

BMJ Open Exploring the role of Microbiome in Susceptibility, Treatment Response and Outcome among Tuberculosis Patients from Pakistan: study protocol for a prospective cohort study (Micro-STOP)

Muhammad Shahzad ^{1,2}, Simon C Andrews,² Zia Ul-Haq³

To cite: Shahzad M, Andrews SC, Ul-Haq Z. Exploring the role of Microbiome in Susceptibility, Treatment Response and Outcome among Tuberculosis Patients from Pakistan: study protocol for a prospective cohort study (Micro-STOP). *BMJ Open* 2022;**12**:e058463. doi:10.1136/bmjopen-2021-058463

► Prepublication history for this paper is available online. To view these files, please visit the journal online (<http://dx.doi.org/10.1136/bmjopen-2021-058463>).

MS and SCA are joint first authors.

Received 17 October 2021
Accepted 11 May 2022



© Author(s) (or their employer(s)) 2022. Re-use permitted under CC BY-NC. No commercial re-use. See rights and permissions. Published by BMJ.

¹Institute of Basic Medical Sciences, Khyber Medical University, Peshawar, Pakistan

²School of Biological Sciences, University of Reading, Reading, UK

³Institute of Public Health & Social Sciences, Khyber Medical University, Peshawar, Pakistan

Correspondence to

Dr Muhammad Shahzad;
shahzad.ibms@kmu.edu.pk

ABSTRACT

Introduction Tuberculosis (TB) caused by *Mycobacterium tuberculosis* is a common infectious disease associated with significant morbidity and mortality, especially in low-income and middle-income countries. Successful treatment of the disease requires prolonged intake (6–8 months) of multiple antibiotics with potentially detrimental consequences on the composition and functional potential of the human microbiome. The protocol described in the current study aims to identify microbiome (oral and gut) signatures associated with TB pathogenesis, treatment response and outcome in humans.

Methods and analysis Four hundred and fifty, newly diagnosed patients with TB from three district levels (Peshawar, Mardan and Swat) TB diagnosis and treatment centres, will be recruited in this non-interventional, prospective cohort study and will be followed and monitored until treatment completion. Demographic and dietary intake data, anthropometric measurement and blood, stool and salivary rinse samples will be collected at baseline, day 15, month-2 and end of the treatment. Additionally, we will recruit age (± 3 years) and sex-matched healthy controls (n=30). Blood sampling will allow monitoring of the immune response during the treatment, while salivary rinse and faecal samples will allow monitoring of dynamic changes in oral and gut microbiome diversity. Within this prospective cohort study, a nested case–control study design will be conducted to assess perturbations in oral and gut microbiome diversity (microbial dysbiosis) and immune response and compare between the patients groups (treatment success vs failure).

Ethics and dissemination The study has received ethics approval from the Ethic Board of Khyber Medical University Peshawar, and administrative approval from Provincial TB Control Programme of Khyber Pakhtunkhwa, Pakistan. The study results will be presented in national and international conferences and published in peer-reviewed journals.

Trial registration number NCT04985994.

INTRODUCTION

Tuberculosis (TB), caused by the bacterium *Mycobacterium tuberculosis* (Mtb), is a global public health issue and the second

STRENGTHS AND LIMITATIONS OF THIS STUDY

- ⇒ Prospective study design with sufficiently large sample size and sampling frequency to ensure capture of an accurate picture of any emerging microbial dysbiosis and immune modulation.
- ⇒ In addition to microbial signatures, collection of sociodemographic, anthropometric, nutrition and comorbidities data will permit further subgroup analysis and meaningful results.
- ⇒ A potential weakness is that not all patients may be able to provide samples for all of the specified time points.
- ⇒ Another weakness is the raised likelihood that some participants will withdraw since the study is carried out in resource poor communities.

most common cause of death in humans by an infectious agent after HIV/AIDS.¹ It is estimated that around one-fourth (24.8%) of the world's population has latent Mtb infection with no clinical symptoms and minimum chances of infecting others.² Of these, 5%–10% may develop active disease (characterised by clinical signs and symptoms of TB or microbiological confirmation of Mtb infection or both) at some stage of their life, especially when the immune system is compromised due to ageing, HIV infections or malnutrition.³ According to a 2020 Global Tuberculosis Report by the WHO, approximately 10.0 million new cases of TB were diagnosed in 2019, of which 1.4 million subsequently died.¹ The TB problem is more severe in lower-income and middle-income countries, including Pakistan. On a global scale, Pakistan ranks fifth in terms of TB prevalence with an estimated 570 000 new cases reported in 2019¹ thus posing a significant burden on the country's already strained healthcare system and economy.

TB pathogenesis is a complex and dynamic process encompassing several stages that include disease susceptibility, infection, latent or active disease and treatment response or failure.⁴ *Mtb* has the ability to evade the immune system over prolonged time periods, even decades after exposure, in the form of latent TB.⁵ Once active, the disease is among the most difficult to treat infections requiring multidrug therapy for as long as 6–9 months. However, treatment failure is frequent (even where adherence to antibiotic therapy is maintained) at 15% for drug susceptible infections and 31% for drug resistant TB cases.^{6,7} Thus, in some population groups, mortality is around 12%.⁸ Furthermore, relapse and TB reactivation are also common public health issues.⁹ Although, a number of factors that modulate the risk of progression from one stage of the disease to another has been documented, the underlying biological modulators of the risk remains elusive especially with regard to the microbiome.¹⁰ Emerging evidence suggests that the microbiome is likely to play a critical role in TB pathogenesis as well as in treatment response and outcome, primarily due to multifaceted interactions between the pathogen, microbiome and host immune response.^{4,11}

The human microbiome consists of various commensal consortia of micro-organisms (bacteria, archaea, viruses and fungi) colonising different habitats of the human body, such as the skin, gut and mucosal surfaces.¹² Recent technological advances spurred by culture-independent metagenomic sequencing techniques coupled with ever declining costs of sequencing (a 200 000-fold decrease since 2001)¹³ have greatly improved our understanding of the microbiome's crucial role in human health and disease. It is believed that humans have co-evolved with their commensal microbes for mutual benefit.¹⁴ The human host provides the microbiota with a warm and nutrient-rich environment in which to reside. On the other hand, the microbiome plays an important role in stimulating the development of a functional immune system (both innate and adaptive immune system), which is crucial in maintaining host–microbe symbiosis. The microbiota of the gut provides essential nutritional factors (such as vitamins) and enhances the host's ability to extract energy from food. Indeed, there is now considerable evidence suggesting that the human microbiome impacts, either directly or indirectly, the development of various host processes including circadian rhythmicity and nutritional, metabolic and immune responses.^{15–17} Furthermore, the microbiome can exert such effects not only locally but also across distant body sites via gut-lung, gut-brain and/or gut-liver axes. Thus, any perturbation in microbiome composition or functional potential may lead to pathogenic infections and altered immune responses, and can also play a crucial role in development of non-communicable diseases ranging from immune-mediated disease to intergenerational obesity

and even cancer.¹⁷ Microbial dysbiosis at distinct body sites, such as the oral cavity and the gut, has also been reported in TB-associated comorbidities such as diabetes mellitus¹⁸ and malnutrition.¹⁹ However, until now, very few studies have focused on the role of key microbiome communities in response to TB infection.

Microbial dysbiosis in the human gut during TB infection was first reported by Dubourg *et al.*²⁰ Using culture-dependent and independent methods, they found impoverished microbial communities residing in the gut of a single TB patient undergoing anti-TB treatment. However, the study included only one human subject (a 63-year-old female) with multidrug-resistant TB. The patient was also on broad-spectrum antibiotic treatment for the preceding 4 months and it is unclear to what degree the reduction in gut bacterial diversity was due to the antibiotic treatment, MDR TB, nutritional status or other comorbid conditions. Another study also found significant changes in gut microbial diversity in a mouse *Mtb* infection model where 16S-rRNA-gene Next Generation Sequencing (NGS) revealed a rapid reduction in gut microbial diversity following infection. It was suggested that this effect arose as a consequence of immune signalling from lung to gut²¹ through the so called 'gut-lung axis' whereby alterations in one microbiome causes an adjusted immune response and altered composition of the other. A more recent study by Hu *et al* found unique gut microbiome signatures and metabolic functions that were predictive of *Mtb* infection in patients with TB.²² Similarly, oral anaerobic bacteria such as *Prevotella* have been found in abundance in the lower respiratory tract of HIV patients undergoing anti-retroviral therapy. Metabolic products from these bacterial population, such as butyrate and other short chain fatty acids, were associated with increased susceptibility to TB infection in these patients.²³ On the other hand, *Helicobacter pylori* infection in latent TB patients is thought to be protective against progression of disease from latent to active TB in humans.²⁴ All these findings may be of relevance to the understanding of the role of the microbiota in TB pathogenesis, recurrence and reactivation. However, until now, there is no strong evidence that indicates whether there is any significant interplay between the microbiome and immune system in humans that affects susceptibility to TB infection, TB progression and response during the course of anti-TB therapy.

Therefore, this study aims to dissect the relationship between the microbiota (oral and gut) and its interaction with the immune system during TB infection and anti-TB therapy in humans.

OBJECTIVES

Primary objective

The primary objective of the study is to explore the effect of TB infection and anti-TB therapy on oral and gut microbiome diversity and functional potential, and the

immune response in newly diagnosed patients with TB from Pakistan.

Secondary objectives

1. To determine oral and gut microbiome diversity and functional potential at baseline and compare with healthy controls.
2. To assess the relationship between the oral and gut microbiome and sociodemographic characteristics and dietary intake in patients with TB at baseline, before the start of anti-TB treatment.
3. To describe any occurrence of oral and gut microbial dysbiosis and its association with adverse reaction and treatment failure in patients with TB.
4. To identify specific oral and gut enterotypes associated with adverse reaction and unfavourable treatment outcomes.

METHODS AND ANALYSIS

Study design and setting

The ‘Microbiome in Susceptibility, Treatment Response and Outcome among Tuberculosis Patients (Micro-STOP)’ study is designed as a population-based, prospective cohort study in newly diagnosed patients with TB in three district-level (Peshawar, Mardan and Swat) TB diagnostic and treatment centres of Khyber Pakhtunkhwa province of Pakistan. These centres have been selected based on the highest TB prevalence in the year 2020. The participating sites approximately treat 2500 patients with TB annually.

The study will be conducted as part of an ongoing collaboration between Khyber Medical University Peshawar, Pakistan, TB Control Programme Khyber Pakhtunkhwa and University of Reading, UK. The TB Control Programme is the main body for developing and implementing policies and strategies for prevention and management of TB, and for governing TB diagnostics and treatment centres across the province. The TB Control Programme is responsible for patient identification, clinical data and collection of study-related samples. Khyber Medical University is the only public sector medical university and centre for healthcare research and education in the province. The University of Reading will analyse the data as explained in the methodology section below. The Micro-STOP study started in August 2021 and data collection is planned till August 2022 to achieve the required sample size.

Sample size

Because of the strict inclusion and exclusion criteria, and absence of similar studies, statistical power cannot be calculated accurately. Therefore, we have followed a pragmatic approach for sample size calculation. Previously published literature suggest that for metagenomic studies, a sample size of 30 is sufficient to observe phenotypic heterogeneity at the molecular level.²⁵ We are aiming to recruit 450 TB patients on the basis of 7% TB treatment failure rate in Pakistan,¹ to achieve sufficient numbers to observe phenotypic differences in the corresponding microbiomes (treatment

failure vs success). Furthermore, we will also recruit 30 healthy controls.

Study population

We are aiming to recruit 450, newly diagnosed, untreated patients with TB from TB treatment facilities in selected centres of Khyber Pakhtunkhwa province of Pakistan. The cohort will be followed during the first line anti-TB therapy at different time points until the treatment ends. After the treatment is completed, a nested case–control study will be conducted between the patients with treatment success and failure. Based on the TB treatment failure rate in Pakistan,¹ we are expected to get sufficient number of patients with treatment failure to compare the microbiome with those having successful treatment outcome. Furthermore, in order to discern the role of microbiome in TB pathogenesis, we will assess microbiome diversity of newly diagnosed TB patients with no history of broad spectrum antibiotics use for 2 month (n=30) and compare with controls (n=30). Controls will be healthy subjects with no history of pulmonary TB, matched for sex and age (± 3 years) with the TB patient group. They will be randomly selected from non-family neighbours of the patients. One person will be selected randomly from 5 to 7 proposed healthy controls.

Inclusion criteria

Participants will be included in the study if they are:

1. Diagnosed with pulmonary TB after detailed history collection, clinical examination and laboratory assessment (sputum culture positive).
2. Aged 18–65 years.
3. Willing to participate in the study.
4. Healthy controls are those who are free of TB symptoms, healthy on physical examination and provide a negative sputum culture result.

Exclusion criteria

Participants will be excluded if they are:

1. Already on anti-TB treatment or were previously treated for TB.
2. Severely anaemic (haemoglobin (Hb) < 10 g/L).
3. Having diarrhoea or other major gastrointestinal disorders.
4. Using a medically prescribed diet or nutritional supplement.
5. Pregnant or lactating women.
6. Patients with liver or renal dysfunction, or having any other chronic disease condition.
7. Extra pulmonary and drug-resistance TB patients.

Study and sampling procedure

An overview of the overall study flow and conduct is presented in [figure 1](#). Patients will be recruited daily by respective site coordinators who are clinical experts in TB diagnosis and treatment provision.

All patients with clinical or radiological signs suggestive of TB will undergo sputum smear microscopy and Xpert MTB/RIF assay to confirm diagnosis of TB following

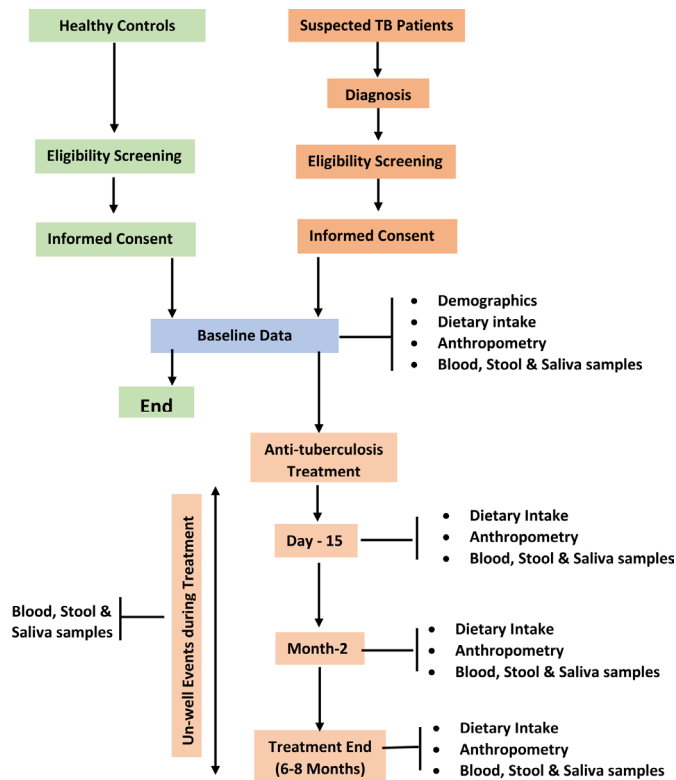


Figure 1 Flow chart of the study. TB, tuberculosis.

the National TB Control Programme revised guidelines for the control of TB in Pakistan. Smear microscopy employing Ziehl-Neelsen (ZN) staining to identify acid-fast bacilli in sputum smears is a fast, easy and cost effective method for diagnosis of pulmonary TB in low-income and middle-income countries.²⁶ Xpert MTB/RIF assay (Cepheid, Sunnyvale, California, USA) is a WHO recommended, PCR-based molecular diagnostic technique for simultaneous identification of Mtb and rifampin resistance (ie, mutation of the *rpoB* gene as surrogate for multidrug resistance) in clinical samples.²⁷ According to the National TB Control Programme guidelines,²⁸ all the patients receive first line anti-TB treatment consisting of 2 months intensive phase and 4 months continuation phase. Biological samples will be collected at baseline, during and at the end of treatment as shown in [figure 1](#). The clinicians will explain the sample collection procedure to the patients in local language. The patients will be asked to provide up to 5 mL of blood-free sputum in sterile, 50 mL plastic jars with screw caps. ZN staining of sputum smears will be performed and reporting will be based on the American Thoracic Society guidelines²⁹ described in [table 1](#).

All the patients meeting the eligibility criteria will be invited to participate in the study by trained research assistants. A participant information sheet containing all the study details and procedures in an easy to understand, local language (Pashto) will be provided to all the participants. The study procedures will also be explained to the participants verbally. Once agreed, the participants

Table 1 Reporting criteria for AFB smear microscopy

No of bacilli	Report
No AFB/200 field	No AFB seen
1–9 AFB/100 field	Scanty
10–99 AFB/100 field	+positive
1–9 AFB/field	++positive
10–99 AFB/field	+++positive
AFB, acid-fast bacilli.	

will be asked to sign a written informed consent form in their preferred language.

Collection of sociodemographic and anthropometric data

Sociodemographic information of the participants such as name, age, gender, ethnicity, education, occupation and household income will be collected by research assistants using structured questionnaire. The data will be collected only at baseline before the start of anti-TB therapy. [Table 2](#) gives an overview of study conduct and timeline. Anthropometric measurements including height and weight will also be recorded following standard methods. In order to record height, patients will be instructed to remove shoes and head coverings (cap, scarf, etc), stand comfortably against a wall-mounted stadiometer (Seca, Hamburg, Germany) with heels, buttocks, shoulder blades and back of the head touching the vertical back board (Frankfurt plane position). Height will be then measured to the nearest 0.1 cm. Similarly for weight measurement, the patients will be asked to remove shoes and any extra piece of cloth and jewellery. Weight will be recorded in kilograms to the nearest 0.1 kg using calibrated electronic scale (Seca). Body mass index (BMI) will be computed as the fraction of weight in kilograms to the squared height in metres (kg/m^2). All the anthropometric data will be collected at all four time points by two fully trained research assistants at each site.

Dietary assessment

Because of the unavailability of a validated food frequency questionnaire for use in Pakistan, dietary intake of the participants will be assessed by trained nutritionist using the 24 hours dietary recall method. The 24-hour dietary recall will be conducted in the form of an in-depth interview using a standardised four stage protocol.³⁰ The procedure typically requires around 20–30 min to complete. Efforts will be made to list all the food items and beverages consumed during the past 24-hour period. This will include recording of information relating to all the food and beverages consumed, ingredients, cooking methods and brand names of commercial foods. The amount of each food or beverage consumed will be estimated in reference to common size containers (bowls, cups and glasses), standard measuring cups and spoons, two dimensional aids (photographs) and three dimensional

Table 2 Study timeline and conduct

Timeline	Baseline	Day 15	Month-2	Unwell episode	Treatment end (6–8 months)
Sputum culture	x	x	x		x
Chest E-ray	x	x	x		x
Drug susceptibility testing	x		x		
Eligibility assessment	x	x			
Informed consent	x				
Sociodemographic data	x				
Medical history	x				
Tobacco use	x				
Anthropometric measurement	x		x		x
Dietary assessment	x	x	x		x
Blood samples	x	x	x	x	x
Sputum samples	x	x	x	x	x
Stool samples	x	x	x		x
Salivary rinse samples	x	x	x		x

food models. Dietary intake assessment will be performed at all four time points.

Sample collection

Blood

Non-fasting, whole blood samples will be collected by a trained phlebotomist using butterfly needles and plastic vacutainers (BD Diagnostics, Switzerland). At least 5 mL of blood will be drawn from an antecubital vein into pre-chilled collection tubes containing silica clot activator. Whole blood will be sent to the local laboratory for complete blood count. For separation of serum, blood samples will be left on ice for 30 min followed by centrifugation. Aliquots of the serum samples (250 µL) will be prepared and stored at -80°C till further processing in KMU main lab.

Stool

All participants will be asked to provide a stool sample at each of the four time points. For this purpose, stool samples collection kits will be provided which contain a collection pot, plastic bag, disposable gloves and an instruction sheet in their preferred language. Participants will be asked to pass an entire bowel movement into the plastic pot and close the lid when finished. After collection, the samples will be handed over to the research associate or delivered in their local study site (TB centre) for further processing. The research associate will then transfer part of the stool samples to 10 mL screw top tubes (Bijoux tubes) with the help of the mini-spoon attached to the lid. Lids will be closed tightly, and tubes will then be placed in the plastic bag and transferred to the main laboratory maintaining the cold chain. In the laboratory, aliquots of the samples (200 mg) will be taken and stored at -80°C for further processing.

Oral rinse

Oral rinse samples will be collected from all the patients at all the time points using standard methods.³¹ Before the sample collection, the patients will be instructed to avoid eating food, drinking fluids (especially flavoured and carbonated drinks), chewing gum and using tobacco products for at least 1 hour before sample collection. They will be asked to swish (not gargle) 10 mL of sterile phosphate buffered saline (PBS) for 1 min, and expectorate the contents of the mouth into a 50 mL centrifuge tube. Collected samples will be vortexed (3×30 s), and each collected sample will be centrifuged at 14 000 x g for 10 min at 4°C to pelletise the corpuscular part and separate it from extracellular soluble components (supernatant). Resultant cell pellets will be frozen at -80°C until use.

LABORATORY ANALYSIS

Haematological and biochemical parameters

Whole blood samples will be used for complete blood counts, and for Hb, haematocrit and mean corpuscular volume determination using an automated haematology analyzer (Sysmex XP-100, Singapore). Serum iron, ferritin, transferrin, C reactive protein and alpha 1-acid glycoprotein will be assayed using commercially available kits on an Abbott Architect ci8200 automated analyser (Abbott, Abbott Park, Illinois, USA) in the Mardan Medical Complex, Mardan, Pakistan. Micro-nutrient status of the patients will also be evaluated at all four time points. Vitamin A and D will be assessed through ELISA while plasma zinc and other minerals will be measured using the inductively coupled plasma-mass spectrometry facility at the University of Reading, UK.

Assessment of immune status

Immune status of the host will be assessed by full blood counts and measuring a panel of proinflammatory (IFN- γ , TNF- α and IL-6) and anti-inflammatory cytokine (IL-10, TGF- β 1) levels in serum. Selection of these cytokines is based at the recently published study where significant differences in their levels were observed between patients with active TB and healthy controls.¹⁷ Serum concentration of cytokines will be measured by the sandwich ELISA technique following manufacturer instructions.

DNA extraction and 16S rRNA sequencing

Bacterial genomic DNA will be extracted from the oral rinse and faecal samples (200 mg) stored at -80°C using a ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Irvine, California, USA) following the manufacturer's instructions. Each sample will be quantified using a Denovix and quality-checked through agarose gel electrophoresis and PCR of the 16S rRNA gene target. To prepare the samples for sequencing, genomic DNA will be used to amplify the V1-V3 regions of the 16S rRNA gene using Golay barcoded primers through conventional PCR.³² The amplified samples will then be subjected to agarose gel electrophoresis to separate the DNA samples from primer dimers. Each DNA band will then be extracted and purified using a QIAquick gel extraction kit (Cat. no. 28705 QIAGEN Germany). After purification, the concentration of each sample will be quantified using a Nanodrop. Concentrations of all samples will then be standardised to a minimum of 2.5 nmol. Pooled samples will then be subject to high throughput sequencing on the Illumina Miseq platform with 2×300 base reads using V3 chemistry as specified by the manufacturer (Illumina, San Diego, California, USA) at the Rehman Medical Institute, Peshawar. Data output will be demultiplexed with the in-built RTA software.

NGS data analysis

Demultiplexed sequence data will be processed and analysed using the open-source software pipeline Quantitative Insights Into Microbial Ecology 2 (QIIME 2) (<http://qiime2.org>)³¹ as follows. The forward and reverse reads will be merged using fastq-join, and will then be subject to quality filtering using minimum Phred quality score of 20. Reads will then be assigned to amplicon sequence variant using the built-in deblur command of QIIME2 at a 99% similarity threshold. Taxonomic assignment will then be achieved using the current bacterial Greengenes database. For alpha and beta diversity tests, all samples will be subsampled to an equal number of reads. β -Diversity analysis will be performed by principal coordinate analysis with Weighted Unifrac similarity matrices and subsequent permutational multivariate analysis of variance using the Microbiome Analyst web platform (<https://www.microbiomeanalyst.ca/>). Univariate analysis will be also be executed using Microbiome Analyst and significant differences selected with Bonferroni correction. Impact of different factors such as age, gender, BMI disease severity

etc on microbiome diversity will be evaluated using generalised linear model.³³ Analysis of statistical differences at different taxonomic and functional levels will be carried out using Statistical Analysis of Metagenomic Profiles that use Fischer's exact test along with Storey's false discovery rate (FDR) correction at 95% CI.³⁴ We will seek to identify any correlations between the collected metadata and microbiome composition using canonical correspondence analysis or redundancy analysis³⁵ and identify any gut/oral microbiota trends associated with unfavourable TB treatment outcome using random forest approach. Sequencing files and associated metadata will be deposited in an appropriate database (eg, the NCBI BioSample database) at the point of publication.

Descriptive data including sociodemographic, clinical and laboratory data will be described as frequency and percentages. Depending on the data type (quantitative and categorical), appropriate statistical tests (Student's t-test and Pearson's and χ^2 test) will be applied to compare differences between the groups.

Patients and public involvement

The community members or the patients were not directly involved in study design, conduct and outcome measures. However, the protocol was reviewed and approved by Provincial TB control programme of Khyber Pakhtunkhwa, Pakistan. Clinicians and programme managers of TB control programme have close liaison with the patients and act as bridge between the researchers and patients. They have substantial contribution in designing data collection questionnaires, informed consent and ensuring confidentiality. The study findings that report a positive impact on clinical practice will be reported in annual health meetings and communicated to the patients through the clinicians. However, individual data will not be reported back to the patients.

ETHICAL CONSIDERATIONS

Ethics approval of the study has already been obtained from the Ethics Board of Khyber Medical University (DIR/KMU-EB/PR/000858). A participant information sheet will be provided to all the patients in their preferred language (Urdu, Pashto). The information sheet contains all the relevant information regarding the exact nature of the study, procedures, potential risks and benefits involved and whom to contact in case of emergency or when further information is required. The study procedures will also be explained verbally by clinical staff and research assistants and any queries will be answered. Once the participants have agreed to be included in the study, written informed consent will be obtained. The informed consent will be dated and signed by both the participant and the research assistant who presented the information to the participant. In cases where the participant is unable (illiterate) to sign the consent form, a thumb print will be obtained. The informed consent clearly indicates that the patient's participation in the study is purely voluntary and

he/she can withdraw from the study anytime without any reason and the decision will not affect the standard of treatment he/she receives.

Dissemination

We intend to publish the results of the study in international, peer-reviewed journals as they become available. The data will also be presented in scientific conferences and seminars.

Contributors MS and SCA conceived the project idea and research methodology. ZU-H is overseeing recruitment of the study participants, samples and data collection. All the authors read and approved the final version of this manuscript.

Funding The study is funded by Higher Education Commission Pakistan National Research Program for Universities fund (No: 10289/KPK/ NRPUR&D/HEC/ 2017).

Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Consent obtained directly from patient(s)

Provenance and peer review Not commissioned; externally peer reviewed.

Open access This is an open access article distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited, appropriate credit is given, any changes made indicated, and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>.

ORCID iD

Muhammad Shahzad <http://orcid.org/0000-0001-6565-1777>

REFERENCES

- World Health Organization. Global tuberculosis report 2020 [Internet]. Geneva, Switzerland, 2020. Available: <https://www.who.int/news-room/fact-sheets/detail/tuberculosis>
- Cohen A, Mathiasen VD, Schön T, et al. The global prevalence of latent tuberculosis: a systematic review and meta-analysis. *Eur Respir J* 2019;54:1900655.
- Jilani TN, Avula A, Zafar Gondal A, et al. Active Tuberculosis. In: *StatPearls*. Treasure Island (FL): StatPearls Publishing, 2021. <http://www.ncbi.nlm.nih.gov/books/NBK513246/>
- Naidoo CC, Nyawo GR, Wu BG, et al. The microbiome and tuberculosis: state of the art, potential applications, and defining the clinical research agenda. *Lancet Respir Med* 2019;7:892–906.
- Orme IM, Robinson RT, Cooper AM. The balance between protective and pathogenic immune responses in the TB-infected lung. *Nat Immunol* 2015;16:57–63.
- Karumbi J, Garner P. Directly observed therapy for treating tuberculosis. *Cochrane Database Syst Rev* 2015;5:CD003343.
- Orenstein EW, Basu S, Shah NS, et al. Treatment outcomes among patients with multidrug-resistant tuberculosis: systematic review and meta-analysis. *Lancet Infect Dis* 2009;9:153–61.
- Shuldiner J, Leventhal A, Chemtob D, et al. Mortality after anti-tuberculosis treatment completion: results of long-term follow-up. *Int J Tuberc Lung Dis* 2016;20:43–8.
- Humbwawali JB, Trujillo NJ, Paim BS, et al. Sputum monitoring during tuberculosis treatment for predicting outcome. *Lancet Infect Dis* 2011;11:160.
- Barry CE, Boshoff HI, Dartois V, et al. The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. *Nat Rev Microbiol* 2009;7:845–55.
- Mori G, Morrison M, Blumenthal A. Microbiome-immune interactions in tuberculosis. *PLoS Pathog* 2021;17:e1009377.
- Gilbert JA, Blaser MJ, Caporaso JG, et al. Current understanding of the human microbiome. *Nat Med* 2018;24:392–400.
- The Cost of Sequencing a Human Genome [Internet]. Genome.gov. Available: <http://www.genome.gov/about-genomics/fact-sheets/Sequencing-Human-Genome-cost> [Accessed 29 Sep 2021].
- Dethlefsen L, McFall-Ngai M, Relman DA. An ecological and evolutionary perspective on human-microbe mutualism and disease. *Nature* 2007;449:811–8.
- Hacquard S, Garrido-Oter R, González A, et al. Microbiota and host nutrition across plant and animal kingdoms. *Cell Host Microbe* 2015;17:603–16.
- Lynch JB, Hsiao EY. Microbiomes as sources of emergent host phenotypes. *Science* 2019;365:1405–9.
- Zheng D, Liwinski T, Elinav E. Interaction between microbiota and immunity in health and disease. *Cell Res* 2020;30:492–506.
- Qin J, Li Y, Cai Z, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* 2012;490:55–60.
- Million M, Diallo A, Raoult D. Gut microbiota and malnutrition. *Microb Pathog* 2017;106:127–38.
- Dubourg G, Lagier JC, Armougom F, et al. The gut microbiota of a patient with resistant tuberculosis is more comprehensively studied by culturomics than by metagenomics. *Eur J Clin Microbiol Infect Dis* 2013;32:637–45.
- Winglee K, Eloë-Fadros E, Gupta S, et al. Aerosol Mycobacterium tuberculosis infection causes rapid loss of diversity in gut microbiota. *PLoS One* 2014;9:e97048.
- Hu Y, Feng Y, Wu J, et al. The gut microbiome signatures discriminate healthy from pulmonary tuberculosis patients. *Front Cell Infect Microbiol* 2019;9:90.
- Segal LN, Clemente JC, Li Y, et al. Anaerobic bacterial fermentation products increase tuberculosis risk in Antiretroviral-Drug-Treated HIV patients. *Cell Host Microbe* 2017;21:530–7.
- Perry S, de Jong BC, Solnick JV, et al. Infection with *Helicobacter pylori* is associated with protection against tuberculosis. *PLoS One* 2010;5:e8804.
- Ardura MI, Banchereau R, Mejias A, et al. Enhanced monocyte response and decreased central memory T cells in children with invasive *Staphylococcus aureus* infections. *PLoS One* 2009;4:e5446.
- Chandra TJ, Alan RR, Selvaraj R, et al. MODS assay for rapid diagnosis of tuberculosis among HIV TB CO infected individuals in a tertiary care Hospital, Andhra Pradesh. *Pak J Chest Med* 2014;20.
- Boehme CC, Nabeta P, Hillemann D, et al. Rapid molecular detection of tuberculosis and rifampin resistance. *N Engl J Med* 2010;363:1005–15.
- National TB Control Program. National Institute of health Islamabad. Available: <https://www.nih.org.pk/national-tb-control-program/> [Accessed 20 Apr 2022].
- Lewinsohn DM, Leonard MK, LoBue PA, et al. Official American thoracic Society/Infectious diseases Society of America/Centers for disease control and prevention clinical practice guidelines: diagnosis of tuberculosis in adults and children. *Clin Infect Dis* 2017;64:e1–33.
- Shim J-S, Oh K, Kim HC. Dietary assessment methods in epidemiologic studies. *Epidemiol Health* 2014;36:e2014009.
- Woo JS, Lu DY, . Procurement, transportation, and storage of saliva, buccal swab, and oral wash specimens. *Methods Mol Biol* 2019;1897:99–105.
- Zheng W, Tsompana M, Ruscitto A, et al. An accurate and efficient experimental approach for characterization of the complex oral microbiota. *Microbiome* 2015;3:48.
- Bolker BM, Brooks ME, Clark CJ, et al. Generalized linear mixed models: a practical guide for ecology and evolution. *Trends Ecol Evol* 2009;24:127–35.
- Parks DH, Tyson GW, Hugenholtz P, et al. Stamp: statistical analysis of taxonomic and functional profiles. *Bioinformatics* 2014;30:3123–4.
- Makarenkov V, Legendre P. Nonlinear redundancy analysis and canonical correspondence analysis based on polynomial regression. *Ecology* 2002;83:1146–61.