecular ions for MBT and TbAd in each positive sputum sample with the AFB smear microscopy scores, mycobacterial growth indicator tube (MGIT) time-to-positivity, and GeneXpert grade for each respective sputum sample. As may be seen in Table 1c, MBT levels showed a significant correlation with AFB smear microscopy and approached significance in its correlation with GeneXpert grade. TbAd levels showed significant correlations with AFB smear microscopy and GeneXpert grades, and approached significance in its correlation with MGIT time-to-positivity. These results suggest that the LC-MS/MS intensities of these 2 small molecule biomarkers track with other commonly used measures of bacterial abundance.

Next, we analyzed cerebrospinal fluid (CSF) from patients with culture-confirmed tuberculosis meningitis, comparing them to control samples from patients with culture-confirmed cryptococcal meningitis. We were able to detect MBT in CSF from 71% (10/14) of tuberculous meningitis cases and TbAd in 7% (1/14) of cases. There was no detectable MBT or TbAd in any of the 12 M. tuberculosis culture-negative, Cryptococcus neoformans culture-positive CSF patient samples tested (Table 1d) (71% sensitivity, 100% specificity for any BSMs being positive). In 11 subjects with culture-confirmed tuberculosis meningitis who also had AFB smear performed on their CSF, 9/11 were
positive for any BSMs and 7/11 were AFB smear-positive (sensitivity of any BSMs being positive 82%, sensitivity of AFB smear 64%).

Last, we tested the BSMs in another paucibacillary form of tuberculosis, tuberculous lymphadenitis. We obtained cervical lymph nodes from 10 South African patients with lymphadenitis
who underwent diagnostic excisions. In addition to histopathology and mycobacterial culture, all samples were evaluated by AFB smear, GeneXpert, and LC-MS/MS. Five patients had lymph nodes which were *M. tuberculosis* culture-positive, while the other 5 had histology consistent with malignancy or reactive lymphadenitis with no evidence of caseation. We detected MBT in 40% (2/5) of the *M. tuberculosis*–positive lymph nodes and TbAd in 20% (1/5) as shown in Table 1e. All *M. tuberculosis*–negative samples also tested negative for the target molecules (sensitivity 40%, specificity 100% for any BSMs being positive). AFB smear was positive in 20% (1/5) of the tuberculous lymph nodes, while GeneXpert was positive in 100% (5/5). Interestingly, 1 of the patients with tuberculous lymphadenitis also provided a blood sample that was GeneXpert positive and was BSM-positive (MBT positive/TbAd negative); however, the blood sample was culture-negative for *M. tuberculosis*. None of the other 9 patients had evidence of mycobacteremia by any of the 3 tests.

Our studies demonstrate that 2 types of BSMs released by *M. tuberculosis*, MBT and TbAd, may be found in blood, sputum, CSF, lung, and lymph node samples from mice and human infected with *M. tuberculosis*. Evidence suggests that these 2 molecules play a role in tuberculosis virulence—MBT in nutrient acquisition and survival inside host cells [6] and TbAd in resistance against macrophage killing [8]. So it is not surprising that they are actively produced during infection. Not only were these molecules found in infected mice and in sputum samples from infected human patients, we were also able to detect them in human CSF and lymph node tissue in which the bacterial burden is low. The potential use of these BSMs in monitoring response to therapy in pulmonary tuberculosis patients warrants further investigation.

Our work has some limitations. To establish proof-of-principle for the presence of the target BSMs in multiple forms of tuberculosis, we studied relatively small numbers of patients. Hence, the current data cannot predict the population sensitivity and specificity of this test in the clinical setting. Moreover, all sputum samples from tuberculosis patients in South Africa were smear-positive, whereas the control samples were obtained from the United States. The fact that no sputum samples from smear-negative tuberculosis patients were used and that the positive and control samples came from different countries might affect test accuracy. Additionally, our detection method, LC-MS/MS, may not at present be amenable to point-of-care diagnosis. While LC-MS/MS–based biomarker monitoring could be valuable for reducing the cost of tuberculosis clinical trials, simpler and less expensive methods of detection would be required for use in routine clinical settings. Although specific antibodies are not currently available, both molecules are readily targetable with enzyme-linked immunoassays.

Our study is unique in focusing on bacterial-derived, secreted, small molecules. Previous studies of bacterial antigens have focused on nonsecreted macromolecules, which may be shed less readily into other body compartments. These include proteins [10], lipoarabinomannan (LAM) [11], dimyocerosates [12], and mycolic acids [13]. LAM, an amphipathic bacterial macromolecule that appears in urine and blood, has been tested in several clinical trials as a tuberculosis biomarker. While in general LAM antigen detection assays have been inferior to traditional tuberculosis diagnostics, some studies have demonstrated good sensitivity in HIV-infected individuals, suggesting that the test may be valuable in certain subpopulations [14]. The introduction of nucleic acid amplification tests for tuberculosis has been an important advance during the past 2 decades. In particular, the GeneXpert platform, which may deliver results in less than 2 hours, has proven useful in tuberculosis control programs [15]. This and other nucleic-acid amplification tests are limited in their ability to detect tuberculosis using blood and urine and also to quantify disease burden over time because the DNA of killed bacteria remains detectable for many days despite effective therapy. Hence, there remains an ongoing need for tuberculosis biomarkers, similar to HIV viral load testing, which quantify disease progression or regression using readily available patient samples such as blood or urine. In this study, we present proof-of-principle evidence that bacterial-derived, pathogen-specific, small molecules—whose presence is independent of host immunity—may prove valuable as novel biomarkers for tuberculosis. Such markers would be valuable supplements to the currently available platform of tools.

**Supplementary Data**

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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**References**


