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TITLE: Urinary Biomarkers of Tuberculosis: Potential for Diagnosis and Beyond

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14. ABSTRACT
Tuberculosis (TB) remains the leading cause of deaths due to an infectious disease. A major barrier to control of the pandemic is the lack of rapid, point-of-care biomarkers. Urinary biomarkers were identified that can both diagnose active TB and decrease or increase, over time, while on anti-TB treatment. The objective of this proposal is to determine the reliability of these as urinary biomarkers, both for diagnosis and assessment of treatment of TB. To do this we have obtained urine samples from two distinct clinical cohorts. The first cohort consists of 37 participants with active pulmonary TB followed over time. We have successfully completed metabolomic analysis of urine samples from this cohort and have identified several urinary metabolites that decrease with treatment response and are associated with mycobacterial burden. The second cohort consists of 100 participants with active pulmonary TB, 100 participants who have latent TB and 100 uninfected controls. We have completed metabolomic analysis for 100 randomized urine samples from this cohort and will complete metabolomic analysis for the additional 200 in the next month. Follow on statistical studies should be completed in the next 1-2 months.

15. SUBJECT TERMS
Tuberculosis, biomarkers, urine, treatment response

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Table of Contents

1. **INTRODUCTION:**

Tuberculosis (TB) remains the leading cause of deaths due to an infectious disease. A major barrier to control of the pandemic is the lack of rapid, point-of-care biomarkers. Urinary biomarkers were identified that can both diagnose active TB and decrease or increase, over time, while on anti-TB treatment. The *objective* of this proposal is to determine the reliability of these urinary biomarkers, both for diagnosis and assessment of treatment of TB. Aim 1 of this proposal aims to prospectively follow urinary biomarker concentrations in participants during treatment**.** The *working hypothesis* is that urinary biomarker concentration will change over time and track with treatment response and mycobacterial load. Aim 2 is to determine whether urinary biomarker concentrations can differentiate between active TB, latent TB infection (LTBI) and un-infected healthy controls**.** The *working hypothesis* is that urinary biomarker concentrations will be increased in cases of LTBI, defined as having a positive interferon-gamma release essay (IGRA), as compared to healthy controls, but decreased when compared to cases of active TB.

2. **KEYWORDS:**

Tuberculosis, biomarker, urine, treatment response

3. **ACCOMPLISHMENTS:**

   - **What were the major goals of the project?**

The major goal of this project was to validate 10 urine metabolites as diagnostic for active tuberculosis and as potential markers of tuberculosis treatment response. Aims were as follows:

   - **Aim 1: Prospectively follow urinary biomarker concentrations in participants during treatment to determine the relationship of the biomarkers to treatment response, bacterial burden and immunologic status.** The *working hypothesis* is that urinary biomarker concentration will decrease over time and will be associated with bacterial burden as measured by sputum acid-fast bacilli (AFB).
   - **Aim 2: Determine whether urinary biomarker concentrations can differentiate between cases of active TB infection, latent TB infection (LTBI) and un-infected healthy controls.** The *working hypothesis* is that urinary biomarker concentrations will be increased in cases of LTBI, defined as having a positive interferon-gamma release essay (IGRA), as compared to healthy controls, but decreased when compared to cases of active TB.

To date we have completed mass spectrometry and statistical analysis on all participant urine samples described in Aim 1. We have completed preparation for all participant urine samples detailed in Aim 2 and should complete all mass spectrometry and statistical analysis in the next 2 months.

The statement of Work is as follows:

STATEMENT OF WORK – February 12, 2018
PROPOSED START DATE Aug 01, 2018

Site 1: Weill Cornell Medicine
413 East 69th Street
PI: Flonza Isa

Site 2: N/A

Specific Aim 1: Determine metabolite concentrations in participants being treated for active TB over time	**Timeline**	**Site 1**	**Status**
Major Task 1: Prepare and test samples on HPLC-MS	Months		
Subtask 1 Prepare samples	2	Dr. Isa	Completed 11.1.18
Subtask 2 Test samples on HPLC	4	Dr. Isa	Completed 12.1.19
Milestone(s) Achieved	*6*	Dr. Isa	Yes
Major Task 2: Analyze metabolite concentrations in TB treated participants over time, subgroup analysis			
Subtask 1 Perform quality control and repeat sample testing	3	Dr. Isa (37 participants being treated for TB over 52 weeks)	Completed 1.30.19
Subtask 2 Analyze metabolite abundances for sialic acid, kynurenine, N-acetylhexosamine and unknown-mass 240 and perform repeated measures ANOVA and linear mixed modeling.	1	Dr. Isa	Completed 2.30.19
Milestone(s) Achieved:	*4*	Dr. Isa	Yes
Specific Aim 2 Determine metabolite concentrations in active TB, LTBI and IGRA negative controls			
Major Task 3: Prepare and test samples on HPLC-MS			
Subtask 1 Prepare samples	5	Dr. Isa	Completed 4.1.19
Subtask 2 Test samples on HPLC	7	Dr. Isa	100 completed 5.1.19, 200 will be completed 1 month
Milestone(s) Achieved: All samples prepared and tested	*12*	Dr. Isa	To be completed

Major Task 4: Analyze metabolite concentrations within each group, subgroup analysis			
Subtask 1 Perform quality control and repeat sample testing	5	Dr. Isa (100 participants with active TB, 100 IGRA+ healthy controls, 100 IGRA- healthy controls.)	100 completed 5.30.19, 200 will be completed 1-2 month
Subtask 2 Analyze metabolite abundances for diacetylspermine, neopterin, sialic acid and N-acetylhexosamine and perform one-way ANOVA analysis and linear regression.	1	Dr. Isa	To be completed
Milestone(s) Achieved: All analysis complete	*6*	Dr. Isa	To be completed
Manuscript writing/preparation/submission	1	Dr. Isa	Manuscript describing results from Aim 1 completed. Manuscript describing results from Aim 2 to be completed in next 2-6 months.
Local IRB/IACUC Approval	3	Dr. Isa	
Milestone Achieved: HRPO/ACURO Approval	6		

- **What was accomplished under these goals?**

*the following is unpublished data

For Aim 1 the specific objectives were to quantify urinary metabolite levels in participant who were cured of TB to identify potential markers of treatment response. To date, we have completed experiments and analysis for longitudinal urine samples from 37 participants taken at the time of diagnosis and at week 2, 4, 8, 17, 26 and 52 after initiation of anti-tuberculosis therapy. Samples were grouped by participant and then randomized. Each urine sample was normalized to an osmolality of 150 mOsm and mixed with methanol 0.2% formic acid in a 1:1 mixture and then analyzed using an Agilent 6230 TOF LC/MS (Liquid chromatography–mass spectrometry), and Profinder B08 and Qualitative Analysis bio-informatic pipeline. All 10 metabolites tested showed a significant decrease during the course of TB treatment (figure 1). Kynurenine and diacetylspermine are significantly decreased at 2 weeks after the start of treatment. Each metabolite was significantly correlated with sputum AFB microscopy score (figure 2) and some metabolites were significantly higher in participants with high initial sputum AFB score when compared to low AFB score.(figure 3). All metabolite abundances were normalized to creatinine concentrations and adjusted for age sex and participant weight.

These findings suggest that several urinary metabolites can be useful as prognostic markers of tuberculosis treatment response, even as early as 2 weeks after the start of treatment. As many of these metabolites are known inflammatory intermediates, these findings need to be validated in a cohort of treatment responders and non-responders to confirm their ability to identify those at risk for treatment failure.

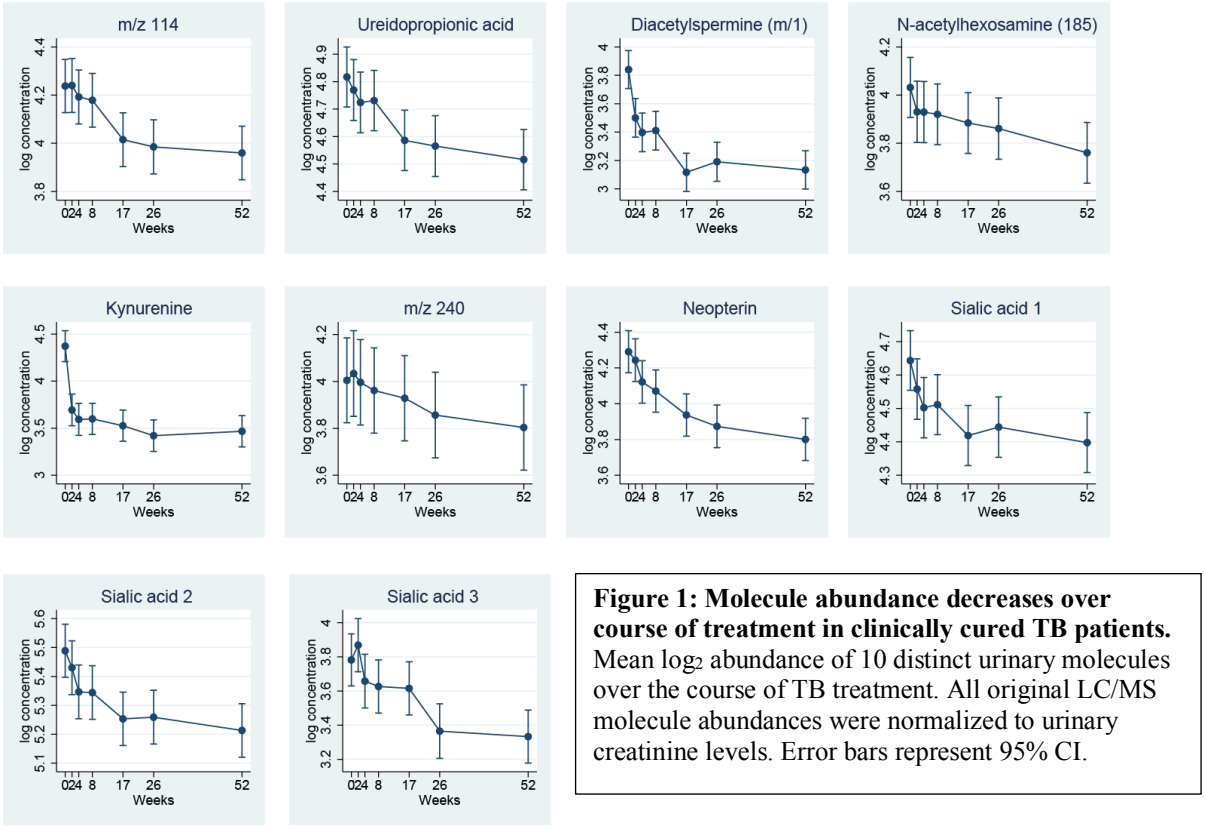


Figure 1: Molecule abundance decreases over course of treatment in clinically cured TB patients. Mean $\log_2$ abundance of 10 distinct urinary molecules over the course of TB treatment. All original LC/MS molecule abundances were normalized to urinary creatinine levels. Error bars represent 95% CI.

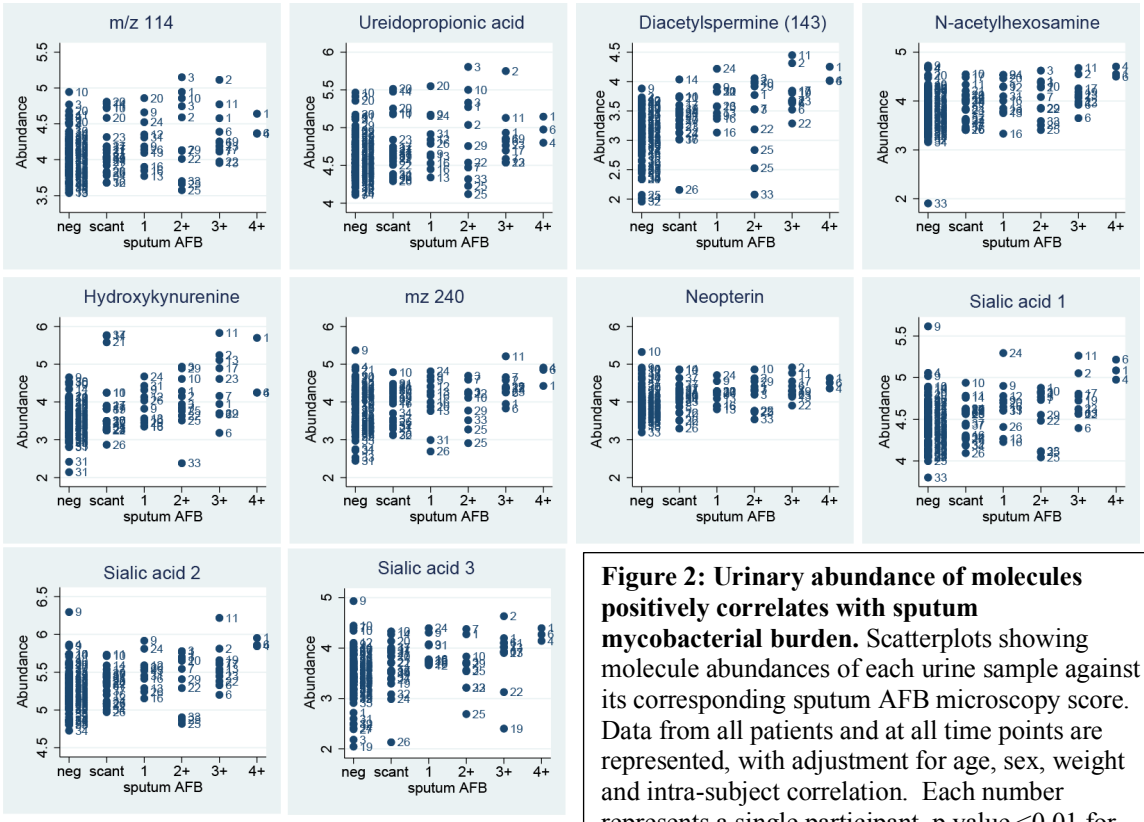
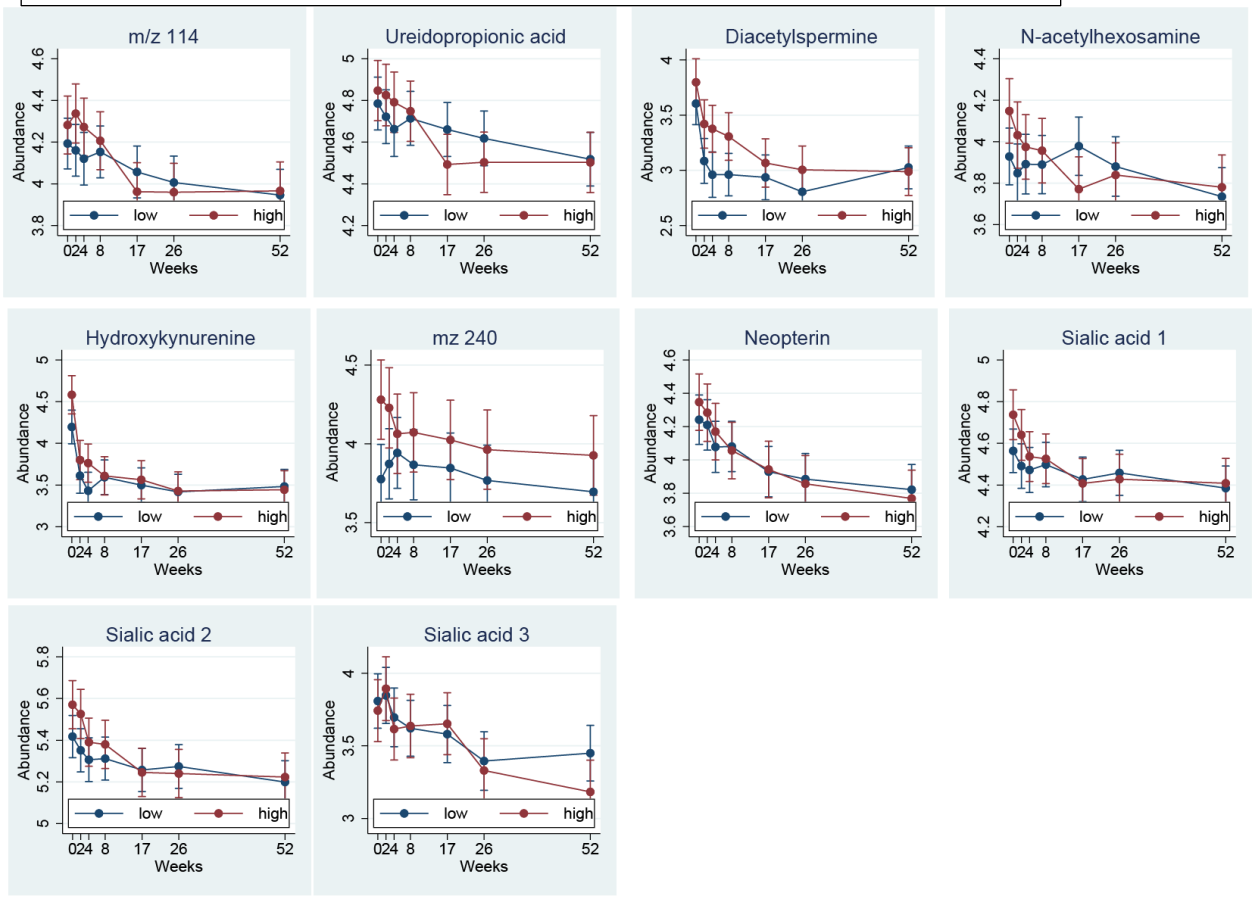


Figure 2: Urinary abundance of molecules positively correlates with sputum mycobacterial burden. Scatterplots showing molecule abundances of each urine sample against its corresponding sputum AFB microscopy score. Data from all patients and at all time points are represented, with adjustment for age, sex, weight and intra-subject correlation. Each number represents a single participant. p value <0.01 for all molecules.

Figure 3: Mean molecule abundance is higher in urine of TB patients with high initial sputum mycobacterial load. Participants were separated by sputum AFB smear score at time of diagnosis (week 0). Initial AFB score of 3+ or 4+ were categorized as "high sputum load"; initial AFB score of 2+ or lower were categorized as "low sputum load". Error bars represent 95% CI, $\log_2$ scale. All samples normalized to creatinine and adjusted for age, sex and weight.



The goals for Aim 2 are to study urinary metabolite levels in 100 participants with active TB, 100 participants with latent TB infection and 100 uninfected controls. To date, all the samples are prepped and 100 randomized samples have been run on the Agilent 6230 TOF LC/MS as described above. Preliminary analysis of this data set suggests that several of these metabolites may be increased in participants with HIV-TB co-infection.

We plan to complete the LC-MS and statistical analysis of these samples in the next 2 months.

- **What opportunities for training and professional development has the project provided?**
    - Nothing to report
- **How were the results disseminated to communities of interest?**

    - We plant to disseminate the findings to the community by 1) publishing in peer reviewed medical journals and 2) presenting the results at international conferences and meetings. To meet these objectives we have already tested, analyzed and prepared a manuscript describing the longitudinal treatment response cohort. We plan to submit this manuscript for publication in a peer reviewed journal in the next month. Additionally, we have been invited to speak about our findings at the international infectious diseases conference IDWeek 2019 this October.

- **What do you plan to do during the next reporting period to accomplish the goals?**

    - During the next 6 months of the discovery award we plan to complete the analysis for the 300 participant urine samples with active TB, latent TB and un-infected controls as described in aim 2. The mass spectrometry analysis will be complete in the next month. The statistical analysis and subgroup analysis should be completed in the next 1-2 months. Follow on experiments and manuscript preparation should be completed in the next 2-6 months.

4. **IMPACT:**

- As a result of this project we have identified potential early markers of TB treatment response. The current WHO recommendations state that TB treatment failure can only be evaluated after continued sputum positivity at 2-3 months after starting treatment. During these crucial months, a person is on ineffective or partially effective treatment, all the while being exposed to drug toxicity, increasing their risk for developing drug resistance and continuing to spread disease. An early marker of treatment efficacy could dramatically alter how TB is treated by identifying treatment failures as early as 2 weeks after starting therapy. Early identification of treatment failures would not only help individual patients, but could also help identify drug resistance, and decrease overall infectivity and thus TB incidence.

- **What was the impact on the development of the principal discipline(s) of the project?**
    - The ten metabolites being tested are thought to be immune intermediates. In addition to their potential as diagnostic and prognostic biomarker of TB, these metabolites could highlight previously unknown mechanisms of disease. As these biomarkers were identified using untargeted metabolomics, several of them have not been previously identified as associated with TB or host immunity. Diacetylspermine, specifically has not been previously associated with TB immunopathogensis. Further studying this molecule and its role in TB-

host interaction could give new insight into novel pathways important in our immune response to TB infection.

- **What was the impact on other disciplines?**
  - Nothing to report
- **What was the impact on technology transfer?**
  - Nothing to report
    - .
- **What was the impact on society beyond science and technology?**
  - The aim of this project is to validate urine biomarkers of TB. If validated this would be one step closer to developing a urine point-of-care test for diagnosing TB or monitoring response to therapy. A urine point of care test for TB would dramatically change how TB is diagnosed and/or treated around the world and especially in resource limited settings, where disease incidence is high, or in difficult to diagnose populations, such as children or extrapulmonary disease.

5. **CHANGES/PROBLEMS:**
   - **Changes in approach and reasons for change**
     - Nothing to report
   - **resolve them**
     - Nothing to report
   - **Changes that had a significant impact on expenditures**
     - Nothing to report. There have been no changes that significantly impact expenditures.
   - **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
     - Nothing to report. There have been no significant changes in the use or care of human subjects, vertebrate animals, biohazards or select agents.
   - **Significant changes in use or care of human subjects**
   - **Significant changes in use or care of vertebrate animals.**
   - **Significant changes in use of biohazards and/or select agents**
6. **PRODUCTS:**
   - **Publications, conference papers, and presentations**
     - **Journal publications.** Nothing to report
     - **Books or other non-periodical, one-time publications.** Nothing to report

- **Other publications, conference papers, and presentations.** Results from this work have been presented at the regional and national Tuberculosis Research Unit meetings and will be presented at the international infectious disease conference IDWeek in Oct 2019.

- **Website(s) or other Internet site(s)**
  Nothing to report. There are no internet sites that disseminate the results of this research.

- **Technologies or techniques**
  Nothing to report. There have been no technologies or techniques that resulted from the research activities to date.

- **Inventions, patent applications, and/or licenses**
  Nothing to report. No inventions, patent applications and/or licenses have resulted from this project.

- **Other Products**

  - data or databases; The metabolomics data generated from the participant urine samples has contributed a urine metabolomics database within the Rhee/Isa lab.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**

Name:	Flonza Isa
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	9
Contribution to Project:	Dr. Isa has performed the sample preparation, metabolomic analysis, statistical analysis and manuscript preparation.
Funding Support:	
Name:	Kyu Rhee MD PhD
Project Role:	Co-investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.6
Contribution to Project:	Dr. Rhee has provided valuable scientific insight and expertise.
Funding Support:	NIH, Gates foundation
Name:	Daniel Fitzgerald
Project Role:	Co-investigator
Researcher Identifier (e.g. ORCID ID):	

Nearest person month worked:	0.12
Contribution to Project:	Dr. Fitzgerald has provided valuable scientific insight and expertise.
Funding Support:	NIH, Fogerty
Name:	Qianjing Xia
Project Role:	Medical Student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	Jenny aided in sample preparation and analysis.
Funding Support:	NIH

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  - Nothing to Report
- **What other organizations were involved as partners?**
  - Nothing to Report

8. **SPECIAL REPORTING REQUIREMENTS**
   - **COLLABORATIVE AWARDS:** N/A
   - **QUAD CHARTS:** N/A
9. **APPENDICES:** N/A