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,[1,6] Alexander Pym,[1] Deepak Almeida,[2]
Afton Dorasamy,[1] Emilie Layre,[7] David C. Young,[7] Ravesh Singh,[8]
Vinod B. Patel,[9] Kristina Wallengren,[10] Thumbi Ndung'u,[1,8,11,12]
Douglas Wilson,[13] D. Branch Moody,[7] and William Bishai[2,4]

[1]KwaZulu-Natal Research Institute for Tuberculosis and HIV (K-RITH), Nelson
R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa;
[2]Center for Tuberculosis Research, Johns Hopkins University School of Medicine,
Baltimore, Maryland; [3]San Francisco School of Medicine, University of California,
Oakland; [4]Howard Hughes Medical Institute, Chevy Chase, Maryland; [5]School of
Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Durban,
South Africa; [6]Division of Pulmonary and Critical Care Medicine, Department of
Medicine, and [7]Division of Rheumatology, Immunology and Allergy, Brigham and
Women's Hospital, Harvard Medical School, Boston, Massachusetts; [8]HIV
Pathogenesis Programme, Doris Duke Medical Research Institute, Nelson
R. Mandela School of Medicine, [9]Department of Neurology, University of
KwaZulu Natal, and [10]Tuberculosis & HIV Investigative Network in KwaZulu-Natal
(THINK), Durban, South Africa; [11]Max Planck Institute for Infection Biology,
Berlin, Germany; [12]Ragon Institute of Massachusetts General Hospital,
Massachusetts Institute of Technology and Harvard University, Cambridge; and
[13]Department 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 com-
partments 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 com-
pared favorably to polymerase chain reaction–based detection.
Secreted BSMs, detectable in serum, warrant further investiga-
tion as a means for diagnosis and therapeutic monitoring in
patients with tuberculosis.**

Keywords. biomarker; diagnostic; mycobactin; small mole-
cule; 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 under-
scores 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 cur-
rent 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 cough-
ing and sputum production resolve many months before cure in
most active tuberculosis patients. Moreover, since human im-
munodeficiency virus (HIV) coinfection and other forms of im-
munocompromise 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, bac-
terial-derived small molecules, which may enter compartments
outside of the lung such as blood or urine.

After phagocytosis by alveolar macrophages, *Mycobacter-
ium tuberculosis* depends on a number of strategies for surviv-
ing intracellularly; these include mechanisms for harvesting
host iron stores and for preventing phagosome maturation.
M. tuberculosis acquires iron through the secretion and subse-
quent reinternalization of siderophores mycobactin T (MBT)
and carboxymycobactin (cMBT) [4]. Species-specific differences
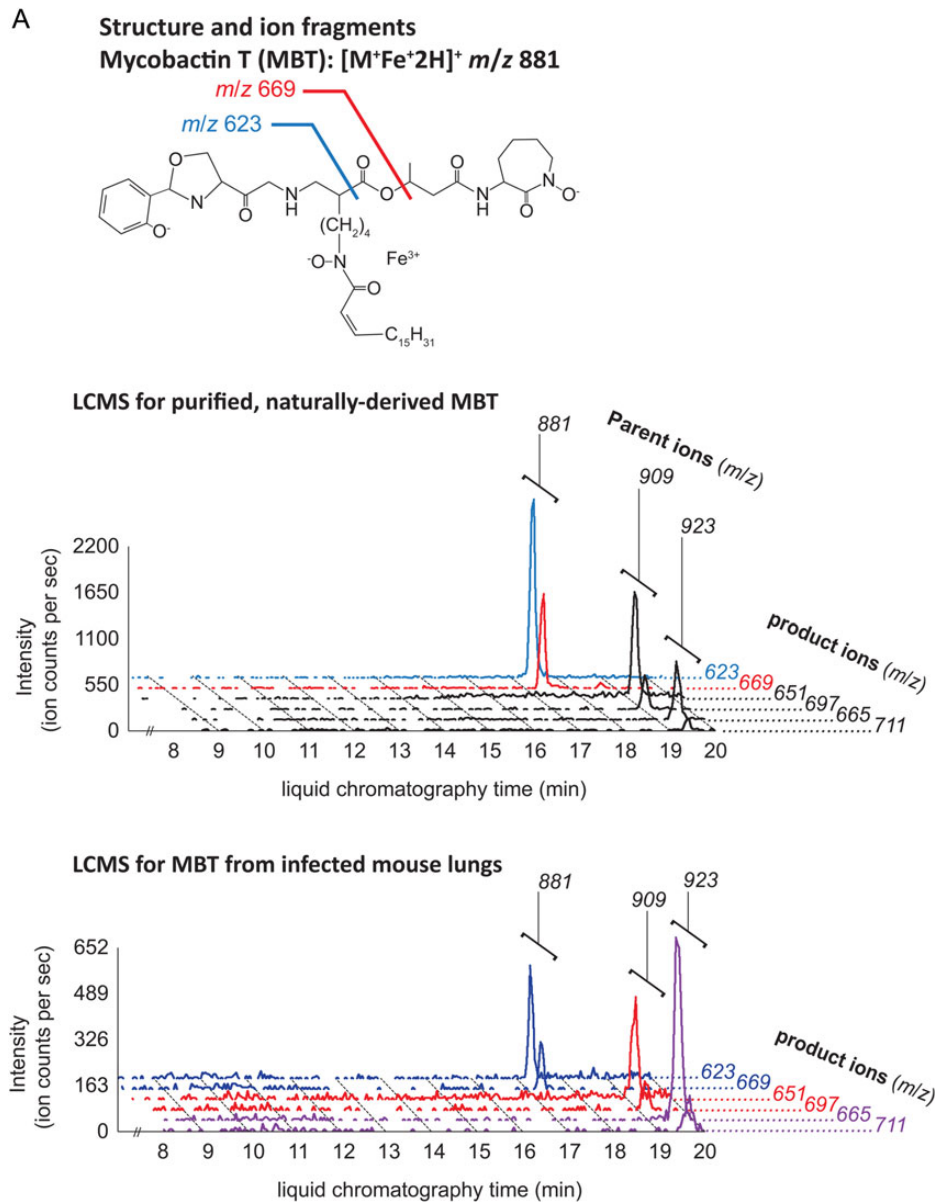
Figure 1. Structures and ion fragments (top), representative ion chromatograms of purified, naturally derived, tuberculosis-specific bacterial small molecules (BSMs), illustrating the characteristic ion intensity peaks with collision-induced liquid chromatography mass spectrometry (LCMS) in positive ion mode at concentrations of 10 nM (middle), and characteristic ion chromatograms of the product ions detected by LCMS analysis from infected mouse lungs (bottom) for mycobactin T (*A*), carboxymycobactin (*B*), and tuberculosinyladenosine in its 2 forms, 1-TbAd and the isomeric N6-TbAd (*C*). For mycobactin T, the 15 methylene aliphatic side chain as shown gives a parent ion *m/z* of 881, but 17- (*m/z* 909) and 18-methylene (*m/z* 923) forms are also produced. For carboxymycobactin, the aliphatic link as shown gives a parent ion *m/z* of 743 but the 2-methylene aliphatic chain shown may extend to 4 (*m/z* 771) or 5 methylenes (*m/z* 785) in length. For mouse infections, LCMS analyses are shown from 1 processed lung tissue sample from a BALB/c mouse sacrificed 21 days after aerosol infection with *M. tuberculosis* H37Rv. Infected mouse lungs showed characteristic peaks for mycobactin T (*A*, bottom panel) and 1-TbAd (*C*, bottom panel), but carboxymycobactin was less abundant with only 1 ion transition observed above the detection limit (*B*, bottom panel). Trace levels of N6-TbAd were observed eluting later than 1-TbAd in the infected mouse lung (*C*, bottom panel). The 1-TbAd used as standard material in this study was fully characterized by mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy. This included accurate mass assignments (elemental composition), collisionally induced dissociation fragment analysis, and 2-dimensional NMR, allowing characterization of the 1-TbAd structure (*D*). Abbreviation: TbAd, tuberculosinyladenosine.

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

B   **Structure and ion fragments**
Carboxymyobactin (cMBT): $[M^+Fe^+2H]^+$ *m/z* 743
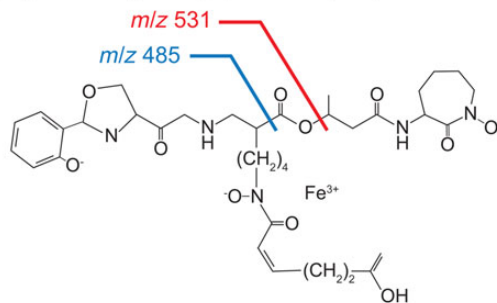
LCMS for purified, naturally-derived cMBT
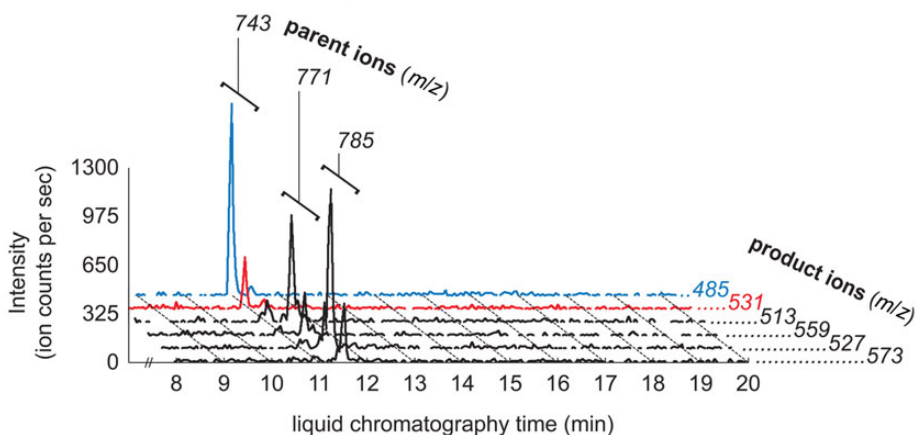
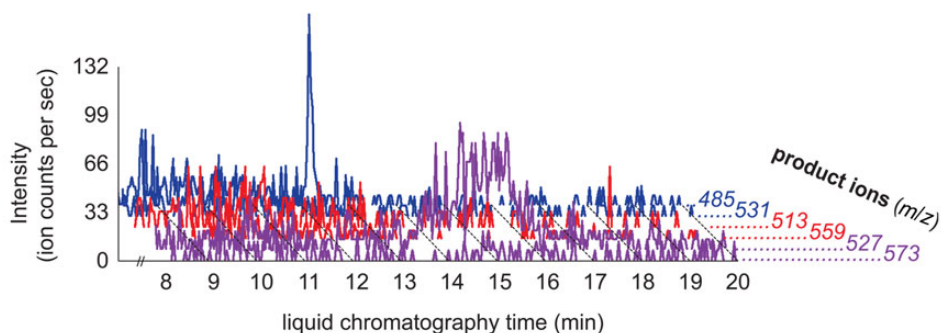*LCMS for cMBT from infected mouse lungs*
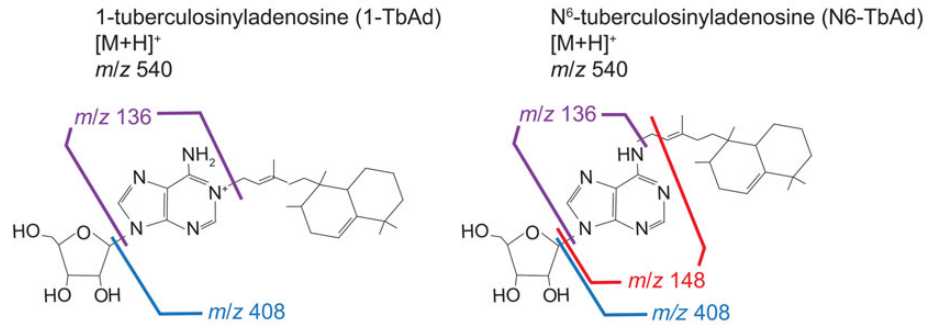
Figure 1   continued.

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 *M. tuberculosis* complex. However, the locus contains loss-of-function mutations in *M. bovis* and *M. bovis* bacillus Calmette-Guérin (BCG), and it appears that *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 *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.
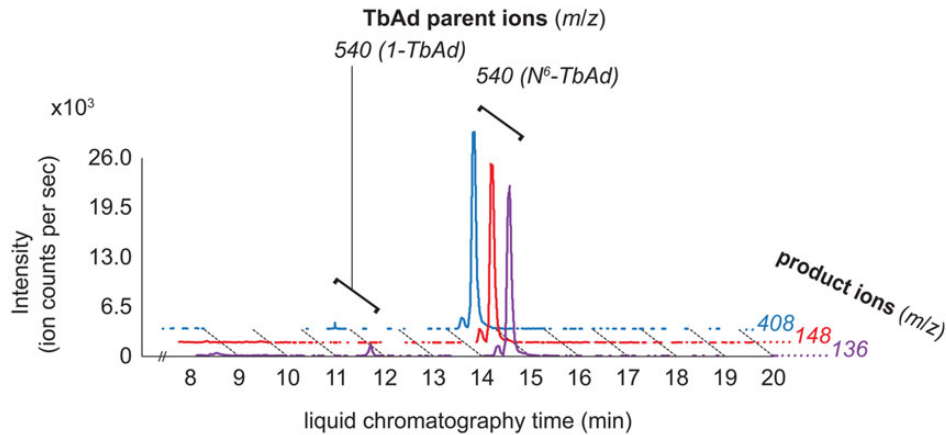
RESULTS AND DISCUSSION

MBT and cMBT were purified from sterilized lipid extracts of *M. tuberculosis* H37Rv cultured in iron-deficient media to

C   Structure and ion fragments

1-tuberculosinyladenosine (1-TbAd)
[M+H]⁺
m/z 540

N⁶-tuberculosinyladenosine (N6-TbAd)
[M+H]⁺
m/z 540

LCMS for purified, naturally-derived TbAd

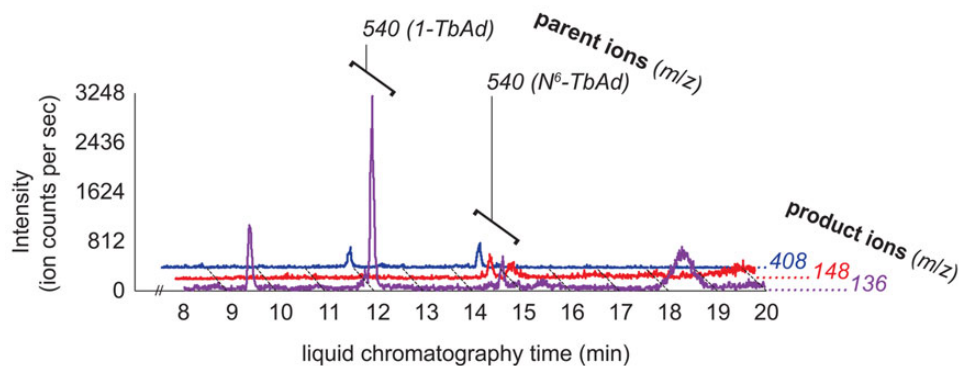**LCMS for TbAd from infected mouse lungs**

Figure 1 continued.

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
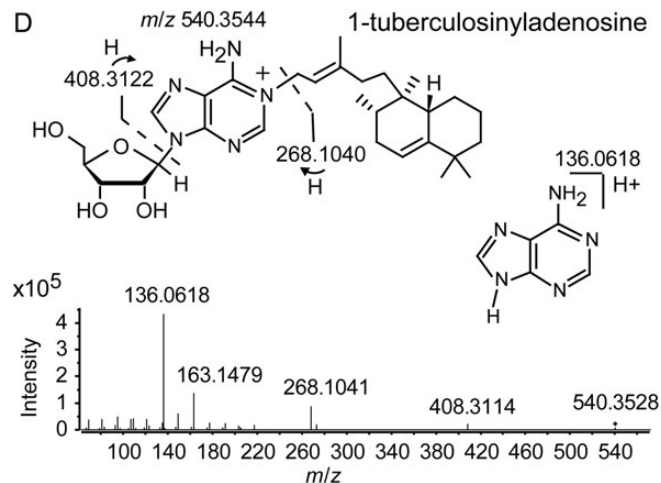
Figure 1 continued.

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 1*A*), and we observed discrete pulmonary lesions in the lungs of mice 21 days postinfection (Supplementary Figure 1*B*).

LC-MS/MS evaluation revealed MBT in lung tissue and serum of 100% (7/7) *M. tuberculosis*–infected mice (Table 1*a* and Figure 1*A*–*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 1.1×10^4 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 1*b*, 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 1*c*, 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 tuberculous 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 1*d*) (71% sensitivity, 100% specificity for any BSMs being positive). In 11 subjects with culture-confirmed tuberculous meningitis who also had AFB smear performed on their CSF, 9/11 were

Table 1. Detection of Bacterial Small Molecules in Mouse and Human Samples

Sample	Molecule Targeted	Detection Among Mice, by Tuberculosis Disease Status (%)		*P* Value
		Infected	Uninfected	
(a)				
Serum	Mycobactin T	7/7 (100)	0/6 (0)	<.001
	Carboxymycobactin	1/7 (0)	0/6 (0)	1.00
	TbAd	3/7 (43)	0/6 (0)	.192
Lung	Mycobactin T	7/7 (100)	0/6 (0)	<.001
	Carboxymycobactin	0/7 (14)	0/6 (0)	1.00
	TbAd	7/7 (100)	0/6 (0)	<.001

Sample	Molecule Targeted	Detection in Patient Sputum Samples, by Tuberculosis Disease Status (%)		*P* Value
		Infected	Uninfected	
(b)				
Sputum	Mycobactin T	9/10 (90)	0/10 (0)	.00058
	Carboxymycobactin	0/10 (0)	0/10 (0)	1.00
	TbAd	6/10 (60)	0/10 (0)	.011

	Correlation Coefficient	*P* Value
(c)		
Mycobactin T		
AFB smear microscopy score	0.8739	.0024
MGIT TTP time	−0.2970	.4069
GeneXpert grade	0.5919	.0952
TbAd		
AFB smear microscopy score	0.8587	.0095
MGIT TTP time	−0.5253	.0989
GeneXpert grade	0.8980	.0079

Molecule Targeted	Detection in Patient Cerebrospinal Fluid Samples, by Culture-Confirmed Meningitis Diagnosis (%)		*P* Value
	Tuberculosis	Cryptococcal	
(d)			
Mycobactin T	10/14 (53)	0/12 (0)	.0002
TbAd	1/14 (7)	0/12 (0)	1.00

Sample	Detection Method	Detection of *M. tuberculosis* in Patient Lymph Node Samples, Fraction Positive (%)		*P* Value
		Infected	Uninfected	
(e)				
Lymph node	Any BSMs	2/5 (40)	0/5 (0)	.44
	AFB smear	1/5 (20)	0/5 (0)	1.00
	GeneXpert	5/5 (100)	0/5 (0)	.008
	Culture	5/5 (100)	0/5 (0)	.008
Blood	Any BSMs	1/5 (20)	0/5 (0)	1.00
	GeneXpert	1/5 (20)	0/5 (0)	1.00
	Culture	0/5 (0)	0/5 (0)	1.00

Abbreviations: AFB, acid-fast bacilli; BSMs, bacterial small molecules; MGIT, mycobacterial growth indicator tube; TbAd, tuberculosinyladenosine; TTP, time to positivity.

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 1*e*. 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], dimycocerosates [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 copyedited. 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

References

1. Wallis RS, Kim P, Cole S, et al. Tuberculosis biomarkers discovery: developments, needs, and challenges. Lancet Infect Dis **2013**; 13: 362–72.
2. Swaminathan S, Rekha B. Pediatric tuberculosis: global overview and challenges. Clin Infect Dis **2010**; 50(suppl 3):S184–94.

3. Chen J, Zhang R, Wang J, et al. Interferon-gamma release assays for the diagnosis of active tuberculosis in HIV-infected patients: a systematic review and meta-analysis. PLOS One **2011**; 6:e26827.

4. Ratledge C. Iron, mycobacteria and tuberculosis. Tuberculosis (Edinb) **2004**; 84:110–30.

5. Snow GA. Mycobactins: iron-chelating growth factors from mycobacteria. Bacteriol Rev **1970**; 34:99–125.

6. Reddy PV, Puri RV, Chauhan P, et al. Disruption of mycobactin biosynthesis leads to attenuation of *Mycobacterium tuberculosis* for growth and virulence. J Infect Dis **2013**; 208:1255–65.

7. Layre E, Lee HJ, Young DC, et al. Molecular profiling of *Mycobacterium tuberculosis* identifies tuberculosinyl nucleoside products of the virulence-associated enzyme Rv3378c. Proc Natl Acad Sci USA **2014**; 111:2978–83.

8. Pethe K, Swenson DL, Alonso S, Anderson J, Wang C, Russell DG. Isolation of *Mycobacterium tuberculosis* mutants defective in the arrest of phagosome maturation. Proc Natl Acad Sci USA **2004**; 101:13642–7.

9. Young DC, Layre E, Pan SJ, et al. Terpene nucleosides are transformed in vivo and serve as specific chemical markers for *M. tuberculosis* infection. Chem Biol **2015**; 22:515–26.

10. Young BL, Mlamla Z, Gqamana PP, et al. The identification of tuberculosis biomarkers in human urine samples. Eur Resp J **2014**; 43:1719–29.

11. Chatterjee D, Bozic CM, McNeil M, Brennan PJ. Structural features of the arabinan component of the lipoarabinomannan of *Mycobacterium tuberculosis*. J Biol Chem **1991**; 266:9652–60.

12. O'Sullivan DM, Nicoara SC, Mutetwa R, et al. Detection of *Mycobacterium tuberculosis* in sputum by gas chromatography-mass spectrometry of methyl mycocerosates released by thermochemolysis. PLOS One **2012**; 7:e32836.

13. Shui G, Bendt AK, Jappar IA, et al. Mycolic acids as diagnostic markers for tuberculosis case detection in humans and drug efficacy in mice. EMBO Mol Med **2012**; 4:27–37.

14. Gounder CR, Kufa T, Wada NI, et al. Diagnostic accuracy of a urine lipoarabinomannan enzyme-linked immunosorbent assay for screening ambulatory HIV-infected persons for tuberculosis. J Acquir Immune Defic Syndr **2011**; 58:219–23.

15. Boehme CC, Nabeta P, Hillemann D, et al. Rapid molecular detection of tuberculosis and rifampin resistance. New Engl J Med **2010**; 363: 1005–15.