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Urinary Biomarkers of Mycobacterial Load and Treatment Response in Pulmonary Tuberculosis

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Abstract

Background: Control of the tuberculosis (TB) pandemic remains hindered, in part, by a lack of simple and accurate measures of treatment efficacy. Current gold standard markers rely on sputum-based assays that are slow and challenging to implement. Previous work identified urinary $N^2, N^{12}$-diacetylperimine (DiAcSpm), neopterin, hydroxykynurenine, $N$-acetylhexosamine, ureidopropionic acid, sialic acid, and $m/z$ 241.0903 as potential biomarkers of active pulmonary TB (ATB). Here, we evaluated their ability to serve as biomarkers of TB treatment response and mycobacterial load.

Methods: We analyzed urine samples prospectively collected from two cohorts with ATB: 34 participants from African countries treated with first line TB therapy (HRZE) and followed for one year, and 35 participants from Haiti treated with either HRZE or an experimental drug followed for 14 days. Blinded samples were analyzed by untargeted high-performance liquid chromatography-coupled-time of flight mass spectrometry.

Results: Urinary levels of all seven molecules exhibited significant decreases by week 26 of successful treatment ($p=0.01$-$p<0.0001$), and positive correlations with sputum mycobacterial load ($p<0.0001$). Urinary levels of DiAcSpm exhibited significant decreases in participants treated with HRZE as early as 14 days ($p<0.0001$) but were unchanged in participants receiving ineffective therapy ($p=0.14$).

Conclusion: Reductions in urinary DiAcSpm, neopterin, hydroxykynurenine, $N$-acetylhexosamine, ureidopropionic acid, sialic acid, and $m/z$ 241.0903 correlated with successful anti-TB treatment and sputum mycobacterial load. Levels of DiAcSpm exhibited reductions capable of differentiating treatment success from failure as early as two weeks after the initiation of chemotherapy, commending its further development as a potentially simple, non-invasive biomarker of treatment response and bacterial load.
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Introduction

Tuberculosis (TB) remains the leading cause of death worldwide due to a single infectious agent, and is the leading cause of death due to drug resistance (1). Control of the TB pandemic remains hindered, in part, by the limited range of clinically relevant diagnostic and treatment response biomarkers. Current gold standard methods for monitoring response to therapy continue to depend on sputum-based assays (2). Such methods are recognized for their ability to indicate disease severity and transmissibility, but are limited by practical challenges associated with the ability to recover sputum, relative insensitivity of microscopy to bacterial burden and viability, and the inherently retrospective nature of culture-based methods which often lag weeks to months behind the need for clinical decision making (3, 4). Recent advances in nucleic acid amplification-based methods such as GeneXpert® (Cepheid, Sunnyvale, USA) have begun to overcome some of these barriers. However, despite their increased speed and sensitivity, such tests continue to require sputum samples, are unable to distinguish live from dead mycobacteria, and remain prohibitively expensive to operate in low and middle-income countries, where more than 90% of TB cases occur (5, 6). These hurdles to the timely diagnosis of disease and verification of treatment efficacy are problematic because while awaiting test results, ineffective or only partially-effective treatments continue to promote clinical progression, continued transmission, and the emergence of drug resistance itself (7, 8).

Sputum mycobacterial load is a widely recognized marker of disease severity that correlates with clinical symptoms, the presence of cavitary lung lesions, and transmission rates (9–11). Novel biomarkers of TB diagnosis and treatment response would ideally enable rapid detection and quantification of sputum bacterial load, and should be inexpensive, simple, non-invasive, and non-sputum based. Such characteristics would increase the ability to reach lower-resource
healthcare systems and reduce cost of diagnostic algorithms. In addition to their potential to
directly impact patient care, such biomarkers would also provide faster readouts of experimental
drug efficacy that could accelerate TB drug development.

Urinary biomarkers have recently begun to emerge as clinically useful diagnostic markers of
infectious disease and prognostic markers of treatment efficacy (12–14). Urine is an easily
obtainable biological sample that is chemically complex, and indicative of host physiologic
states, including infection (15). Moreover, growing evidence has demonstrated that TB may
elicit specific patterns of immune activation, including unique transcriptional signatures and TB-
specific T cell populations, some of which may be in the metabolic profiles of the blood and
urine of afflicted patients (16–21).

In previous work, we identified N1, N12-diacetylspermine (DiAcSpm), hydroxykynurenine,
neopterin, N-acethylhexosamine, ureidopropionic acid, sialic acid, and an uncharacterized
molecule with mass-to-charge ratio \((m/z)\) of 241.0903 as potential urinary biomarkers of active
pulmonary tuberculosis (ATB) (Table 1) (22). Levels of these urinary molecules decreased after
60 days of anti-TB treatment in 20 participants, supporting their utility for indicating active TB
disease (22).

In this report, we set out to investigate the potential of these same urinary metabolites to also
serve as biomarkers of TB treatment response and mycobacterial load.
Results

Levels of Urinary Molecules Decrease during TB Treatment

We first characterized the urine metabolic profiles of 34 participants treated for ATB using a blinded set of prospectively collected longitudinal urine samples from the REMoxTB trial (23) obtained through the Consortium for TB Biomarkers (CTB2) (Figure 1). All participants received either 8 weeks of isoniazid, rifampin, pyrazinamide, and ethambutol (HRZE), followed by 18 weeks of isoniazid and rifampin, or received moxifloxacin in combination with isoniazid or ethambutol as detailed in the study description (23). Available participant characteristics are listed in Table 2. All participants tested positive for TB by sputum culture, AFB smear, or GeneXpert assay at the time of enrollment. All participants had negative AFB smears and sputum cultures at treatment termination (26 weeks) (Table S1). Participant urine samples were obtained prior to initiation of treatment (week 0) and at weeks 2, 4, 8, 17, 26 and 52 of the study. Samples were blinded, randomized and normalized by dilution to 150 milliosmoles (mOsm) prior to untargeted metabolomic profiling by high performance liquid chromatography-coupled high-resolution time-of-flight mass spectrometry (HPLC/MS).

Urinary levels of DiAcSpm, hydroxykynurenine, neopterin, N-acetylhexosamine, ureidopropionic acid, sialic acid, and \( m/z \) 241.0903 all exhibited significant decreases by the end of treatment at 26 weeks, after adjusting for age, sex, and BMI (linear mixed model \( p=0.01 \) - \( p<0.0001 \)) (Figure 2). Levels of DiAcSpm and hydroxykynurenine exhibited significant decreases after only 2 weeks of TB treatment (\( p<0.0001 \), \( p<0.0001 \), respectively) (Figure 2A, 2B) while levels of neopterin, N-acetylhexosamine, ureidopropionic acid, sialic acid, and \( m/z \) 241.0903 exhibited significant decreases from baseline between 4 and 26 weeks of treatment (\( p=0.01 \)-\( p<0.0001 \)) (Figures 2C-2G).
Urinary Molecule Abundance Correlates with Sputum Mycobacterial Loads

We next investigated whether urinary levels of any of these seven molecules correlated with sputum mycobacterial burden. Molecule abundance data from all CTB2 urinary samples with their corresponding sputum AFB scores are presented on scatter plots, regardless of participant identification or treatment time point, in Figure 3. Panel linear regression analysis was conducted for each of the 7 molecules accounting for intra-participant correlation and batch-effect of mass spectrometry. Regression slopes identified strong positive correlations between sputum AFB score and molecule abundance for all seven molecules (p<0.0001).

To further investigate the relationship between the levels of these molecules and treatment-induced declines in sputum bacterial load, we assigned each of the 34 CTB2 cases into “high” or “low” mycobacterial burden groups based on sputum AFB scores at the time of diagnosis (week 0). Participants with initial AFB scores of 3+ or 4+ were categorized as “high sputum load” (n=11), and participants with initial AFB scores of 0, scanty, 1+ or 2+ were categorized as “low sputum load” (n=23) (Table S1). At baseline, levels of N-acetylhexosamine, sialic acid, and m/z 241.0903 differed significantly between high and low sputum groups (Figure 4A-C). In all cases, higher initial mycobacterial burden was associated with higher urinary molecule abundance. In contrast, by the time of treatment termination at week 26, levels of all molecules from both “high” and “low” initial sputum load groups converged to similar levels, consistent with their shared clinical endpoint of cure. DiAcSpm exhibited a similar trend to the three previously highlighted molecules, although the difference at baseline did not reach statistical significance (Figure 4D).
DiAcSpm Correlates with Early Treatment Response Outcomes and Mycobacterial Load

To independently validate our findings, we obtained and analyzed urine samples from a second cohort of 35 participants with ATB enrolled in an early bactericidal activity (EBA) study at the GHESKIO Centers in Port-au-Prince, Haiti. The study was designed to determine if the in vitro activity of the FDA-approved anti-parasitic agent nitazoxanide (NTZ) against *Mycobacterium tuberculosis* (*Mt*) could serve as a mycobactericidal agent for drug-sensitive pulmonary TB (24). Participants were recruited at the GHESKIO Centers and allocated into two treatment arms: 19 participants received NTZ for 14 days, while 16 participants received standard TB treatment of HRZE (Figure 1). Participant demographics are shown in Table 3. Inclusion criteria included either a sputum smear AFB score of at least 2+, or GeneXpert® MTB/RIF positivity for MTB at the medium or high level at the time of enrollment. Urine samples were collected before treatment on day 0, and on days 2, 4, and 14 after treatment initiation. All urine samples were blinded, randomized and normalized to 150 mOsm prior to untargeted metabolomic analysis by HPLC/MS. Additionally, overnight sputum samples were collected from each patient upon diagnosis, and continuously collected every two days for 14 days to monitor for changes in culture time to positivity (TTP) as a measure of treatment efficacy (24). TTP data from this study were mathematically converted to colony forming unit (CFU) (25).

Treatment with NTZ yielded no change in sputum culture CFU after 14 days, whereas treatment with HRZE resulted in the expected decrease in sputum culture CFU (Figure 5A). These findings thus revealed NTZ to lack clinical anti-mycobacterial activity, making it possible to evaluate the performance of our urinary biomarkers in relation to treatment efficacy by comparing urine samples obtained at the start and end of treatment from each arm of this study.
Linear mixed modeling of these data demonstrated that HPLC/MS-measured mean urinary DiAcSpm decreased significantly in the HRZE arm (p<0.0001), but not in the NTZ arm (p=0.14), and reached statistical significance at the study endpoint of treatment day 14 (p<0.0001) (Figure 5B). This trend was also observed on the individual patient level when comparing changes in urinary DiAcSpm between days 0 and 14 (Figure 5C). Moreover, these reductions could be detected using an analytically independent, and commercially available, monoclonal antibody-based ELISA (Trans Genic Inc., Kobe, Japan) developed for clinical use (26) (Figure 6A and B).

Levels of urinary hydroxykynurenine, N-acetylhexosamine, ureidopropionic acid, and m/z 241.0903 showed similar significant decreases in mean abundance in participants treated with HRZE over the first two weeks, but did not achieve statistical significance when comparing the two treatment arms by the day 14 endpoint (Figure S1). Linear mixed modeling of maximum daily axillary temperatures taken on corresponding treatment days (0, 2, 4, 14) similarly failed to demonstrate significant difference between treatment arms (Figure S2).

Given DiAcSpm’s ability to differentiate effective (HRZE) from ineffective (NTZ) therapy, we further investigated the association between DiAcSpm and mycobacterial burden in this cohort. To do so, we plotted baseline (day 0) calculated culture CFUs against corresponding urinary levels of DiAcSpm. As shown in Figure 7, DiAcSpm concentrations correlated positively with mycobacterial burden in both HPLC/MS and ELISA results, as indicated by higher corresponding CFU values (p=0.0001 & 0.0003, r²=0.3812 & 0.3318 respectively). This positive association was further validated using urine samples obtained from a third cohort reported by Dupnik et al. (16) (Figure 1, Figure S3).
In order to assess the predictive value of declining DiAcSpm concentration for treatment response, we constructed a Receiver Operator Characteristic (ROC) curve using DiAcSpm concentration fold change at 14 days in NTZ and HRZE-treated groups (Figure 8). Area under the ROC curve (AUC) values were 85.76% (95% CI = 72.05, 99.48) and 83.82% (95% CI = 70.01, 97.64) for mass spectrometry- and ELISA-based data, respectively.
Discussion

Current measures of TB treatment response and disease burden remain rooted in sputum-based assays that are prohibitively slow, complex, and are often incompatible with the healthcare settings in which TB is most frequently seen. Clinicians are thus often forced to rely on more subjective measures of symptom resolution while waiting several weeks or months for confirmation by sputum AFB and culture. Fast, sensitive and affordable point-of-care tests of treatment response thus constitute a major unmet clinical need that is critical for control of TB at both the individual and population levels (27).

Urinary biomarkers decrease with treatment and correlate with mycobacterial load

Biomarkers from human biofluids are useful reporters of host physiology in different pathological states, but are understudied in the context of TB treatment response. Our study demonstrates that urinary levels of DiAcSpm, hydroxykynurenine, neopterin, N-acetylhexosamine, ureidopropionic acid, sialic acid, and m/z 241.0903 all decreased over the six-month course of treatment in 34 successfully treated ATB cases. Levels of these molecules predominantly stayed low or even continued to decrease after treatment termination at 6 months. These findings thus suggest that the molecular markers are not merely measuring pharmacological actions of medication on host metabolism, but more importantly are reporting on disease activity itself. Previous work reported that urinary levels of these seven molecules were elevated in ATB cases compared to cases of non-TB pulmonary disease with an overall sensitivity and specificity of over 80% before initiation of treatment (22). Levels of kynurenine, neopterin, and sialic acid have also been previously reported to be increased in various human biofluids of ATB (28–33).
The rates at which each of these seven molecules decreased over 26 weeks of treatment varied. In all participants, some molecules dropped precipitously within the first two weeks, while others declined more gradually over the course of treatment. However, all were associated with significant reductions by the end of treatment, with some, such as hydroxykynurenine, exhibiting reductions as large as eight-fold (Figure 2B). These preliminary data suggest a potential role for one or a combination of these seven urinary molecules to be developed into surrogate biomarkers of TB treatment response. Molecules exhibiting overall early declines could have a role in determining suitability of medical regimens, while molecules with overall slower kinetics might serve as longer term reporters of treatment efficacy. Binary classification of participants into “high” and “low” initial sputum burden groups also highlighted the differing rates of molecule decline based on initial disease severity (Figure 4). These findings thus raise the intriguing possibility of developing a number of clinically useful quantitative metrics of disease state and treatment response based on baseline levels of bacterial load, and absolute or relative reductions in biomarker levels.

Urinary levels of all seven target molecules were also positively associated with sputum mycobacterial load. Levels of N-acetylhexosamine, sialic acid, and m/z 241.0903 were initially significantly higher in participants with high sputum AFB scores at diagnosis, but eventually converged with levels from participants with low sputum AFB by 26 weeks. Acetylated sugars such as N-acetylhexosamine are known components of the Mtb cell wall, and sialic acids are often expressed by pathogens in order to enhance intracellular survival and reduce host immune response (34). It is thus possible that these molecules may reflect specific Mtb-derived products. Biological origin notwithstanding, changes in mycobacterial burden may be the best current indicator of treatment outcome for TB, and are routinely used in clinical practice to document
treatment response (35). Correlative data between urinary molecule levels and corresponding sputum TB load presented in this study show promise for these urinary compounds to serve as surrogate markers of *Mtb* bacillary load.

**DiAcSpm’s potential role as a marker of antimycobacterial activity**

Polyamines, including spermine, spermidine, and putrescine, are present in all organisms. They play important roles in major cellular processes such as growth and proliferation, and normally have tightly regulated intracellular levels (36). Current knowledge of polyamine metabolism is shown in Figure 9, which illustrates the ability of spermine to undergo catabolism either through direct oxidation by spermine oxidase (SMOX), or acetylation by spermidine/spermine *N*-acetyltransferase (SSAT). *N*-acetylspermine can undergo a second acetylation step via SSAT, forming DiAcSpm. Acetylated polyamines are then exported from the cell via an ATP-dependent polyamine transporter (36).

Considerable evidence has implicated polyamines in the pathogenesis of various mammalian bacterial diseases. In addition to DiAcSpm, several other metabolites in the polyamine catabolic pathway have previously been reported to be increased in TB states, including *N*-acetylisoputreanine (37). Moreover, several bacteria have been shown to upregulate polyamine catabolism in infected host tissues, and it has been suggested that acetylated end products of polyamine catabolism facilitate cellular export (36, 38). Macrophages have conversely been implied as a source of DiAcSpm. A study by Hamaoki and Nagata revealed that peritoneal macrophages from lymphoid tumor-bearing mice produced DiAcSpm in the presence of exogenous spermine (39). Most interestingly, an in vitro study from the 1950s demonstrated that exogenous spermine exhibited antimycobacterial properties after an unidentified enzymatic
alteration (40). However, very little is known about the biological role of polyamines in TB immunopathogenesis (41, 42).

Previous work from this and other groups showed elevated levels of urinary DiAcSpm in ATB cases (22, 43). Our study demonstrates that levels of DiAcSpm also decrease rapidly with effective TB treatment. This could indicate that polyamine catabolism increases in TB infected lung tissues and subsequently decreases with resolution of the infection. An alternative explanation could involve an increased conversion of spermine to DiAcSpm by macrophages during active infection, in an attempt to produce antimycobacterial effects similar to those observed by Hirsch et al. in vitro (40). In this scenario, levels of DiAcSpm would decrease with treatment since macrophages would face decreasing Mtb bacterial burden as the infection clears.

Our study also demonstrates the potential ability of DiAcSpm levels to differentiate treatment success from failure during the first 14 days of anti-mycobacterial therapy. The predictive value of a decrease in urinary DiAcSpm concentration was depicted using ROC curves, which showed AUCs of over 80% in data obtained from two distinct detection modalities. The parameter used to construct ROC curves was the fold-change in DiAcSpm concentration over time, which is independent of any specific concentration at any single time point. This finding highlights the predictive power of rate of change alone, regardless of starting molecule concentration, the latter of which has been shown to vary among individuals based on disease burden (Figures 3 and 7).

Interestingly, recent work has begun to suggest that clinical cure of TB may not be strictly equated with microbiologic sterilization (44-46), suggesting the presence of varying levels of residual bacteria among cured individuals. DiAcSpm’s potential ability to be used as an early marker of treatment efficacy could thus both significantly improve clinical medication
management through its ability to detect potential treatment failures prior to the availability of
drug susceptibility test results, as well as play an important role in facilitating EBA drug trials,
which are currently the frontline standard for determining in vivo bactericidal activity of new TB
drugs, and still rely on a time-consuming method of counting amounts of viable CFUs from
sputum cultures.

From a translational perspective, DiAcSpm is a regular constituent of human urine, consistently
accounting for 0.5% of total excreted urinary polyamines (47). DiAcSpm is not reabsorbed by
the glomerular filtration system, and there is minimal diurnal variation in its urinary content
among healthy individuals, suggesting tight control of its secretion (47, 48). These remarkable
qualities thus commend further investigation of urinary DiAcSpm as a candidate biomarker of
treatment response.

**Conclusion**

We have identified several candidate prognostic biomarkers of TB treatment response. Levels of
urinary DiAcSpm specifically show early and significant decrease in cases of successful TB
treatment, suggesting its potential for development into an early biomarker of TB treatment
efficacy.
Methods

Study Design

Longitudinal urine samples from 34 participants successfully treated for ATB were obtained from the CTB2 biorepository for urinary metabolite analysis over the course of treatment. Sputum mycobacterial data were made available in order to study correlation between urinary metabolites and \textit{Mtb} burden. Additional urine samples were obtained from the GHESKIO Centers in Port-au-Prince, Haiti from 35 participants enrolled in an EBA trial, and were used for urinary metabolite analysis in cases of ineffective treatment (24).

CTB2 Longitudinal Cohort

The CTB2, comprised of the Global Alliance for TB Drug Development, the TB Trials Consortium, and the AIDS Clinical Trials group, has created a collaborative biobank in order to accelerate biomarker discovery and validation for the diagnosis and treatment of TB. In collaboration with CTB2, we obtained prospectively collected longitudinal urine samples from 34 participants treated for confirmed ATB (Table 2). By request, clinical information for these samples were blinded to us until completion of metabolite analysis. Participants were recruited for two separate studies in unspecified African countries and followed over one year. Treatment consisted of either 8 weeks of rifampin, isoniazid, pyrazinamide, and ethambutol (HRZE), followed by 18 weeks of isoniazid and rifampin, or was replaced in part by moxifloxacin as outlined in the REMox Trial (23). Information on the specific treatment regimen corresponding to each participant, medication compliance, and drug susceptibility was not provided to us. Urine samples from each participant were collected at baseline (week 0), and at weeks 2, 4, 8, 17, 26, and 52 post-treatment. Sputum culture and AFB data were obtained at weeks 0, 4, 8, 26, and 52.
post-treatment (Table S1). Chest X-rays (CXRs) and GeneXpert data were recorded at the time of diagnosis for 33 and 28 patients, respectively. All participants had either sputum AFB, culture, or GeneXpert positivity at time of diagnosis. All patients showed no culture or AFB positivity at treatment termination (26 weeks).

**Urine sample collection, storage, and shipment (CTB2)**

Clean-catch urine samples were stored at -80°C in the Fischer BioServices facility in Bishop’s Strotford, England. Samples were shipped via PDP Couriers on dry ice, with constant temperature monitoring using a United Technologies Sensitech TempTale4 system, to the Belfer Research Labs at Weill Cornell Medicine and stored at -80°C until time of analysis.

**Assignment to sputum mycobacterial load group (CTB2)**

AFB seen under smear microscopy are classified as 4+, 3+, 2+, 1+, scanty, or 0, with greater numbers denoting higher bacillary loads. To create a dichotomous variable for mycobacterial load, the 34 CTB2 cases were assigned into “high” or “low” mycobacterial burden groups based on sputum AFB scores at the time of diagnosis (week 0). Participants with initial AFB scores of 3+ or 4+ were categorized as “high sputum load” (n=11), and participants with initial AFB scores of 2+, 1+, scanty, or 0 were categorized as “low sputum load” (n=23).

**GHESKIO Cohort**

Urine samples were collected from 35 participants with confirmed drug-sensitive ATB at the GHESKIO Centers as part of a 14-Day EBA study of NTZ for the treatment of pulmonary TB (Table 3) (24). Urine was collected from each participant pre-treatment on day 0, and on days 2,
Participants were allocated into two treatment groups: 19 participants were treated with a 14-day course of NTZ, and 16 participants were treated with the standard HRZE therapy as defined by the WHO. Of the 16 HRZE-treated participants included in our analysis, 10 were randomized control participants from the clinical trial, and 6 were additional control participants enrolled in a pilot phase of the trial to validate laboratory assays. Overnight sputum samples from this cohort were collected every two days and cultured using the Mycobacterial Growth Indicator Tube (MGIT) automated liquid culture system (BACTEC; BD, Franklin Lakes, NJ) to generate time to positivity (TTP) data. TTP data were subsequently mathematically converted to CFU values in this study, using the formula derived by Diacon et al.

**Urine sample collection, storage, and shipment (GHESKIO)**

Clean-catch urine samples were collected in sterile cups and immediately refrigerated at -4°C for 1-7 hours. Urine was then aliquoted on ice and stored at -80°C in GHESKIO facilities in Port-au-Prince, Haiti, until shipment to New York City. Shipments were sent on dry ice via World Courier from GHESKIO to the Weill Cornell Center for Global Health laboratory in New York City, and stored at -80°C until time of analysis.

**Sample Preparation**

Samples from all cohorts were stored in a -80°C freezer at the Belfer Research Building at Weill Cornell Medicine until testing. Samples were blinded, randomized and prepared in sets of 20 to 25. The osmolality of each sample was measured using an Advanced Instruments model 3250 Micro-Osmometer. Samples were then centrifuged for 10 minutes at 10,000rpm in PALL nanosept centrifuge devices. Filtered substrate was diluted with MilliQ water to 150mOsm in
order to standardize the salt concentration within each sample. All samples below 150mOsm
prior to dilution were excluded from analysis. Diluted samples were mixed with LC/MS grade
methanol containing 0.2% formic acid at a 1:1 sample to solvent ratio for HPLC/MS analysis.
Each set of 20 to 25 samples was run with a standard solution that consisted of 10µM of
glutamate, succinate, lysine and nicotinic acid that served as analytical quality control standards
for the LC/MS. Pooled urine samples were included periodically throughout each set to allow for
normalization of peak intensities and monitoring of mass spectrometer sensitivity within each
run. A third of the total urine samples from both CTB2 and GHESKIO cohorts were randomly
selected for replicate runs to ensure data reproducibility. Replicate runs were performed using
previously un-thawed urine aliquots, independently randomized, and run in sets of 20 to 25
color with the above standard solutions.

HPLC/MS Analysis:
Samples were analyzed using an Agilent Technologies 6230 TOF LC/MS. Liquid
chromatography (LC) separation was achieved using a Cogent 4 Diamond Hydride column with
an initial gradient of 85% LC/MS grade acetonitrile containing 0.2% formic acid, followed by a
gradual increase in hydrophilicity to 95% LC/MS grade water containing 0.2% formic acid.
Detected ions were indexed and characterized using their ion mass to charge ratio (m/z) and
chromatographic retention time. Data was analyzed using Agilent Technologies Qualitative
Analysis B.07, Agilent Technologies MassHunter Profinder B.08, and the XCMS software.
Compound identification was achieved using known m/z and retention time coupled to chemical
standards of targeted compounds run with each set of urine samples. Identity of DiAcSpm was
further confirmed using MS/MS fragmentation analyses of chemical standards and random
patient urine samples. DiAcSpm chemical standards at five known concentrations (50nM, 100nM, 500nM, 1µM, and 5µM) were included within each run to create standard curves for urinary DiAcSpm concentration calculation.

Urinary DiAcSpm ELISA kits

Previously unthawed urinary samples were used for this portion of the analysis. 50µL of vortexed urine was centrifuged for 5 minutes at 1500rpm. Urine was serially diluted 4 to 9 times accordingly in order for resulting concentrations to remain within ELISA kit detection range. Absorbance was measured using a Spectramax M2 microplate reader at 490nm. Each sample was measured in duplicates, and all measured DiAcSpm concentrations were within range of the standard curve. Final results were adjusted for initial dilution ratio, and further normalized to respective urinary creatinine concentrations, with a final unit of nmol/g creatinine.

Creatinine Normalization:

All molecule abundances were additionally normalized to creatinine concentrations of corresponding urine samples using a creatinine colorimetric assay kit (Sigma Aldrich Catalog number MAK080). Absorbance was measured using a Spectramax M2 microplate reader at 570nm. Each sample was measured in duplicates, and all measured creatinine concentrations were within range of the standard curve.

Statistical Methods

All normalized molecule abundances were log₂ transformed for analysis and visualization. Data analysis was performed using STATA SE version 15 and GraphPad Prism 6. For all statistical analyses, a p-value of less than 0.05 was considered significant.
**Longitudinal Analysis**

Longitudinal trends of target urinary molecules were fitted using a mixed model. We estimated the effects of treatment at each time point as fixed effects while incorporating subject-specific abundances as random effects in the model. Hypotheses of factor variables and their interactions were assessed using the Wald test provided by the STATA margins command.

**CTB2 cohort:** We used a binary variable (high vs. low initial sputum AFB) to estimate the effect AFB had on longitudinal abundance profiles in this cohort. We included interaction terms between AFB and time in order to account for confounding trends over time. Time and AFB effects were calculated while adjusting for BMI, age, and sex.

**GHESKIO cohort:** We used the two treatment arms of NTZ and HRZE as binary variables in our statistical model. Interactions between treatment and time were included and assessed. Treatment and time effects were calculated while adjusting for age and baseline weight.

**Molecule abundance correlation with Sputum AFB Score (CTB2)**

We conducted panel linear regression analyses using per patient trend profiles of sputum AFB as covariates (Figure S4), and corresponding per patient trend profiles of each biomarker as outcomes (examples shown in Figures S5 and S6). This per patient aggregate analysis of the correlation between AFB and urinary molecule levels allowed us to account for within patient correlations and mass spectrometer technical batch effects. Data from all available CTB2 cohort samples and time points were included.

**GHESKIO cohort TTP and CFU**

Effect of treatment (NTZ vs HRZE) was assessed by fitting a mixed model on longitudinal arrays of CFU data. CFU values were mathematically converted from clinically measured patient
TTP data, using the formula $\log_{10}(CFU) = 16.41 - 5.17 \times \log_{10}(TTP)$ derived by Diacon et al (25).

Graphical representation of the original TTP data was shown in a study by Walsh et al (24).

Dupnik cohort urine DiAcSpm correlation with GeneXpert sputum load

We obtained banked urine samples collected from participants originally described by Dupnik et al. in a study looking at blood transcriptomic markers of sputum mycobacterial load (16). This cohort consisted of 51 individuals with active pulmonary TB and 21 community controls with no signs or symptoms of TB and no prior history of TB. Of the 51 participants diagnosed with TB, 19 had low TB load and 32 had high TB load as determined by GeneXpert MTB/RIF C_T values.

Further details on participant selection, sample collection, and experimental group assignments are described in the Methods section of (16). Urine samples obtained as part of this study were stored at -80°C in the Belfer Research Labs at Weill Cornell Medicine until time of analysis. Sample preparation and analysis on the HPLC/MS were completed as described in above sections. We used a one-way analysis of variance (ANOVA) test to determine whether a statistically significant difference existed between DiAcSpm concentrations of participants within the control, low sputum, and high sputum groups. We further used two-tailed Welch-corrected t-tests to determine differences between each two adjacent categories, i.e. control vs. low sputum group, and low vs high sputum group.

Study Approval

Consent was obtained from all participants by local health workers during meetings conducted in the local language. All participants provided written informed consent prior to inclusion in the clinical cohort studies. IRB approval was obtained for this present study at Weill Cornell
Medicine, New York, NY. Studies from which CTB2 and GHESKIO samples were obtained have IRB approval at their respective institutions.

Author Contributions

F.I. and Q.X. designed and conducted the experiments, acquired and interpreted MS data, and evaluated urinary molecule performance as markers of successful TB treatment. M.H.L. performed statistical analyses. J.M.B., K.F.W., K.M., and K.M.D. collected and provided clinical samples and clinical data. K.Y.R. provided reagents and machinery for the study. Q.X., F.I., and K.Y.R. wrote the manuscript. K.Y.R. and D.F. supervised and coordinated the work. All authors reviewed the manuscript, agreed with the results, and provided insight.

Acknowledgements

We thank the CTB2 and Weill Cornell Center for Global Health for generously providing urine samples for analysis. We thank the volunteers who participated in this study and the staff at GHESKIO and all involved African institutions for helping to enroll, consent, and collect samples from participants. We gratefully acknowledge Robert Jansen and Travis Hartman for help with mass spectrometry data acquisition and interpretation. We thank the Rhee Lab, Matthew Wipperman, Selin Somerson, Carl Nathan, the TB researchers at Weill Cornell Medicine, and the Tri-I TBRU for critical input and guidance.
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Figure 1. **Study design flow chart of each study cohort.** Two main longitudinal cohorts, CTB2 and GHESKIO, and one supplemental cohort, were included in this study.
Figure 2 Molecule abundance decreased over course of treatment in clinically cured TB patients from CTB2 cohort (n=34). Mean fold change of each urinary molecule from baseline levels (week 0) are depicted in log₂ scale. Red line represents no change from baseline. All original HPLC/MS molecule abundances were normalized to corresponding urinary creatinine levels. Error bars represent 95% CI. Statistical difference between adjacent time points was determined using the Wald test and represented by *. (**, p<0.01; ****, p<0.0001)
Figure 3 Urinary molecule abundance positively correlates with sputum mycobacterial burden in CTB2 cohort. Scatterplots depict molecule abundances of each urine sample against its corresponding sputum AFB score. Vertical axes represent HPLC/MS-measured molecular abundances in log₂ scale after creatinine normalization. Data from all CTB2 participants (n=34) and at all time points are represented. Regression lines adjusted for within-patient correlation and technical batch effects are represented in red (all p<0.0001, r² = 0.0928 – 0.2505).
Figure 4 Mean urinary molecule abundance is higher in TB patients with high initial sputum mycobacterial load in CTB2 cohort. Mean HPLC/MS abundance in log$_2$ scale separated by initial sputum mycobacterial load of sialic acid (A), N-Acetylhexosamine (B), $m/z$ 241.0903 (C), and diacetylspermine (D). Participants were separated by sputum AFB smear score at time of diagnosis (week 0). Initial AFB scores of 3+ or 4+ were categorized as “high sputum load” (n=11, in red); initial AFB scores of 2+ or lower were categorized as “low sputum load” (n=23, in blue). Error bars represent 95% CI.
Figure 5. Urinary $N^1$, $N^{12}$-diacetylsperrme levels measured by mass spectrometry differentially decrease in successfully treated patients within the first 14 days in GHESKIO cohort. (A) Sputum culture CFUs show no change in bacterial burden of TB patients treated with 14 days of NTZ (n=19, in red). CFUs decrease during treatment with rifampin, isoniazid, pyrazinamide, and ethambutol (HRZE) (n=16, in blue), demonstrating decreased bacterial burden. (B) HPLC/MS-measured urinary DiAcSpm decreases significantly in participants treated with HRZE (blue) but not in those treated with NTZ (red). Solid circles represent mean fold change from baseline levels in log$_2$ scale. Error bars represent 95% CI and do not overlap at day 14. Dotted red line represents no change from baseline. (C) HPLC/MS-measured urinary DiAcSpm levels of individual participants. Each line represents an individual participant. Dotted line represents no change from baseline. All values have been normalized to corresponding urinary creatinine concentration.
Figure 6. Change in urinary $N^1, N^{12}$-diacetylsperrmine levels in GHESKIO cohort confirmed using ELISA. (A) ELISA-measured urinary DiAcSpm levels demonstrate significant concentration decreases in participants treated with HRZE ($n=16$, in blue) over 14 days, but not in those treated with NTZ ($n=19$, in red). Solid dots represent mean fold change from baseline levels in log$_2$ scale. Error bars represent 90% CI and do not overlap at day 14. (B) Changes in ELISA-measured urinary DiAcSpm levels of individual participants over 14 days. Each line represents a single participant. Dotted line in each graph represents no change from baseline. All values have been normalized to corresponding urinary creatinine concentration.
Figure 7. Urinary $N^1$, $N^{12}$-diacetylspermine concentration correlates with culture measures of mycobacterial burden in GHESKIO cohort. Scatterplots with regression lines show correlation between calculated CFU upon diagnosis (Day 0) and DiAcSpm concentration (n=35). Increasing DiAcSpm concentration is associated with an increase in CFU, which is in turn a microbiological measure of mycobacterial burden. DiAcSpm concentrations were determined using (A) HPLC/MS chemical standard abundances ($p = 0.0001$, $r^2 = 0.3812$) and (B) ELISA ($p = 0.0003$, $r^2 = 0.3318$). All values have been normalized to corresponding urinary creatinine concentrations.
Figure 8. ROC curves show predictive value of urinary \( N^1, N^{12}\)-diacetylspermine using concentration fold change over 14 days. ROC curves of participants in the GHESKIO cohort (n=35) were plotted using urinary DiAcSpm concentration fold change between days 0 and 14 as classifiers to known participant treatment group (NTZ vs HRZE). Area under the ROC curve (AUC) values are 85.76% (95% CI = 72.05, 99.48) and 83.82% (95% CI = 70.01, 97.64) for mass spectrometry and ELISA-based readouts, respectively.
Figure 9. **Polyamine synthetic and catabolic pathway.** Circled enzyme spermidine/spermine N\(^{\prime}\)-acetyltransferase (SSAT) is hypothesized to be responsible for the production of DiAcSpm through a second acetylation process. SMOX – spermine oxidase. Solid lines represent known pathways; dotted line represents postulated pathway.
**Table 1.** Characteristics of molecules analyzed in urinary samples of ATB cases

<table>
<thead>
<tr>
<th>Mass-to-Charge Ratio $(m/z)$</th>
<th>Retention Time (min)</th>
<th>Predicted Formula</th>
<th>Preliminary Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>133.0600</td>
<td>1.56</td>
<td>C$_4$H$_8$N$_2$O$_3$</td>
<td>Ureidopropionic acid</td>
</tr>
<tr>
<td>144.1241</td>
<td>14.92</td>
<td>C$<em>{14}$H$</em>{30}$N$_4$O$_2$</td>
<td>$N^2,N^{12}$-diacetylspermine</td>
</tr>
<tr>
<td>186.0762</td>
<td>2.08</td>
<td>C$<em>8$H$</em>{15}$NO$_6$</td>
<td>N-Acetylhexosamine</td>
</tr>
<tr>
<td>225.0845</td>
<td>6.22</td>
<td>C$<em>{10}$H$</em>{12}$N$_2$O$_4$</td>
<td>Hydroxykynurenine</td>
</tr>
<tr>
<td>241.0903</td>
<td>1.77</td>
<td>C$<em>9$H$</em>{12}$N$_4$O$_4$</td>
<td>Unknown</td>
</tr>
<tr>
<td>254.0859</td>
<td>3.32</td>
<td>C$<em>9$H$</em>{11}$N$_5$O$_4$</td>
<td>Neopterin</td>
</tr>
<tr>
<td>310.1148</td>
<td>2.56</td>
<td>C$<em>{11}$H$</em>{19}$NO$_9$</td>
<td>Sialic acid</td>
</tr>
</tbody>
</table>

*All molecules were previously elucidated by Isa et al. 2018*
### Table 2. Clinical characteristics of participants from the CTB2 longitudinal cohort

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Participant Characteristics (n=34)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Age, years (range)</td>
<td>33.9 (18-59)</td>
</tr>
<tr>
<td>Sex (% female)</td>
<td>21M 13F (38.2%)</td>
</tr>
<tr>
<td>Mean BMI (range)</td>
<td>18.7 (14.4-25.2)</td>
</tr>
<tr>
<td>HIV+ (%)</td>
<td>4 (11.8%)</td>
</tr>
<tr>
<td>GeneXpert + (%)</td>
<td>28 (100%) ^A</td>
</tr>
<tr>
<td>Cavitation on CXR (%)</td>
<td>31 (93.9%) ^B</td>
</tr>
<tr>
<td>Culture and AFB neg by 8wks (%)</td>
<td>19 (55.9%)</td>
</tr>
<tr>
<td>Culture and AFB neg by 26wks (%)</td>
<td>34 (100%)</td>
</tr>
</tbody>
</table>

^A 6 patients did not undergo GeneXpert testing at time of diagnosis

^B 1 patient did not undergo chest X-ray testing at time of diagnosis
### Table 3. Clinical characteristics of participants from the GHESKIO cohort

<table>
<thead>
<tr>
<th></th>
<th>Participants with ATB under Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HREZ Treatment (n=16)</td>
</tr>
<tr>
<td>Mean Age, years (range)</td>
<td>32.1 (18-52)</td>
</tr>
<tr>
<td>Sex (% female)</td>
<td>9M 7F (43.8%)</td>
</tr>
<tr>
<td>Mean Weight, pounds (range)</td>
<td>119.0 (101-137.2)</td>
</tr>
<tr>
<td>HIV+ (%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>GeneXpert + (%)</td>
<td>16 (100%)</td>
</tr>
<tr>
<td>Cavitation on CXR (%)</td>
<td>8 (50%)</td>
</tr>
</tbody>
</table>