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A Grant Proposal to Evaluate the Effect Antibiotic TB Treatment has on the Gut Microbiota and on Metabolic Functions of Pediatric TB Patients in Dekalb County

Oluwatobiloba Adeola Akingbade
Georgia State University

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ABSTRACT

A Grant Proposal to Evaluate the Effect Antibiotic TB Treatment has on the Gut Microbiota and on Metabolic Functions of Pediatric TB Patients in Dekalb County

By

Oluwatobiloba Akingbade

July 28, 2017

INTRODUCTION: This capstone project is modeled after a National Institutes of Health R21 grant application to evaluate the relationship between tuberculosis (TB) antibiotic treatment and 1) gut microbiota and 2) long-term metabolic functions among pediatric patients less than five years old or greater than five years old, residing in Dekalb county.

AIM: The proposed study specific aims are to 1) determine the relationship between standard drug-sensitive antibiotic TB regimens on the gut microbiota (total density and taxa) at the time of TB treatment completion and one-year after treatment completion; 2) determine the relationship between standard drug-sensitive antibiotic TB regimens and metabolic biomarkers (Short Chain Fatty Acids (SCFAs), amino acids (Branched Chain and Aromatic), cholesterol, and glucose) at the time of treatment and one-year follow-up.

SIGNIFICANCE: In the last ten years, antibiotic use has increased substantially, correlating with the increase incidence of childhood obesity and diabetes. This trend may partially be explained by an association between broad-spectrum antibiotic usage during childhood and the dysbiosis of the gut microbiome. The disruption of the gut microbiota induces the dysregulation of metabolic pathways, which may lead to the increased risk of developing obesity and diabetes. In 2015, TB accounted for 1 million incident cases in children. Given antibiotic treatment for TB requires exposure to multiple antibiotics for more than 6 consecutive months, this proposal intends to understand the extent to which exposure to TB treatment may impact the gut microbiome and whether antibiotic induced dysbiosis in the gut microbiome has long-term impact on pediatric metabolic function.

APPROACH: Eligibility criteria includes all TB pediatric patients (< 15 years of age) residing in DeKalb county, receiving care for drug-susceptible pulmonary TB by the DeKalb Refugee Clinic within the Dekalb County Board of Health. Patients with previous history of diabetes, obesity, or HIV will be excluded.

STUDY DESIGN: We will perform a prospective cohort study of N=50 pulmonary drug-susceptible pediatric TB patients from 2018-2020. At the time of baseline, TB treatment completion, and one-year follow-up the primary measures collected will be total density and taxa distribution of the gut microbiome. We will also collect SCFA, branched chain and aromatic amino acids, fasting glucose, A1c, and fasting cholesterol. Baseline measures will act as an internal comparison group for each patient.

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By

Oluwatobiloba Akingbade

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APPROVAL PAGE

A Grant Proposal to Evaluate the Effect Antibiotic TB Treatment has on the Gut Microbiota and on
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By

Oluwatobiloba Akingbade

Approved:

Dr. Matthew Magee, PHD, MPH, Assistant Professor
Committee Chair

Dr. Alawode Oladele, MD, MPH
Committee Member

July 24, 2017

Date

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Author's Statement Page

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SPECIFIC AIMS

In 2015, tuberculosis (TB) in children accounted for 1 million incident cases with 210,000 deaths worldwide¹. With the recently improved recommendations for antibiotic TB drug dosages for children² and the creation of solid fixed dose combination (FDC) child-friendly formulated TB drugs¹, adherence and completion of antibiotic TB treatment will increase. Subsequently, since treatment can take anywhere from 6 months to 24 months³, active pediatric TB patients are exposed to prolonged antibiotic TB treatment during crucial developmental stages. This research will determine the extent to which antibiotic TB treatment affects the gut microbiome.

Currently, little is known about the impact antibiotic TB treatment has on the gut microbiome of pediatric TB patients. Studies on mice have found that broad-spectrum antibiotic use disrupts the development of early life gut microbiota causing biodiversity loss (i.e. loss of bacteria strains and a decrease of overall bacteria abundance)^{4,5}. Biodiversity loss impacts the metabolic functions of the gut which in turn increases adipose tissue and the risk of obesity^{6,7}. This proposal will focus on understanding the relationship between antibiotic TB treatment and four metabolic biomarkers: Short Chain Fatty Acids (SCFA's), Amino Acids (Branched Chain and Aromatic), cholesterol, and glucose. The overarching goal of this study is to understand the impact antibiotic TB treatment has on the gut microbiome and on metabolic functions.

Human studies reveal that infants who received antibiotics had a reduced frequency of phylum Firmicutes and a higher frequency of phylum Proteobacteria, two of the eight bacterial divisions in the gut, than infants who did not receive any antibiotics⁸. SCFAs are bacterial waste products produced from two phyla's, Bacteroides and Firmicutes. Since the gut dysbiosis, induced by antibiotics, decreases the occurrence of Firmicutes, a production of SCFAs will also decrease⁹. This leads to a decline in overall health since SCFAs regulate the homeostasis of various metabolic pathways, such as cholesterol and glucose, which are associated with the protection and improvement of type 2 diabetes^{10,11}. Subsequently, phyla Proteobacteria and Firmicutes are gut bacteria that produce the most abundant amount of amino acids¹². The gut microbiota as a whole, through fermentation of complex carbohydrates, increases the specific production of circulating Branch Chain Amino Acids (BCAA) and Aromatic Amino Acids (AAA)¹³. Currently, it is known that BCAA and AAA play a role in glucose homeostasis and have been identified as predictors for developing diabetes¹⁴.

This study hypothesizes that culture confirmed pediatric patients (<5 years old), compared to pediatric patients (>5 years to 15 years old), who undergo 6 months of antibiotic TB treatment will see a decline in gut biodiversity (total density, and taxa distribution) and irregular levels of SCFA, amino acids, cholesterol, and glucose one year after antibiotic TB completion. In 2015, DeKalb's Health District had the highest TB case rates (7.9 per 100,000) in Georgia¹⁵, making it a suitable setting to evaluate the proposed hypothesis. The study will be conducted in one of Georgia's well-established TB clinic, the DeKalb Refugee Health Clinic in Decatur, Georgia. We will enroll culture confirmed newly diagnosed TB (n=50) pediatric patients (who are <5 [n=25] or >5 years [n=25]) and collect measures of biodiversity (total density and taxa distribution) of the gut microbiota, levels of SCFA's, amino acids, cholesterol, and glucose before, at TB treatment completion, and at one-year follow-up.

Aim 1: Determine the relationship and impact of antibiotic TB treatment on the gut microbiota (total density and taxa distribution) in pediatric patients (<5 and >5 years) at the time of TB treatment completion, and at one-year follow-up.

Hypothesis 1: Pediatric patients <5 years old, compared to patients >5 to 15 years old, will 1a) have reduced gut biodiversity (total density and taxa distribution) at the time of TB treatment completion and 1b) persistent gut dysbiosis one year after TB treatment completion.

Aim 2: Determine the relationship between antibiotic TB treatment and metabolic biomarkers (SCFA's, amino acids, cholesterol, and glucose) at the time of treatment and one-year follow-up.

Hypothesis 2: Pediatric patients <5 years old, compared to patients >5 to 15 years old, will 2a) have irregular levels of SCFA's, amino acids, cholesterol, and glucose at the time of TB treatment completion and 2b) one year after TB treatment completion.

A. SIGNIFICANCE

The global burden of infectious diseases, such as tuberculosis, has driven an unanticipated rise in irrational antibiotic use^{16,17}. With the global rise in non-communicable diseases, such as obesity and diabetes¹⁸, this intersection may be explained by the dysbiosis of the gut microbiota. The global estimated incidence of childhood TB has increased from 663,990 in 1990 to 1 million in 2014¹⁹. Due to the financial increase of developing countries and the unregulated over the counter trade of antibiotics, individuals have improved access to antibiotics without any medical consultation or written prescription^{16, 20, 21}. Between 2000 and 2010, antibiotic use increased 35% worldwide²², and within pediatric patients pre-school aged children had a 72% higher prevalence of antibiotic use than school age children and adolescents²³. Subsequently, during the same time, the global prevalence of childhood obesity increased from 4.2% in 1990 to 6.7% in 2010 with about 81% of these individuals residing in developing countries^{24, 25}. Although, for over a century epidemiological studies have focused on the effect diabetes mellitus (DM) has on TB susceptibility and have concluded that DM increases the risk and severity of TB²⁶, there is an alternative hypothesis that could better explain these trends. Numerous studies have shown an association between antibiotic use and the alteration in the gut microbiota^{4, 6} which influences the development of type 2 diabetes²⁷. This proposal intends to explore whether antibiotic TB treatment alters the gut microbiome, which may consequently increase the risk of non-communicable diseases such as obesity and diabetes in children.

The duration and the amount of dosages taken during antibiotic TB treatment in pediatric patients is a cause for concern on its effects on the gut microbiota and on what that implies for the future of pediatric TB treatment. Antibiotic TB treatment is divided into two phases. The first phase, called the intensive phase, lasts about two months and the second phase, called the continuous phase, lasts either four to seven months²⁸⁻³⁰. The purpose of the intensive phase is to kill off most of the TB bacteria as quickly as possible, which is why more antibiotic drug combinations are utilized in this phase than the continuous phase^{29, 30}. The continuous phase then intends to eliminate any dormant TB bacteria^{29, 30}. The recommended regimen for pediatric patients newly diagnosed with pulmonary drug-susceptible TB is a combination of 50 mg of isoniazid (INH), 75 mg of rifampin (RIF), and 150 mg of pyrazinamide (PZA) for seven or five days a week during the intensive phase²⁹. During the continuous phase, a combination of 50 mg of INH and 75 mg of RIF is taken²⁹. This regimen is about 182 to 130 dosages for the duration of the TB treatment and is only utilized for drug susceptible TB²⁸. Other forms of TB, like TB meningitis, add more antibiotics to the regimen²⁸⁻³⁰. Currently, little is known about the effects of consuming this amount of antibiotic dosages for TB has on the gut microbiota. If TB treatment does significantly cause the dysbiosis of the gut microbiota science may need to 1) create treatments, like probiotics, that are implemented after treatment to bring the gut microbiota to its proper equilibrium and/or 2) the transplantation of the gut microbiota from one healthy individual to an individual who endured TB treatment. As for now, by deducing from studies on broad spectrum antibiotic use, this proposal intends to conceive how antibiotic TB treatment causes dysbiosis in the gut microbiota.

The colonization and configuration of an infant's gut microbiota are important for its overall health. The introduction of antibiotic treatment, even fleeting, can cause metabolic perturbations leading to increased adipose tissue⁴, and early childhood obesity³¹. Upon delivery, most neonates are colonized by maternal bacteria through the skin, vaginal tract, and feces³². This colonization molds the compositions of the infants gut microbiota³³ which increases in richness and diversity to adult-associated configuration by the age of 3 years³⁴. The adult composition of the gut microbiota consists of the highest bacteria cell density of any ecosystem recorded but the lowest diversity of 8 bacterial divisions, with the dominant phyla Firmicutes, Bacteroides, Actinobacteria, and Verrucomicrobia^{35, 36}. The gut microbiota is critical in regulating the permeability of the gastro-intestinal mucosa lining, which is important in preventing the colonization of pathogens. It is also important in the synthesis of amino acids, production of vitamins, and the fermentation and absorption of dietary polysaccharides³⁷. Since infants are still developing their gut microbiota, their ecosystem is dependent on their diet and environment,³⁸ which is why any form of antibiotic use, especially for the duration in which antibiotic TB treatments are applied, can cause damage to the equilibrium of the gut microbiota and lead to the health decline of the host. Various human studies have shown that infants who were given broad spectrum antibiotics had a reduction of phyla Actinobacteria and Firmicutes, and were dominated by phylum Proteobacteria even eight weeks after antibiotic treatment^{39, 40}. Studies agree that the gut microbiota 1) promotes the fermentation of indigestible polysaccharides into SCFA^{35, 41} and 2) at persistent dysbiosis increases levels of amino acids⁴², consequently leading to the production of body fat and insulin resistance⁴¹.

Scientific Premise: The bacterial waste products produced during colonic fermentation⁴³, SCFA, can potentially protect against diet induced obesity¹¹ and diabetes⁴⁴. SCFA consist of acetate, propionate, and butyrate, and are produced by Bacteroides (acetate and propionate producing organism) and Firmicutes (butyrate producing organisms)^{43, 45}. Their main role is to be a source of energy for colonic epithelial cells, which receive 60-70% of their energy from mostly Butyrate⁴⁶. SCFAs, acetate, and butyrate⁴⁶, regulate the metabolism of fatty acids by activating fatty acid oxidation and inhibiting lipolysis. This consequently leads to the reduction of free fatty acids and the body weight of an individual⁴⁷. SCFAs also regulate the metabolism of glucose and lowers the production of plasma glucose levels in the blood. Propionate and acetate regulate cholesterol metabolism and lower cholesterol plasma and serum concentrations respectively⁴⁷. In a human study by Yamashiro et al. reduced levels of the phylum Firmicutes, acetate, and propionate was associated with higher levels of HbA1c and LDL cholesterol⁴⁸. These findings implicate that the decrease production of SCFA could potentially lead to hyperglycemia and hyperlipidemia, which could predispose individuals to Type 2 diabetes⁴⁶.

The gut microbiota also produces amino acids from energy created by the fermentation of dietary carbohydrates¹³ particularly those of Branch-chain (BCAA) (isoleucine, leucine, and valine) and aromatic amino acids (AAA) (tyrosine, and phenylalanine) which have been shown to be predictive of obesity and diabetes^{12, 49}. Antibiotic induced dysbiosis of the gut microbiota have shown to increase phylum proteobacteria³⁹ and phylum proteobacteria is one of two phyla's that produce the most abundant amount of amino acids¹², consequently increasing circulating amino acids. Amino acids aid with glucose homeostasis by controlling insulin and glucose secretion¹⁴. A study conducted by Wang et al. followed non-diabetic individuals for a 12-year period and discovered that all cases who had high concentrations of either isoleucine, leucine, valine, tyrosine, and phenylalanine, developed diabetes. Likewise, individuals who had a combined increased circulating concentration of Isoleucine, tyrosine, and phenylalanine had a 5- to 7-fold higher risk of developing diabetes⁴⁹. A proposed mechanism by Krebs et al. found that increased concentration of amino acids induces insulin resistance by impairing glucose transport and/or phosphorylation activity⁵⁰. The defect in glucose transport and/or phosphorylation activity would then impair glycogen synthesis and lead to obesity⁵¹ and type 2 diabetes⁵². Another possible mechanism by which increased concentrations of amino acids promote obesity and type 2 diabetes is through insulin secretion⁴⁹. Studies infusing BCAA into healthy human subjects decreases insulin sensitivity and studies depriving BCAA in animal models improved insulin sensitivity¹⁴. Through the chronic elevation of amino acids, individuals could develop hyperinsulinism causing pancreatic beta-cell failure^{49, 53}.

B. Innovation

1). **Clinical trials focusing on tuberculosis treatment and its effects on the gut microbiota have not been previously conducted.** Our proposal is unique because we aim to gain a better understanding of the long-term health implications of TB treatment. Currently, there has only been one study that examines the relationship between TB and gut microbiota. It focuses on how the alteration of the gut microbiota can cause TB⁵⁴. Our study will bring insight into the composition of gut microbiota in TB burdened individuals, negative consequences of TB treatment on bacterial phyla's, and the long-term effects of TB treatment on gut microbiota.

2). **Currently, epidemiological and clinical studies are focused on TB susceptibility of Diabetes Mellitus (DM) patients²⁶.** Our studies hypothesis chooses to break from current consensus of thinking and prospectively follow individuals through a cohort study to observe if and/or how TB treatment leads to the increased risk of diabetes. This approach will give our study a unique understanding of the time frame in which individuals would need medical intervention to rectify the effects of TB treatment on their risk of diabetes. This approach will be executed by detecting metabolic biomarkers that are indicative of obesity.

C. APPROACH

C1. Overall goal: The primary motivation for this R21 proposal is to evaluate the hypothesis that antibiotic TB treatment in pediatric patients (children <5 years) causes the dysbiosis of gut microbiota by disrupting total density, and taxa distribution. We will also evaluate the hypothesis that the dysbiosis of gut microbiota leads to a disruption of metabolic functions which could be indicative of increased risk of obesity and diabetes in the future.

C2. Study Design Overview: We will conduct a prospective cohort study at the Dekalb County Board of Health consisting of two groups: 1) pediatric patients less than five years old successfully completing drug susceptible TB treatment, and 2) pediatric patients greater than five to fifteen years old successfully completing drug susceptible TB treatment. SCFA, fasting BCAA and AAA, fasting glucose levels, A1c, and fasting total cholesterol levels will be measured at the time of enrollment, at the time of TB treatment completion (6 months), and one year after TB treatment completion (18 months) (**Figure 1**).

Figure 1: Study Overview Timeline



C3. Study Team: We have gathered a strong team of clinicians with a notable background in epidemiologic research addressing TB and/or the intersection between TB and non-communicable disease. Dr. Matthew Magee (PI) is an Assistant Professor of Epidemiology and Biostatistics at Georgia State University and has countless work with the Dekalb Board of Health. Dr. Alawode Oladele (Co-PI) is the medical director of county-wide services for Dekalb County Board of Health. A clinical physician practicing family medicine in Decatur, Georgia, who works closely with the Dekalb Refugee Clinic.

C.4 Participant Eligibility: All study participants will be pediatric patients (<15). Patients will be grouped by age brackets (<5 years or >5 to 15 years), reside in Dekalb county in Georgia with no history of HIV and diabetes. Eligibility criteria will be the same for the two study groups. All participants must have drug susceptible TB: culture confirmed pediatric TB patients who are susceptible to isoniazid or rifampin antibiotics at the time of TB diagnosis would be eligible for enrollment (see section C6 for enrollment details).

C5. Feasibility: This study will work closely in collaboration with the Dekalb refugee Health Clinic which evaluates ten new families (2500 adults and children) every week with about 40% of these individual's younger than the age of 18. The refugee health clinic provides health and mental screenings, immunizations, and follow-up and management services for tuberculosis. Dekalb County Board of Health supports a pediatric clinic as an extension of the Refugee Health Program which provides refugee children medical treatments referrals. Since most pediatric patients who are suffering from TB are just resettling from a different country, the Dekalb County Board of Health obtains medical information and assessments. Blood, urine, and stool samples are collected to screen for metabolic diseases, nutritional status, and infectious diseases.

C6. Participant enrollment: All drug-susceptible pediatric TB patients (<15 years old) who are enrolled in the TB clinic within the Dekalb Board of Health will have any clinical, laboratory, and hospital records reviewed for history of HIV, TB, and diabetes. Eligible participants will then be approached to enroll before the start of antibiotic TB treatment.

C7. Primary study measures: All primary study measures will be evaluated before, at time of TB treatment completion, and one year after TB treatment with added information from clinical evaluations and questionnaires. Drug Susceptible TB: Pediatric patients whose TB disease is easily treatable by isoniazid or rifampin antibiotics at the time of diagnosis. Patients will either be culture confirmed using BACTEC-MGIT or genetically confirmed using GeneXpert assay to measure drug susceptibility. During the initial time of enrollment, families will be approached for consent by study team members. Instructions for stool sample collection and supplies will be explained and delivered to parents at this time. Subsequent stool samples will be collected during clinic follow-up appointments. After stool samples are collected, they will be sent to the Institute of Biomedical Sciences at Georgia State University. Samples will be transported in plastic bags containing a disposable oxygen-absorbing and carbon dioxide-generating agent inside a container kept at 4°C. This will allow anaerobes sensitive to oxygen a chance to survive the trip to the laboratory⁵⁵. The gut

microbiota: Once stool samples are transported to a laboratory they will be divided in two. One-half of the sample will undergo nucleic acid extraction, using AllPrep PowerFecal DNA/RNA extraction kits (Qiagen) for 16S rRNA sequencing (microbiome assay to characterize the composition of the gut microbiota)⁵⁶. 16S rRNA will be generated and sequenced by the Georgia Genomics Facility at University of Georgia. 16s libraries will be generated by using V3 – V5 primer regions. For data analysis purposes, the Shannon-Wiener diversity index and the amount of 16s rRNA genes belonging to phyla's Proteobacteria, Actinobacteria and Firmicutes will be enumerated. **SCFA, BCAA, and AAA:** The second half of the stool sample will be used to derivatize metabolites with 100ul of propyl chloroformate in a reaction system of water/propanol/pyridine at pH 8.0 before a two-step extraction with hexane. Analysis of derivative samples will then be performed using an Agilent 7890A gas chromatography system coupled to an Agilent 5975C inert XL EI/CI mass spectrometric detector (GC-MS)(MSD, Agilent Technologies, Santa Clara, CA)⁴². Any remaining stool sample material and/or nucleic acid extracts will be frozen at 80°C and stored in the laboratory for future ongoing studies. **Fasting glucose, fasting total cholesterol, and A1C:** At the time of enrollment and during clinic follow-up appointments, blood samples will be collected from each individual. A point of care diagnostic device, Alere Cholestech LDX® Analyzer (Waltham, USA), will be used to obtain fasting glucose and fasting total cholesterol levels. DCA Vantage® Analyzer (Siemens, USA) will be used to obtain A1c levels. According to the American Diabetes Association, guidelines for: 1) Fasting glucose are <100mg/dl normal, 100 -120 mg/dl prediabetes, and ≥126 mg/dl diabetes and 2) A1c are <5.7% is normal, 5.7% - 6.4% is prediabetes, and ≥6.5% is diabetes⁵⁷. Individuals who have fasting glucose levels ≥100mg/dl or A1c levels ≥5.7% will be categorized as hyperglycemic. According to the National Cholesterol Education Program, guidelines for total cholesterol are: <200 mg/dl is desirable, 200 -239 mg/dl is borderline high, and ≥240 mg/dl is high⁵⁸. Individuals who either have borderline high or high will be categorized as hyperlipidemic.

C8. Key covariates: Study team members will conduct questionnaires and extract information from medical records. Enrolled patients will be able to divulge information on previous demographic characteristics, diet, and cigarette exposure. Key covariates, shown in Table 1, will be collected at the time of enrollment, at the time of TB treatment completion, and one year after TB treatment completion (**Table 1**).

Table 1. Key study measures, measurement methods and timeline		
Measure	Method	Study Month†
Gut biodiversity (total density and taxa distribution)	GC-MS)(Agilent)	0,6,18
SCFA (acetate, propionate, and butyrate)	GC-MS (Agilent)	0, 6, 18
(BCAA) (isoleucine, leucine, and valine)	GC-MS (Agilent)	0, 6, 18
(AAA) (tyrosine, and phenylalanine)	GC-MS (Agilent)	0, 6, 18
Fasting Glucose	FPG (Cholestech)	0, 6, 18
A1c	HbA1c (Vantage)	0, 6, 18
Fasting Cholesterol	Total Cholesterol (Cholestech)	0, 6, 18
Smoking Exposure	Self-report	0,18
Country of Origin	Self-report	0
Age at entrance into U.S.	Self-report	0
Age at enrollment	Self-report	0
Gender	Self-report	0
TBF	Height/ Weight measurement	0, 6, 18
Previous Antibiotic use	Self-report & Medical Chart Abstraction	Pre Study*
HIV status	Self-report & Medical Chart Abstraction	Pre Study*
Previous Diabetes	Self-report & Medical Chart Abstraction	Pre Study*
History of TB	Self-report & Medical Chart Abstraction	Pre Study*

† 0 indicates time of study enrollment which is time of TB diagnosis.

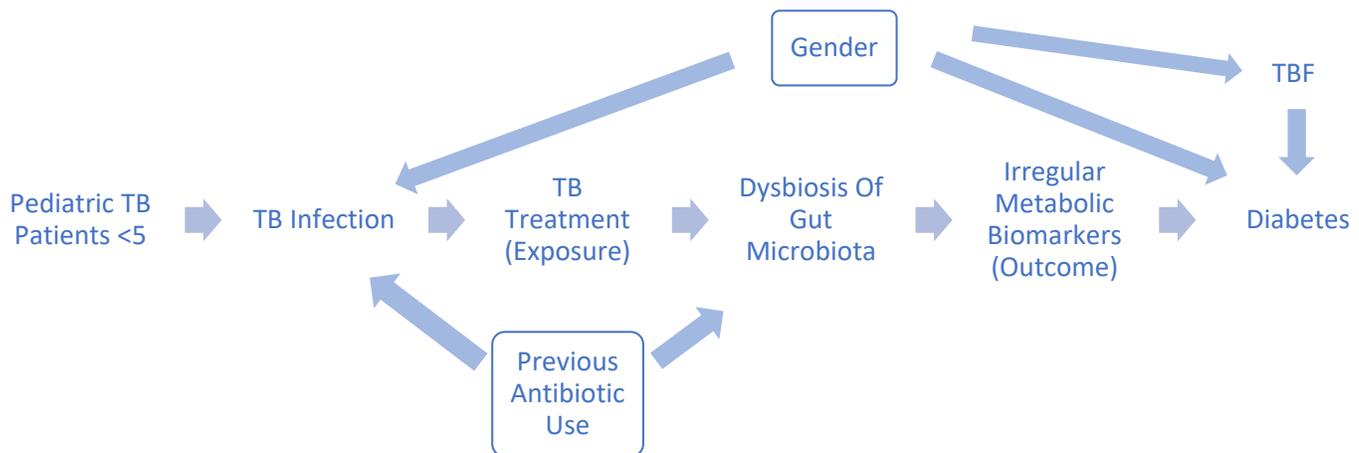
Abbreviations: SCFA – short chain fatty acid, BCAA – branch chain amino acids, AAA – aromatic amino acids, TBF – Total Body Fat. *Pre-study means that measurements will be collected from medical records before TB treatment.

C9. Data analysis: Aim 1 To determine the association between TB treatment (categorized as 2-level categorical variables, pediatric patients <5 years old and >5 to 15 years old) and median levels of gut

biodiversity (total density and taxa distribution) at time of 1a) TB treatment completion (6 months), and 1b) one year after TB treatment completion. Two linear regression models will be used to measure the association between the two comparison groups (age of pediatric patients) on median levels of gut biodiversity (on total density and on taxa distribution) at the time of TB treatment completion and one-year after TB treatment completion. The F-test and R-squared will be reported to assess if age predicts dysbiosis and how much variance in dysbiosis can be accounted by age. A t-test will be conducted to determine the significance of age. A beta coefficient will be used to determine magnitude and direction of the relationship between age and gut dysbiosis. We will also examine the relationship between the two comparison groups, on total density and taxa distribution, controlling for three covariates (smoking exposure, country of origin, and age at entrance) by using two multiple linear regression models. We will test total body fat (TBF) as an interaction variable due to the possibility that higher TBF induces undiagnosed prediabetes, which may contribute to an increased chance of developing diabetes after TB treatment (**Figure 2**). We will also control for confounding variables, previous history of antibiotic use and sex. Individuals history of previous broad-spectrum antibiotic usage may induce gut dysbiosis before study enrollment and males tend to have a higher prevalence of metabolic and intestinal inflammatory diseases compared to women⁵⁹. Aim 2 To determine the association between TB treatment (categorized as 2-level categorical variable, pediatric patients <5 years old and >5 to 15 years old) and metabolic biomarkers SCFAs, BCAA, AAA, cholesterol, and glucose 2a) at the time of TB treatment completion and 2) one-year after TB treatment completion. We will evaluate acetate, butyrate, propionate, isoleucine, leucine, valine, tyrosine, and phenylalanine by using 9 linear regression models to evaluate the association between the comparison groups and all the continuous metabolic biomarkers at TB treatment completion and one-year after TB treatment completion. The same multiple linear regression model, interaction variable, and control of confounding variables will be used to evaluate the relationship between age and SCFA, BCAA, and AAA. To evaluate the categorical dependent variables, cholesterol and glucose, two logistic regression models will be utilized. The chi-squared, R-squared, and beta coefficient will be assessed.

Odds ratios and 95% confidence intervals will be ascertained from each of these regression models. Known confounders (HIV, history of TB, and previous diabetes) have been controlled for by screening individuals with this variable out of the study. Additional covariates that are identified to be confounders will be adjusted for in the regression models.

Figure 2: Directed Acyclic Graph (DAG) of Central Hypothesis



C10. Sample size: The enrollment goal will be to collect three specimens at each of the time points from 50 pediatric patients. There will be a total of 150 stool specimens collected over the 24-month study period. With the same logic, we will also be collecting 150 blood samples. We will collect specimens at baseline, six months, and eighteen months.

C11. Limitations: Due to the small sample size, this proposed study will not be generalizable to all populations. Since pediatric patients will be enrolled from a refugee clinic, the enrollment population is representative of foreign countries. Also, the lack of controls makes it difficult to differentiate if the dysbiosis of the gut microbiota is due to TB treatment or TB disease. Another limitation of this study is the difficulty in

controlling the diet of each individual, which can impact the gut microbiota significantly. This proposal did not account for gender and other factors such as medications that individuals may take during the study period.

C12. Future directions: Results of this prospective cohort R21 proposal will provide novel information for future studies on the relationship between TB treatment and the dysbiosis of the gut microbiota as well as metabolic functions. At the time of enrollment, parents of patients, will be asked if they will consent to being contacted in the future, with the aim to reach the same patients for further studies. Future studies will collect medical record information and questionnaires to ascertain whom within the groups developed or has an increased risk of obesity and diabetes. We believe that pediatric patients younger than five years old with persistent gut dysbiosis at one year after TB treatment will develop an increased risk of a non-communicable disease (obesity and/or diabetes).

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