THE DUAL BURDEN OF METABOLIC ABNORMALITIES AND TUBERCULOSIS AMONG POPULATIONS WITH LOW BODY MASS INDEX

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Elaine Ann Yu

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THE DUAL BURDEN OF METABOLIC ABNORMALITIES AND TUBERCULOSIS AMONG POPULATIONS WITH LOW BODY MASS INDEX

Elaine Ann Yu, Ph. D., M.P.H.

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Background: Globally, metabolic abnormalities affect at least 20-25% of the adult population; active TB disease is the ninth leading cause of death. Our overall goal was to assess key human host factors, including vitamin D and the gut microbiome, which affect this dual burden and have bi-directional effects on inflammation. Specifically, our objectives were to:

1) assess the predictive performance of common anthropometric cut-offs utilized in diabetes population screening;

2) examine the association between vitamin D and metabolic indicators;

3) assess gut microbiota differences based on serum 25-hydroxyvitamin D (25[OH]D) status;

among a population with a high prevalence of suspected or confirmed active TB disease and low or normal body mass index (BMI).

Methods: At a rural hospital in South India, adult outpatients were enrolled after providing informed consent. Data collection included interviews, clinical examinations, anthropometry, and biological samples. Three study participant subsets were included

in different analyses. Cornell University and the hospital institutional review boards approved study protocols.

Results: Most study participants had BMI <25 kg/m^2 (88.2%) or waist circumference (WC) < diabetes screening cut-offs among South Asian populations (79.3%), which would be considered low risk in diabetes screening. However, one-third of study participants either had glycated hemoglobin (HbA1c) ≥ 6.5% (12.3%) or between 5.7% and <6.5% (20.3%). BMI ≥25.0 kg/m^2 demonstrated low sensitivity (0.21; 95% CI: 0.06, 0.35) as a screening indicator for HbA1c ≥ 6.5%. Median 25(OH)D was 51.8 nmol/L (IQR 36.0-70.0). Serum 25(OH)D was inversely associated with glycated hemoglobin and WC (both $p<0.05$), respectively, though not hypertension or gut microbiota diversity (all $p>0.05$). Most bacterial sequences in rectal swab samples were from the Firmicutes and Bacteroidetes phyla.

Conclusions: Our findings indicate a high prevalence of elevated HbA1c (≥ 5.7%), and suggest the need for population-specific BMI and WC cut-offs in diabetes screening. Vitamin D status was associated with HbA1c, however not gut microbiome diversity. Further studies are needed to elucidate the potential roles of vitamin D and the gut microbiome in mitigating the dual burden of metabolic abnormalities and active TB disease among populations with low BMI in resource-limited settings.

BIOGRAPHICAL SKETCH

Elaine Ann Yu focuses on alleviating the dual burden of disease, including the comorbidity of tuberculosis and diabetes mellitus, particularly in resource-limited environments. Prior to the doctoral program in nutritional sciences at Cornell University, her educational background included a Master of Public Health (global epidemiology) at Emory University (Atlanta, Georgia), Bachelor of Arts (molecular and cell biology) at the University of California at Berkeley (Berkeley, California).

In dedication to my parents

who largely inspired and contributed to my interest in public health

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

CHAPTER II

CHAPTER IV

APPENDIX

LIST OF TABLES

CHAPTER I

CHAPTER II

CHAPTER III

CHAPTER IV

CHAPTER I: A brief literature review of the dual burden of disease from metabolic abnormalities and tuberculosis among populations with a high prevalence of low body mass index

Overview

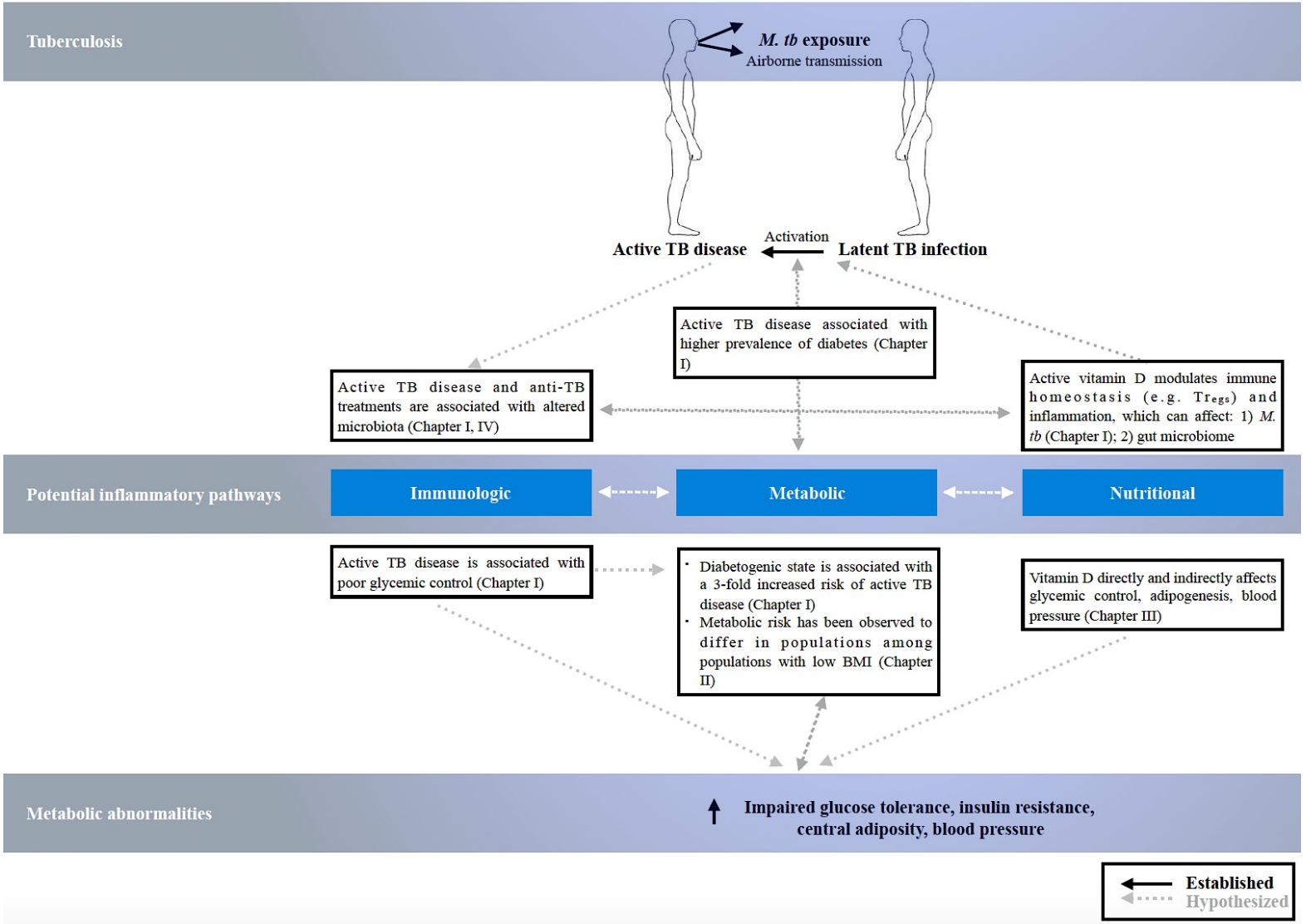
The overarching goal of this dissertation was to elucidate the dual burden of disease from active tuberculosis (TB) disease and metabolic abnormalities such as elevated glycated hemoglobin and abnormal blood pressure among populations that predominantly have low body mass index (BMI; <25 kg/m^2; ***Chapter I***). This included assessing key human host factors, including micronutrient status (vitamin D) and the gut microbiome, which have bi-directional effects on inflammation.

Specifically, the objectives were to:

1) Examine how common anthropometry-based (waist circumference, BMI) population screening cut-offs for diabetes and pre-diabetes perform compared to laboratory assays (***Chapter II***);
2) Assess the association between vitamin D and metabolic abnormalities (***Chapter III***); and
3) Characterize the gut microbiome (composition, diversity) and compare differences by serum 25-hydroxyvitamin D concentration (***Chapter IV***);

among a clinical population with a high prevalence of suspected or confirmed active TB disease and low BMI (***Figure 1***) in South India.

Figure 1. Potential pathways involving inflammation in the dual burden of metabolic abnormalities and active tuberculosis disease



Dual burden from metabolic abnormalities and tuberculosis

Metabolic disorders and abnormalities affect 20-25% of adults globally [1]. Metabolic syndrome, a cluster of metabolic abnormalities including central adiposity and blood pressure, is associated with a three-fold increased risk of diabetes and two-fold increased risk of cardiovascular diseases [1]. Annually, 17.5 million deaths are related to cardiovascular diseases [2]; and 1.5 million deaths are caused by diabetes [3]. Four hundred and twenty-two million people have diabetes currently; the number of individuals with diabetes has quadrupled since 1980 [3]. In India, the prevalence of diabetes is 9.1% among adults between 20-79 years, which is equivalent to more than 66 million adults [4].

Globally, active TB disease is the ninth leading cause of death, and also the single infectious agent that causes the most number of deaths [5]. There were an estimated 10.4 million incident active TB disease cases in 2016 [5]. India had a 37% increase of incident active TB cases between 2013 and 2016 [5].

At the population-level, many geographic regions have the dual burden of disease from metabolic abnormalities as well as TB (active TB disease, latent TB infection) [3, 5]. Moreover, there has been an observed synergistic interaction: 1) diabetes is associated with a three-fold increased risk of active TB disease; 2) active TB disease is associated with a higher prevalence of diabetes and impaired glucose tolerance [6]. However, a key research gap is elucidating how to mitigate the dual burden of disease from metabolic abnormalities and active TB disease at the individual-level.

Among populations with a high prevalence of low body mass index

At the individual-level, one major challenge is the early detection and treatment of metabolic abnormalities, given that metabolic abnormalities differ among populations with low BMI [7-18]. Central adiposity and visceral fat are more strongly associated with insulin resistance and type 2 diabetes mellitus, compared to overall obesity [7-10]. As an example, compared to Caucasian populations, Asian populations have a greater tendency for central fat accumulation [19] and also a higher risk for T2DM at a lower BMI [15-18]. Another study found that reduction of body mass (from gastric bypass) was associated with higher brown and beige adipose tissue activity with cold exposure [20].

Certain fat tissues have higher proportions of brown or beige (synonymously inducible, brown-in-white, brite) adipocytes, while other fat tissues have greater percentage of white adipocytes (including visceral fat) [21-23]. White, brown, and beige adipocytes differ in terms of phenotype and functionality [23-28], including metabolism [21-23]. Brown (or beige) adipocytes are involved in thermogenesis via the mitochondrial uncoupling protein (thermogenin) [21-23]. Furthermore, studies have shown that brown adipocytes are protective against T2DM indicators and associated with lower BMI [22, 29]. In contrast, white adipocytes were associated with visceral fat, which is linked with a higher risk of insulin resistance and thus, diabetes [30]. Given prior evidence, one key research questions is: Should recommendations for the screening and prevention of metabolic abnormalities differ in populations with low BMI (***Chapter II***)?

Role of inflammation

Inflammation is closely interrelated to metabolic function and regulation [31], as well as the host immune response to *Mycobacterium tuberculosis (M. tb)* [32]. A major research gap is understanding how to support the critical balance of pro- and anti-inflammatory responses [31]. This encompasses understanding the potential roles of key human host factors, including nutritional status (e.g. micronutrients [vitamin D]) and the gut microbiome, which have been shown to have bi-directional interactions with inflammation [33].

Vitamin D

Vitamin D is a steroid hormone that is consumed (diet, supplements) and endogenously produced through photochemical synthesis after sunlight exposure (ultraviolet rays) [34]. Vitamin D is converted to the major circulating form (25-hydroyxvitamin D; 25[OH]D) by a cytochrome P450-based hydroxylase (CYP) 2R1 in the liver; and then to the active form (1,25-dihydroxyvitamin D; 1,25[OH]2D) by CYP27B1 in the kidney [34]. Subsequently, 1,25(OH)2D has genomic and non-genomic effects, which affect skeletal and potentially extraskeletal health outcomes [34-41]. Vitamin D is anti-inflammatory and an immunoregulator [42-48], including in the context of metabolic abnormalities (***Chapter III***) as well as active TB disease (***Chapter IV***), respectively [33, 49].

In terms of metabolic abnormalities, vitamin D has been observed to affect energy

homeostasis (including glucose metabolism), body composition, and blood pressure [34, 50]. Potential mechanisms include: 1) Vitamin D receptors (VDRs; nuclear hormone receptors) and binding proteins (DBPs) are in pancreatic tissues [34]. 2) Vitamin D deficiency causes increased phosphorous and reduced calcium, which increases parathyroid hormone [34]. Secondary hyperparathyroidism causes abnormal glucose metabolism [34]. 3) Active vitamin D (1,25[OH]2D) regulates adipocyte metabolism, including adipogenesis and apoptosis [34]. 4) Active vitamin D has been demonstrated to decrease renin gene expression, which affects the renin-angiotensin-aldosterone system and could regulate blood pressure [51-54]. Observational human studies have found inverse associations between suboptimal vitamin D status and glycated hemoglobin (HbA1c) (***Table 1***) as well as increased type 2 diabetes mellitus [55-58], insulin resistance [59]; and reduced insulin sensitivity [60, 61]. Most cross-sectional studies showed an inverse association between low 25(OH)D serum (or plasma) concentration and greater waist circumference (***Table 2***) [62-70]. Based on meta-analyses, vitamin D (25(OH)D) was protective against high blood pressure in two meta-analyses (***Table 3***) [71, 72]; several found null findings [73-76]. Despite the previous evidence, there are limited studies among individuals with low BMI and residing in resource-limited settings (***Chapter III***).

Table 1. Studies assessing the association between vitamin D and glycated hemoglobin [a]

Study Design	Sample Size	Study Population	Location	Exposure			Outcome	Key Findings	Ref
				Biomarker	Biological Sample	Assessment Method			
Cross-sectional	127	Children, adolescents with high BMI (>95th percentile for age)	United States	25(OH)D (nmol/L)	Serum	RIA	HbA1c (%)	• Serum 25(OH)D inversely correlated with HbA1c (r=-0.23, p<0.01)	[50]
Cross-sectional	124	Patients with T2DM	Jordan	25(OH)D (nmol/L)	Serum	LC-MS/MS	HbA1c (%)	• 25(OH)D (log) inversely correlated with HbA1c	[52]
Cross-sectional	216	Adults at 20 clinics	United Arab Emirates	25(OH)D (nmol/L)	Serum	Multiple	HbA1c (%)	• HbA1c ≥6.5% associated with 25(OH)D ≤30 nmol/L	[53]
Cross-sectional	524	Patients with high BMI (≥ 35 kg/m^2)	Italy	25(OH)D (ng/mL)	Blood	Autoanalyzer	HbA1c (%)	• 25(OH)D inversely associated with HbA1c (β=-0.10; p<0.05)	[54]
Cross-sectional	1,175	Patients with T2DM	Korea	25(OH)D (ng/mL)	Serum	LC-MS/MS	HbA1c (%)	• Mean HbA1c inversely associated with 25(OH)D	[55]
Cross-sectional	160	Pregnant women (3rd trimester)	Egypt	25(OH)D (nmol/L)	Serum	RIA	HbA1c (%)	• Inverse correlation of HbA1c and 25(OH)D (r=-0.49; p<0.05)	[56]
Cross-sectional	4,391	Adults (>18 years) with high BMI (obese)	Iran	25(OH)D (ng/mL)	Serum	RIA	HbA1c (%)	• Serum 25(OH)D inversely associated with HbA1c	[57]
Cross-sectional	4,591	Adults	United States	25(OH)D (ng/mL)	Blood	CLIA	HbA1c (%)	• Deficient 25(OH)D associated with increased odds of elevated HbA1c (OR 2.47 [95% CI: 1.95, 3.13]	[58]
Cross-sectional	1,941	Adolescents (12-17 years)	United States	25(OH)D (nmol/L)	Serum	RIA	HbA1c (%)	• No significant association between 25(OH)D and HbA1c	[71]
Cross-sectional	84	Patients at diabetes clinic with BMI between 18.5-29.9 kg/m^2	Iran	25(OH)D (ng/mL)	Serum	ELISA	HbA1c (%)	• 25(OH)D not associated with HbA1c (%)	[72]
Cross-sectional	2,038	Adults (≥ 65 years)	United Kingdom	25(OH)D (nmol/L)	Serum	RIA	HbA1c (%)	• HbA1c (≥ 6.5%) was independently associated with low vitamin D levels (25[OH]D <25.0 nmol/L; OR 2.30 [95% CI 1.20, 4.42])	[59]
Cross-sectional	60	Adults (18-80 years) with DM and stage 1-4 CKD	Canada	25(OH)D, 1,25$(OH)_2$D (nmol/L)	Serum	LC-MS/MS	HbA1c (%)	• No association between vitamin D (25(OH)D, 1,25$(OH)_2$D) and HbA1c (≥ 7.0%)	[73]
Cross-sectional	3.520	Individuals with HbA1c between 5.8-6.9%	Norway	25(OH)D (nmol/L)	Serum	LC-MS/MS	HbA1c (%)	• HbA1c (%) differed across 25(OH)D quartile	[60]

Cross-sectional	8.643	Adults (30-87 years)	Norway	25(OH)D (nmol/L)	Serum	ECLIA	HbA1c (%)	• 25(OH)D and HbA1c (%) were correlated ($p<0.01$)	[157]
Cross-sectional	7,189	Adults (45 years; born in 1st week of March, 1958)	England, Scotland, Wales	25(OH)D (nmol/L)	Serum	Enzyme immunoassay	HbA1c (%)	• 25(OH)D associated with HbA1c	[158]
Cross-sectional	141	Patients with T2DM	Pakistan	25(OH)D (ng/mL)	Serum, plasma	Immunoassay	HbA1c (%)	• 25(OH)D (<20 ng/mL) associated with HbA1c >7.0%	[61]
Cross-sectional	8.655	Non-institutionalized civilian individuals	United States	25(OH)D (nmol/L)	Serum	RIA	HbA1c (%)	• 25(OH)D inversely associated with HbA1c (%)	[62]
Cross-sectional	245	Patients with T2DM and metformin medication	France	25(OH)D (ng/mL)	Plasma	CLIA	HbA1c (%)	• Mean HbA1c (%) differed by 25(OH)D status (sufficiency ≥ 30 ng/mL, insufficiency 21-29 ng/mL, deficiency ≤ 20 ng/mL; $p<0.01$)	[159]
Cross-sectional	15,169	Adults (>20 years) without diabetes	Korea	25(OH)D (ng/mL)	Serum	RIA	HbA1c (%)	• 25(OH)D was not correlated with HbA1c ($p>0.05$)	[74]
Cross-sectional	9,773	Adults (≥ 18 years)	United States	25(OH)D (ng/mL)	Serum	RIA	HbA1c (%)	• 25(OH)D inversely associated with HbA1c ($p<0.01$)	[63]
Cross-sectional	477	Patients with DM	United States	25(OH)D (ng/mL)	Serum	RIA	HbA1c (%)	• Vitamin D insufficiency (<20 ng/mL) associated abnormal HbA1c (OR 1.56 [95% CI 1.03, 2.37]; ≥ 6.5%)	[160]
Cross-sectional	120	Patients with T2DM	Greece	25(OH)D (ng/mL)	Serum, plasma	RIA	HbA1c (%)	• 25(OH)D inversely associated with HbA1c (%) overall (and in stratified groups of DM patients and controls; $p<0.01$)	[64]
Cross-sectional	147	Pregnant women in third trimester and attending gestational DM clinic	Australia	25(OH)D (nmol/L)	Serum	RIA	HbA1c (%)	• 25(OH)D inversely associated with HbA1c (%) among women with GDM	[65]
Cross-sectional	100	Patients with T2DM and CKD (stages 3-4)	Malaysia	25(OH)D (nmol/L)	Serum	CLIA	HbA1c (%)	• Each decrease of 10-nmol/L in serum 25(OH)D associated with 0.2% HbA1c increase	[66]
Cross-sectional	3,210	Adults (50-70 years)	China	25(OH)D (nmol/L)	Plasma	RIA	HbA1c (%)	• Vitamin D modified the effect of rs6902123 (SNP of PPAR-delta gene) on HbA1c (p for interaction = 0.0347)	[161]
Cross-sectional	109	Chinese patients with T2DM	Australia	25(OH)D (nmol/L)	Blood	Nicoll's method	HbA1c (%)	• Hypovitaminosis D not associated with HbA1c	[75]
Cross-sectional	1,139	Adults (Malays, Chinese, Indians)	Singapore	25(OH)D (ng/mL)	Serum	ECLIA	HbA1c (%)	• HbA1c (%) associated with suboptimal 25(OH)D concentration (<30 ng/mL; $p<0.05$)	[162]
Cross-sectional	250	Adults with high BMI (overweight, obese)	New Zealand	25(OH)D (nmol/L)	Serum	RIA	HbA1c (%)	• Inverse association between 25(OH)D and HbA1c (%; r=-0.16; p=0.01)	[67]

Cross-sectional	63	Patients with T2DM	Spain	25(OH)D (nmol/L)	Serum	EIA	HbA1c (%)	• Improved HbA1c (9.4% versus 7.3%) associated with decreased 25(OH)D (72.7 nmol/L to 59.0 nmol/L; p<0.05)	[163]
Cross-sectional	606	Patients with T2DM, pre-diabetes, or normal glucose tolerance	India	25(OH)D (ng/mL)	Serum	Chemiluminescence	HbA1c (%)	• No association between vitamin D deficiency (<30 ng/mL) and HbA1c (%)	[76]
Cross-sectional	2,877	Inuit adults	Greenland	25(OH)D (nmol/L)	Serum	LC-MS/MS	HbA1c (%)	• Increasing 25(OH)D (per 10 nmol/L) associated with HbA1c (0.39%; p<0.01)	[164]
Cross-sectional	121	Children and adolescents (11-21 years) treated for T1DM and T2DM	United States	25(OH)D (ng/mL)	Blood	CLIA	HbA1c (%)	• Patients with T2DM (but not T1DM) had an inverse association between HbA1c and 25(OH)D	[68]
Cross-sectional	128	Patients with T2DM	Turkey	25(OH)D (ng/mL)	Blood	Multiple	HbA1c (%)	• 25(OH)D < 20 ng/mL not associated with HbA1c >7% (p>0.05)	[77]
Cross-sectional	280	Individuals without diabetes and older	Netherlands	25(OH)D (nmol/L)	Blood	RIA	HbA1c (%)	• 25(OH)D not associated with follow-up HbA1c (beta coefficient -0.09, p>0.05)	[165]
Cross-sectional	141	Patients with T1DM (>12 months after diagnosis)	Italy	25(OH)D (nmol/L)	Serum	CLIA	HbA1c (%)	• 25(OH)D inversely associated with HbA1c (p<0.01)	[69]
Cross-sectional	286	Study participants without DM history	Italy	25(OH)D (ng/mL)	Plasma	CL microparticle IA	HbA1c (%)	• HbA1c between 5.7-6.4% associated with 25(OH)D	[166]
Cross-sectional	715	Patients with T2DM	Italy	25(OH)D (ng/mL)	Serum	CLIA	HbA1c (%)	• Serum 25(OH)D inversely associated with HbA1c (r=-0.12, p<0.01)	[70]
Prospective cohort study	6,565	Volunteer study participants	Canada	25(OH)D (nmol/L)	Serum	CLIA	HbA1c (%)	• Compared to those without 25(OH)D nmol/L increase during follow-up, those with a 25(OH)D nmol/L increase ≥50 nmol/L were 0.74 times more likely to have elevated HbA1c (p=0.03)	[78]
Prospective cohort study	197	Pregnant women	Thailand	25(OH)D (ng/mL)	Plasma	--- [b]	HbA1c (%)	• HbA1c independently associated with low 25(OH)D ng/mL among women with GDM (n=70)	[167]
Prospective cohort study	266	Pregnant women (24-28 weeks gestation) screened for GDM	Spain	25(OH)D (ng/mL)	Serum	--- [b]	HbA1c (%)	• Inverse correlation between 25(OH)D and HbA1c	[79]
Prospective cohort	171	Children and adolescents with T2DM	Turkey	25(OH)D (ng/mL)	Plasma	LC-MS/MS	HbA1c (%)	• Vitamin D deficiency (<20 ng/mL) associated with HbA1c (p<0.05)	[168]
Retrospective cohort study	191	Patients with high BMI (obese)	United States	25(OH)D (ng/mL)	Serum	LC-MS/MS	HbA1c (%)	• 25(OH)D inversely associated with HbA1c	[80]
Retrospective cohort study	301	Children, adolescents with T1DM	Saudi Arabia	25(OH)D (nmol/L; ≤37.5 nmol/L)	Serum	HPLC	HbA1c (%)	• No correlation between HbA1c and vitamin D deficiency	[169]

Retrospective cohort study	1,074	Men at outpatient clinics	United States	25(OH)D (ng/mL)	Serum	CLIA	HbA1c (%)	• 25(OH)D inversely associated with HbA1c among African American men, though not Caucasian American men	[81]
Retrospective cohort study	110	Post-menopausal women with T2DM	Mexico	25(OH)D (ng/mL)	Serum	LC-MS/MS	HbA1c (%)	• No significant linear relationship between HbA1c and 25(OH)D ($p>0.05$)	[170]
Case control study	129	Patients with T2DM (n=69); matched controls (n=60)	Kuwait	25(OH)D (nmol/L)	Serum	HPLC	HbA1c (%)	• No association between 25(OH)D and HbA1c	[171]
Case control study	50	Male patients with (n=30) or without T2DM (n=20; age- and socioeconomic status-matched)	Egypt	25(OH)D (ng/mL)	Serum	RIA	HbA1c (%)	• Serum 25(OH)D inversely associated with HbA1c ($p<0.05$)	[82]
Case control study	100	Adolescents with T1DM (n=60) or controls (n=40)	Poland	25(OH)D (ng/mL)	Serum	ECLIA	HbA1c (%)	• Among those with T1DM, low 25(OH)D negatively correlated with HbA1c ($r=-0.43$, $p<0.01$)	[83]
Case control study	436	Patients with T2DM (n=276) and controls (n=160)	Korea	25(OH)D (ng/mL)	Serum	CLIA	HbA1c (%)	• High HbA1c associated with 25(OH)D <20 ng/mL	[84]
Nested case control (in intervention)	160	Patients with intervention (Vitamin D3 supplement of 20,000 IU twice weekly for 6 months)	Norway	25(OH)D (nmol/L)	Serum	LC-MS/MS	HbA1c (%)	• High 25(OH)D associated with lower HbA1c	[85]
Intervention	80	Patients with T1DM and 25(OH)D <50 nmol/L at baseline with intervention (4000 IU vitamin D; 1200 mg calcium intake)	Saudi Arabia	25(OH)D (nmol/L)	Serum	CPBA	HbA1c (%)	• Significant difference in mean HbA1c (%) between vitamin D status groups at study endline (25[OH]D <35.4, ≥35.4-51, >51.0 nmol/L; $p=0.02$)	[172]
Intervention	840	Patients with intervention (Vitamin D2 supplement of 50,000 IU/week for 8 weeks)	United States	25(OH)D (ng/mL)	Serum	Multiple	HbA1c (%)	• 25(OH)D inversely associated with HbA1c ($r=-0.29$; $p=0.03$)	[173]

[a] PubMed search strategy was based on the following terms: "("vitamin d"[MeSH Terms] OR "vitamin d"[All Fields] OR "ergocalciferols"[MeSH Terms] OR "ergocalciferols"[All Fields]) AND ("hemoglobin a, glycosylated"[MeSH Terms] OR "glycosylated hemoglobin a"[All Fields] OR "hba1c"[All Fields])" and restricted by publication date (on or prior to October 1, 2017). Exclusion criteria included: 1) not primary data source (including reviews, editorials); 2) no measurement of glycated hemoglobin; 3) no assessment of vitamin D status in blood; 4) no humans. Among the 379 studies initially identified, 52 studies were within the scope of this review and included.

[b] Only abstract accessed; information not available in abstract

Table 2. Studies assessing the association between vitamin D and waist circumference [a]

Study Design	Sample Size	Study Population	Location	Exposure			Outcome	Key Findings	Ref
				Biomarker	Biological Sample	Assessment Method			
Cross-sectional	87	Healthy female study participants	Saudi Arabia	25(OH)D (ng/mL)	Plasma	HPLC	Waist circumference	• 25(OH)D negatively associated with waist circumference	[100]
Cross-sectional	1,205	Adults (18-80 years)	Qatar	25(OH)D (ng/mL)	Serum	CLIA	Waist circumference	• Waist circumference associated with vitamin D deficiency (20 ng/mL)	[101]
Cross-sectional	1,361	Adolescents	Malaysia	25(OH)D (nmol/L)	Serum	ECLIA	Waist circumference	• Greater waist circumference associated with vitamin D deficiency (OR 2.64 [95% CI 1.65, 4.25])	[102]
Cross-sectional	62	Postmenopausal women	Rome	25(OH)D (ng/mL)	Serum	HPLC	Waist circumference	• 25(OH)D correlated with waist circumference ($r=-0.54$, $p<0.01$) • Adjusted for covariates, 25(OH)D not associated with waist circumference	[103]
Cross-sectional	255	Infants (9 months)	Denmark	25(OH)D (nmol/L)	Plasma	CLIA	Waist circumference	• 25(OH)D negatively associated with waist circumference ($p<0.01$)	[104]
Cross-sectional	570	Outpatients with T2DM (n=420) and non-diabetic obese (n=150)	Italy	25(OH)D (ng/mL)	Serum	CLIA	Waist circumference	• 25(OH)D (age-adjusted) was inversely associated with WC ($p<0.01$) in both genders	[105]
Cross-sectional	100	Renal transplant recipients without diabetes	Brazil	25(OH)D (ng/mL)	Serum	CLIA	Waist circumference	• Median waist circumference higher in the group with vitamin D deficiency	[106]
Cross-sectional	2,096	Participants from two other completed clinical trials of neoplasia	United States	25(OH)D (ng/mL)	Blood	CLIA	Waist circumference	• 25(OH)D inversely associated with waist circumference (p trend <0.04)	[107]
Cross-sectional	435	Prepubertal children (~7 years)	Chile	25(OH)D (ng/mL)	Serum	CLIA	Waist circumference	• 25(OH)D < 30 ng/mL associated with high waist circumference ≥ 75th percentile among girls (OR 2.4 [95% CI 1.4, 4.3]) and boys (OR 2.2 [95% CI 1.2, 4.0])	[108]
Case-control	335	Asian Indians: 162 NAFLD cases, 173 controls (age- and sex-matched)	India	25(OH)D (ng/mL)	Serum	RIA	Waist circumference	• Lowest quartile of 25(OH)D associated with high waist circumference	[174]

[a] The studies in this table were identified from a PubMed search strategy was based on the following terms: "("vitamin d"[MeSH Terms] OR "vitamin d"[All Fields] OR "ergocalciferols"[MeSH Terms] OR "ergocalciferols"[All Fields]) AND ("waist circumference"[MeSH Terms] OR ("waist"[All Fields] AND "circumference"[All Fields]) OR "waist circumference"[All Fields])" and restricted by publication date (on or prior to October 9, 2017). Exclusion criteria included: 1) no waist circumference measurement; 2) no assessment of vitamin D status in blood or vitamin D supplementation; 3) no humans; 4) not systematic review and/or meta-analyses; 5) not in English language.
[b] RCT unless otherwise noted
[c] Range unless otherwise noted

Table 3. Reviews assessing the association between vitamin D and hypertension [a]

Exposure	# of RCTs [b]	# of Study Participants	Study Population	Follow-Up (months) [c]	Outcome(s)	Key Findings	Ref
Vitamin D status	10 prospective studies	58,262	Healthy, disease states	1.3-15	Hypertension	• Reduced risk of incident hypertension (RR 0.76 [95% CI 0.63, 0.90]) among those in top (versus bottom) category of 25(OH)D	[113]
	19 cross-sectional studies	90,535		---		• Reduced odds of hypertension (OR 0.79 [95% CI 0.73, 0.87]) among those in top (versus bottom) category of 25(OH)D	
Vitamin D status	3 cohorts	3,295	Healthy, disease states	364-416	Hypertension	• Lower 25(OH)D associated with incidence hypertension (RR 1.8 [95% CI 1.3, 2.4])	[114]
Vitamin D supplementation	10	37,162	Healthy, disease states (HTN, heart failure, T2DM, overweight and obesity)	1-364	Blood pressure	• Vitamin D supplementation reduced systolic blood pressure (weighted mean difference -1.9 mm Hg [95% CI -4.2, 0.4 mm Hg]) but did not change diastolic blood pressure	
Vitamin D supplementation	8	917	Non-CKD, pre-HTN, HTN, African Americans	3-84	Hypertension, blood pressure	• No differences in systolic (-0.08 mm Hg, p=0.2) or diastolic (0.09 mm Hg, p=0.16) blood pressure among those with vitamin D supplementation, relative to placebo	[115]
Vitamin D supplementation	46	4,541	Healthy, DM, HTN, older, CKD, HIV, HF	1-18	Hypertension, blood pressure	• No differences in systolic (-0.5 mm Hg, p=0.27) or diastolic (0.2 mm Hg, p=0.38) blood pressure among those with vitamin D supplementation, relative to placebo	[116]
Vitamin D supplementation	14	751 patients, 767 controls	Healthy, disease states (e.g. T2DM, osteoporosis, TB, obese or overweight)	1-108	Blood pressure	• No differences in systolic or diastolic blood pressure among those with and without vitamin D supplementation	[117]
Vitamin D supplementation	30	4,744	Healthy, disease states	Mean 5.6 (SD 4.0)	Blood pressure	• No effect of vitamin D3 supplementation on systolic blood pressure (-0.68 mmHg, 95%CI: -2.19 to 0.84) and diastolic (-0.57 mmHg, 95%CI: -1.36 to 0.22) • Subgroup analyses: Daily vitamin D3 supplementation (>800 IU/day) for <6 months among individuals ≥ 50 years reduced systolic and diastolic blood pressure; vitamin D supplementation decreased systolic and diastolic blood pressure among those with low or normal BMI (not overweight or obese)	[118]

[a] The reviews and meta-analyses in this table were identified from a PubMed search strategy was based on the following terms: "(("vitamin d"[MeSH Terms] OR "vitamin d"[All Fields] OR "ergocalciferols"[MeSH Terms] OR "ergocalciferols"[All Fields]) AND ("hypertension"[MeSH Terms] OR "hypertension"[All Fields]) AND ("blood pressure"[MeSH Terms] OR ("blood"[All Fields] AND "pressure"[All Fields]) OR "blood pressure"[All Fields] OR "blood pressure determination"[MeSH Terms] OR ("blood"[All Fields] AND "pressure"[All Fields] AND "determination"[All Fields]) OR "blood pressure determination"[All Fields] OR ("blood"[All Fields] AND "pressure"[All Fields]) OR "blood pressure"[All Fields] OR "arterial pressure"[MeSH Terms] OR ("arterial"[All Fields] AND "pressure"[All Fields]) OR "arterial pressure"[All Fields] OR ("blood"[All Fields] AND "pressure"[All Fields]))) AND Review[ptyp]" and restricted by publication date (on or prior to October 9, 2017). Exclusion criteria included: 1) no blood pressure measurement; 2) no assessment of vitamin D status in blood or vitamin D supplementation; 3) no humans; 4) not systematic review and/or meta-analyses; 5) not in English language.

[b] RCT unless otherwise noted

[c] Range unless otherwise noted

While early studies indicated that vitamin D upregulated anti-microbial activities of *M. tb*-infected macrophages [77-79], more recent data suggest that the more biologically-significant activity of vitamin D is the downregulation of the inflammatory response to *M. tb* [49] and anti-TB treatments [33]. A murine model demonstrated that dietary vitamin D3 attenuated the inflammatory response in mice, however did not affect *M. tb* bacterial burden [49]. In a longitudinal study among 95 patients with active pulmonary TB disease, vitamin D supplementation was associated with decreased proinflammatory cytokines and attenuated the suppressive effects from anti-TB treatment [33]. Additionally, vitamin D supplementation was associated with faster sputum smear conversion [33].

Gut microbiome

The human microbiome is defined as the ecological community of (commensal, symbiotic, pathogenic) microorganisms residing in our body space [80]. The gut microbiome has bi-directional effects on inflammation, including in the context of metabolic abnormalities and active TB disease [81-85]. Certain gut bacteria species produce short-chain fatty acids, including butyrate [81]. Butyrate is a microbial fermentation product, which has been demonstrated to regulate T regulatory (T_{reg}) cell differentiation [86]. T_{reg} cells have an essential role in immunological homeostasis through maintaining peripheral tolerance and controlling inflammation [87]. Butyrate-producing bacteria (e.g. *Roseburia intestinalis, Faecalibaterium prausnitzii*) were lower among individuals with T2DM in one study [81]. Conversely, dysbiotic intestinal bacterial species are hypothesized to cause systemic metabolic endotoxemia, which

subsequently leads to metabolic inflammation, insulin resistance, and dyslipidemia [81-84]. Prior studies found decreased gut microbiota diversity associated with active TB disease (and post-*M. tb* infection in mice) [88]. However preliminary evidence is limited, particularly among humans and in populations with low BMI (***Chapter IV***).

Vitamin D regulates innate and adaptive immune responses [44, 78, 79, 89-93], including in the gastrointestinal tract and among intestinal microbiota [94-97]. Therefore, prior studies have assessed the interactions between vitamin D and the gut microbiome (Table 4) [94, 98]. Two murine studies demonstrated that VDR and CYP27B1 knockout mice had altered intestinal microbiota [94, 99]. Additionally, 1,25(OH)2D treatment in CYP27B1 knockout mice was protective against colitis severity and decreased Helicobacteraceae in fecal samples [94]. Further studies among human populations are needed to confirm these findings (***Chapter IV***), and to assess the respective roles of different microbiomes (such as gut versus lung microbiome) as well as potential cross-talk between different microbiomes.

Table 4. Studies assessing the association between the gut microbiome and vitamin D (25[OH]D, VDR) [a]

Study Design	Sample Size		Location	Exposure	Outcome				Key Findings	Ref
					Assessment	Biological Specimen	Microbial Assessment Method(s)	Hypervariable Regions [b]		
	Cases	Controls								
Murine Models	5 VDR-/- C57BL6 mice	3 wild type C57BL6 mice	Illinois, United States	VDR knockout	Bacterial abundance, functional pathways	Fecal and cecal stool samples	454 pyrosequencing	V4-V6	• In fecal stool of VDR -/- mice, *Lactobacillus* decreased; *Clostridium* and *Bacteriodetes* increased • VDR -/- mice (vs WT): Cecal microbiomes had 26 increased and 14 decreased functional modules; fecal microbioms had 41 increased and 31 decreased functional modules ($p<0.05$)	[140]
	2 VDR-/-, 4 CYP27B1 -/- C57BL6 mice	2 wild type (for VDR -/-) and 4 (for CYP25B1 -/-) C57BL6 mice (age and sex matched)	Pennsylvania, United States	VDR, CYP27B1 knockouts	Bacterial abundance	Fecal samples	454 pyrosequencing	V3	• CYP and VDR -/- mice had increased bacteria in Bacteroidetes and Proteobacteria phyla; and decreased in Firmicutes and Defferibacteres phyla, compared to WT • CYP -/- mice had increased bacteria in Helicobacteraceae family, relative to WT • CYP and VDR -/- mice had higher number of bacteria in the Lactobacillaceae and Lachnospiraceae families, compared to WT	[139]
Cohort (GWAS)	1,812 humans		Kiel, Germany	VDR gene	Bacterial variation	Fecal samples	16S rRNA sequencing	V1-V2	• VDR gene associated with differences in overall microbial variation	[155]
Cross-sectional	150 young adults		São Paulo, Brazil	25(OH)D concentration	Bacterial composition	Fecal samples	16S rRNA sequencing	V4	• PCR, E-selectin; and abundances of *Coprococcus* and *Bifidobacterium* were inversely correlated with 25(OH)D	[175

Intervention	16 healthy humans	Graz, Austria	Vitamin D3 supplementation (8 weeks; weekly dosage of 980 IU/kg bodyweight)	Bacterial composition	7 sites (e.g. stomach, small bowel, colon, stool)	16S rRNA sequencing	V1-V2	• Vitamin D3 supplementation: Decreased relative abundance of Gammaproteobacteria (including Pseudomonas spp., Escherichia/Shigella spp.); increased bacterial richness • Increased CD8+ T cell fraction in terminal ileum • No changes in terminal ileum, appendiceal orifice, ascending colon, sigmoid colon, stools	[176]

[a] PubMed search strategy was based on the following terms: "("vitamin d"[MeSH Terms] OR "vitamin d"[All Fields] OR "ergocalciferols"[MeSH Terms] OR "ergocalciferols"[All Fields]) AND ("microbiota"[MeSH Terms] OR "microbiota"[All Fields] OR "microbiome"[All Fields])" and restricted by publication date (on or prior to October 1, 2017). Exclusion criteria included: 1) not primary data source (including reviews, editorials); 2) no whole microbiome sequencing techniques; 3) no assessment of vitamin D status, genes involved in vitamin D metabolism (e.g. VDR, CYP27B1), or related intervention. Among the 134 studies initially identified, 5 studies were within the scope of this review and included.

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CHAPTER II: Characterizing the source population for a randomized controlled trial of vitamin D supplementation in southern India

Elaine A. Yu,[1] Julia L. Finkelstein,[1] Patsy M. Brannon,[1] Wesley Bonam,[2] David G. Russell,[3] Marshall J. Glesby,[4] Saurabh Mehta[1,5*]

[1]Division of Nutritional Sciences, Cornell University, Ithaca, New York, United States

[2]Arogyavaram Medical Centre, Madanapalle, Andhra Pradesh, India

[3]Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, New York, United States

[4]Weill Cornell Medical College, New York, New York, United States

[5]Institute for Nutritional Sciences, Global Health, and Technology, Cornell University, Ithaca, New York, United States

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Abbreviations

25(OH)D: 25-hydroxyvitamin D

AFB: acid-fast bacilli

ATBD: active tuberculosis disease

aOR: adjusted odds ratio

aRR: adjusted risk ratio

AUC: area under the receiver operating characteristic curve

BMI: body mass index

CI: confidence interval

DEQAS: Vitamin D External Quality Assessment Scheme

ESR: erythrocyte sedimentation rate

HbA1c: glycated hemoglobin

HIV: human immunodeficiency virus

IDF: International Diabetes Federation

INR: Indian rupees

IQR: interquartile range

MCH: mean corpuscular hemoglobin

MCV: mean corpuscular volume

MUAC: mid-upper arm circumference

NPV: negative predictive value

OR: odds ratio

PPV: positive predictive value

RCT: randomized controlled trial

ROC: receiver operating characteristic

T2DM: Type 2 diabetes mellitus

US: United States

WC: waist circumference

WHO: World Health Organization

ABSTRACT

Background: Determining nutritional status among the source population is critical to the design and conduct of randomized controlled trials. This "phase 0" is necessary to evaluate the burden of disease and potential to benefit, and appropriately target interventions.

Objective: Our study objective was to evaluate the nutritional profile of a clinical population in India, from which study participants will be recruited for a randomized controlled trial of vitamin D supplementation (ClinicalTrials.gov #: NCT01992263).

Methods: In this cross-sectional study, nutritional status of outpatients (n=834) was assessed by biochemical indicators, anthropometry, and clinical examination during their hospital visit. Anemia was defined by hemoglobin, per World Health Organization recommendations.

Results: Overall, 71.9% of study participants had anemia (females 82.1%, males 68.2%); among these, 29.2% had hypochromia and microcytosis. Median 25-hydroxyvitamin D was 51.5 nmol/L (IQR 36.6-69.6). Most study participants had low or normal body mass index (BMI; <25 kg/m^2; 88.2%) or waist circumference (WC; < International Diabetes Federation diabetes screening cut-offs among South Asian populations; 79.3%), which would be considered low risk in diabetes screening. However, one-third of study participants either had glycated hemoglobin (HbA1c) ≥ 6.5% (12.3%) or ≥ 5.7% and <6.5% (20.3%). BMI ≥25.0 kg/m^2 demonstrated low sensitivity (0.21; 95% CI: 0.06, 0.35) as a screening indicator for HbA1c ≥ 6.5%.

Conclusions: This "phase 0" investigation yielded three key findings: 1) Most

individuals had anemia but not microcytosis or hypochromia, indicators of iron deficiency. 2) Despite the median BMI of 18.8 kg/m,^{2} nearly one-third of study participants had HbA1c ≥ 5.7%. 3) Current population screening cut-offs (based on BMI or WC) demonstrated low sensitivity in pre-diabetes and diabetes screening, suggesting the need for population-specific cut-offs. These results indicate the suitability of this source population for a nutritional intervention trial, and facilitate intervention tailoring and identifying appropriate potential confounders for trial outcomes.

Key words: Nutrition, vitamin D, micronutrients, anthropometry, assessment

INTRODUCTION

One-third of the global population is estimated to be malnourished (1). Key interventions (2), a strategic framework (3), and goals (4, 5) were recently outlined to achieve improved nutrition, in order to support human health and development. Despite public health and policy efforts, numerous obstacles still impede our ability to effectively target malnutrition (1, 2, 4, 6).

One major challenge is the need for comprehensive nutritional assessment to identify appropriate target populations for conducting intervention trials to establish efficacy and inform the development and scale-up of existing policies and programs. Defining the nutritional profile of the source population (7, 8) is a critical preparation step for intervention studies, in order to evaluate the suitability of study treatment regimens and the likelihood to benefit of potential study participants. Similarly, many global clinical and public health recommendations require information about the target population to inform implementation in specific settings.

In this study, "source population" was defined as the underlying study population from which the participants will be recruited and enrolled in a randomized controlled trial (9). Many factors affect the question of whether study interventions are appropriate among a specific source population. These relate primarily to ethical considerations, characterization of disease burden, and biological plausibility and include:

1) Ethical considerations: *Will proposed interventions (dosage, duration, frequency) cause as minimal risks as possible for study participants (e.g.,*

avoiding toxicity and exceeding tolerable upper limits)? Is there a state of clinical equipoise?

2) Characterization of disease burden: *What is the prevalence of micronutrient deficiency in the source population? What is the burden of disease from related comorbidities?*
3) Biological plausibility: *What is the potential to benefit among potential study participants?*

The goal of this study was to conduct a "phase 0" nutritional assessment of a clinical source population, from which study participants will be recruited and enrolled for a randomized controlled trial (RCT) of vitamin D supplementation in adults with active tuberculosis disease (ATBD) in southern India (ClinicalTrials.gov identifier: NCT01992263). The objectives were to assess the prevalence of malnutrition, based on biochemical (i.e., vitamin D, hemoglobin, anemia) and anthropometric indicators. We also evaluated the relative diagnostic performance of common anthropometric cut-off values (i.e., body mass index [BMI], waist circumference [WC]) in population-based screening for diabetes, given the increasing epidemiological and nutritional transition in similar low- and middle-income settings.

METHODS

Ethical conduct of research

The study protocol was approved by the Institutional Review Board at Cornell

University, and the Institutional Ethics Committee at Arogyavaram Medical Centre. All study participants provided informed consent to participate prior to data collection.

Study population

This cross-sectional study was conducted at Arogyavaram Medical Centre in Andhra Pradesh, India. The clinic is part of a hospital with inpatient facilities, and regularly receives a number of patients with suspected or confirmed ATBD. Hospital physicians referred their patients to study staff. Study participants (n=834) were recruited and enrolled during their hospital visits between September 2014 and May 2016. Exclusion criteria were: age <18 years, severe illnesses or complications requiring hospitalization (i.e., apart from preventative tuberculosis control measures), and no hemoglobin data (***Figure 2***).

Data collection

Structured interviews were administered to collect sociodemographic, clinical, nutritional, and anthropometric data by trained research assistants in the local language, Telugu. Sociodemographic covariates included: age, sex, educational level, monthly household income, and cigarette use.

Sputum and blood samples were collected from a subset of study participants (***Figure 2***), based on clinical assessment by hospital physicians and standard of care. For ATBD assessment, patients were requested to provide a sputum sample at the time of his or her initial hospital visit, and a second sputum sample on the following morning. Trained

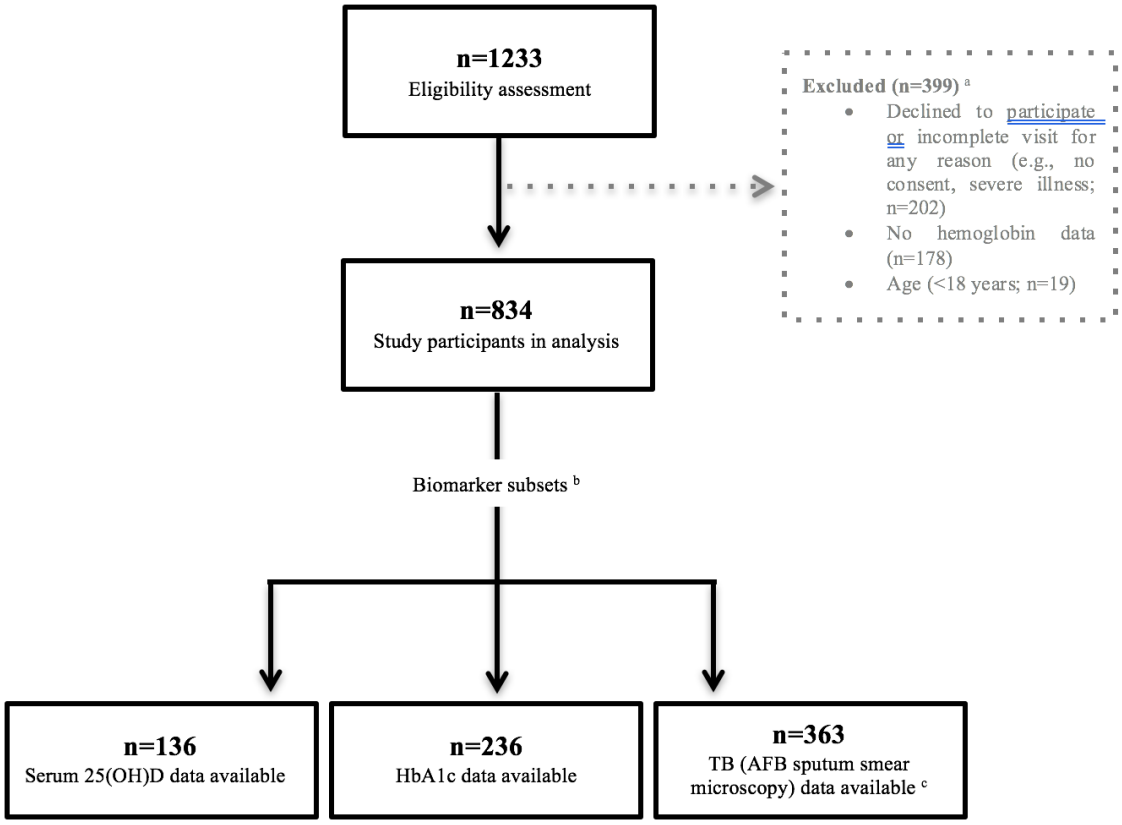

Figure 2. Study participant flow chart

phlebotomists collected blood samples using standard clinical protocols.

Laboratory analyses

Each sputum sample was assessed for ATBD by the detection of standard acid-fast bacilli (AFB) with Ziehl-Neelsen staining and conventional light microscopy. Blood samples were assayed for glycated hemoglobin (HbA1c [%]) by high-performance liquid chromatography (D-10; Bio-Rad Laboratories, Hercules, California, United States [US]). Complete blood counts were assessed by an automated hematology analyzer (BC-2800; Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, People's

Republic of China). Serum 25-hydroxyvitamin D (25[OH]D) was assayed by chemiluminescence immunoassay (LIAISON; DiaSorin Inc., Stillwater, Minnesota, US). The chemiluminescence immunoassay instrument was operated by a laboratory that participated in the Vitamin D External Quality Assessment Scheme (DEQAS), which used National Institute of Standards and Technology (Department of Commerce, US) references.

Anthropometry

Anthropometric measurements were assessed, based on World Health Organization (WHO) recommendations and other commonly utilized methods (10, 11). Height, WC, mid-upper arm circumference (MUAC), and skinfold thickness were measured to the nearest 0.1 centimeter; weight was assessed to the nearest 0.1 kilogram. Biologically unlikely values were considered missing. Total body and trunk fat (kg) were assessed by bioelectrical impedance analysis (BC-418 MA; Tanita Corporation, Tokyo, Japan; 8 electrode).

Definitions

Educational level was categorized based on self-reported completion of formal coursework (i.e., no formal education or illiterate; primary [grades 1-5], secondary [grades 6-12, including higher secondary], and any higher education [college, graduate, post-graduate]). Self-reported monthly household income was dichotomized as < 5000 Indian rupees (INR) or ≥ 5000 INR) (12). This cut-off was rounded from the cut-off of 4860 INR, based on the estimated monthly consumption expenditure for a family of five

residing in a rural area (2011-2012 prices) and the national poverty line of India (Government of India, Planning Commission, 2014 Report) (12). Cigarette use was categorized as current, previous, or never.

ATBD was defined as at least one positive AFB sputum smear result. This included patients with one positive AFB result (regardless of first or second sputum sample), as well as two positive AFB results, according to the standard ATBD diagnostic guidelines in India (13). HbA1c was categorized by common cut-off values (≥ 6.5%, ≥ 5.7% to < 6.5%, and <5.7%) that are recommended for clinical diagnosis of diabetes and pre-diabetes (14, 15). Low vitamin D status was defined with several cut-off values (25[OH]D <25.0 nmol/L (16), <40.0 nmol/L (17), <50.0 nmol/L (17, 18), <75.0 nmol/L (18)).

Hemoglobin (g/L) was considered as continuous and categorical values (including quintiles). Anemia and severe anemia were defined by hemoglobin cut-offs, per WHO recommendations (***Table 11 - Supplemental***) (19). Hemoglobin was adjusted for smoking by subtracting 0.3 g/L among any individuals who self-reported as currently smoking (19). We also considered other factors that affect anemia (e.g. pregnancy, residential elevation above sea level, smoking) (19). However, no study participants self-reported pregnancy; and all study participants visited the hospital study site, which has an elevation that does not require altitude adjustment (19).

Red blood cell morphology were categorized to assess abnormalities, which reflect

different causes of anemia and commonly assist in evaluating different types of anemia (20). Microcytosis (<80 femtoliters/cell) was defined as mean corpuscular volume (MCV; femtoliters/cell; ***Table 11 - Supplemental***) (20). Mean corpuscular hemoglobin (MCH; picograms/cell) was categorized as hyper-, normo-, and hypochromia (***Table 11 - Supplemental***) (20). Hypochromic microcytic anemia was defined by hemoglobin (men <130 g/L, women <120 g/L (19)) as well as MCV <80 femtoliters/cell and MCH <27 picograms/cell (***Table 11 - Supplemental***) (20). Elevated erythrocyte sedimentation rate (ESR; mm/hr) was categorized according to age- and sex-specific cut-off values (***Table 11 - Supplemental***) (21, 22). Biologically unlikely values were considered missing; these included outliers (<2.5th and >97.5th percentiles).

Anthropometric measurements (BMI, WC) were considered as continuous and categorized variables. Body mass index was defined as per standard WHO cut-offs (underweight < 18.5 kg/m^2; normal weight ≥ 18.5 and < 25.0 kg/m^2; overweight ≥ 25.0 and < 30.0 kg/m^2; and obese ≥ 30.0 kg/m^2) (11). Additionally, we considered BMI cut-offs for Asian populations (≥ 18.5 to < 23.0 kg/m^2; ≥ 23.0 to < 27.5 kg/m^2; ≥ 27.5 kg/m^2), based on a WHO expert consultation (23). Waist circumference was considered as a continuous variable and tertiles based on distribution in this population. Elevated waist circumference was also defined based on International Diabetes Federation (IDF) cut-offs (men ≥90 cm, women ≥80 cm) recommended for individuals in South Asia (24).

Statistical analyses

Continuous variables were assessed for normality using the Shapiro-Wilk test. For

descriptive statistics, continuous variables were reported as medians (interquartile ranges [IQRs]); categorical variables were reported as percentages. Subgroup comparisons were based on tests for continuous (i.e., Kruskal-Wallis) and categorical variables (i.e., likelihood ratio test). Spearman's rank correlation coefficients assessed the respective bivariate associations in preliminary analysis.

We assessed associations with univariate and multivariate regressions (log-binomial and logistic for categorical outcomes of interest) with a complete case analysis approach. Associations between anthropometric screening indicators (BMI, WC) and HbA1c ≥ 6.5% were assessed by multivariate log-binomial regressions; key covariates (age, sex) were accounted for in these models.

The association between anemia and ATBD was evaluated by multivariate logistic regressions. Confounding assessment was based on the Rothman and Greenland approach (9). Potential confounders were determined *a priori* from previous literature (9, 19, 25). Based on univariate logistic regressions between each covariate with a respective outcome of interest, we included all covariates with $p<0.25$ in initial full models (9); key independent variables and covariates (age, sex) remained in multivariate models regardless of p-values, according to prior literature. Variables with $p<0.05$ were retained in the final multivariate models, according to a backwards stepwise regression approach (9).

The predictive performance of anthropometric screening indicators (i.e., BMI, WC; categorical variables) for elevated HbA1c (≥ 6.5%) were assessed by sensitivity,

specificity, positive predictive value (PPV), negative predictive value (NPV), and receiver operating characteristic (ROC) curve analysis (26). Observed area under the ROC curves (AUCs) were compared to AUC for the null hypothesis (0.5), based on the contrast matrices of differences between the areas under the ROC curves (ROCCONTRAST in SAS statistical software) (27). We also examined the predictive performance of anthropometric screening indicators, considering a cut-off of HbA1c ≥ 5.7% as the outcome of interest.

Statistical analyses were conducted with SAS statistical software (version 9.4; SAS Institute Incorporated, Cary, North Carolina). All comparisons were two-sided; and considered statistically significant with α value of 0.05.

RESULTS

Sociodemographic characteristics

The median age of study participants was 48 years (IQR 35, 60; ***Table 5***), including individuals ranging from 18 to 87 years of age. Almost three-fourths of study participants were male (n=610; ***Table 5***). Over half of study participants (54.0%) had a self-reported monthly household income less than 5000 INR (***Table 5***).

Clinical morbidities: elevated glycated hemoglobin, tuberculosis

Overall, 12.3% of study participants had HbA1c ≥ 6.5%; and 20.3% of participants had HbA1c ≥ 5.7% and < 6.5% (***Table 5***). Median HbA1c was 5.4% (IQR 5.0, 5.8; ***Table 5***), and was not statistically different between men and women (p>0.05; ***Table 5***).

Among study participants with AFB assessment (n=363), 24.2% had ATBD (***Table 5***).

Biochemical indicators: vitamin D, anemia

Among study participants, median serum 25(OH)D concentration was 51.5 nmol/L (IQR 36.6-69.6; ***Table 6***). The prevalence of low vitamin D ranged between 9.6-80.9%, depending on the 25(OH)D cut-off values: 80.9% (< 75.0 nmol/L), 47.1% (<50.0 nmol/L), 30.2% (<40.0 nmol/L), and 9.6% (<25.0 nmol/L) of study participants had low vitamin D status (***Table 6***).

Median hemoglobin concentrations were 115.0 g/L (IQR 99.0, 129.7; ***Table 6***). A total of 71.9% of study participants (n=600) had anemia, and 6.4% had severe anemia (n=53; ***Table 6***). The prevalence of anemia was 82.1% in women (n=184), and 68.2% in men (n=416; ***Table 6***). Approximately a quarter of study participants had microcytosis (n=203), and none had macrocytosis. Nearly half of study participants were hypochromic (48.0%). A total of 59.0% of study participants had elevated ESR (n=492; median 30 mm/hr; IQR 15, 55; ***Table 6***).

Table 5. Sociodemographic and clinical characteristics of study participants [a]

	Total n=834	Men n=610 (73.1%)	Women n=224 (26.9%)	p
		Median (IQR) or n (%)		
Sociodemographic characteristics				
Age (years)	48 (35, 60)	51 (37, 64)	40 (30, 53)	<0.01 [b]
Education (completed level/class)				
Illiterate	404 (48.4%)	272 (44.6%)	132 (58.9%)	0.03 [c]
Primary [e]	209 (25.1%)	183 (30.0%)	26 (11.6%)	
Secondary, middle	152 (18.2%)	103 (16.9%)	49 (21.9%)	
Graduate, diploma, post-graduate	69 (8.3%)	52 (8.5%)	17 (7.6%)	
Monthly household income (INR)				
< 5000 [f]	354 (54.0%)	231 (50.7%)	123 (61.5%)	0.01 [c]
Self-reported current cigarette smoking	41 (4.9%)	41 (6.7%)	0 (0.0%)	--- [d]
Clinical characteristics				
ATBD [g]	88 (24.2%)	80 (27.7%)	8 (10.8%)	<0.01 [c]
Coughing [h]	693 (85.9%)	516 (87.9%)	177 (80.5%)	<0.01 [c]
With sputum production [h, i]	511 (74.2%)	394 (77.0%)	117 (66.1%)	<0.01 [c]
With blood [h, i]	72 (10.5%)	59 (11.6%)	13 (7.4%)	0.10 [c]
Fever	331 (41.2%)	246 (42.1%)	85 (39.0%)	0.43 [c]
Night sweats	127 (15.7%)	91 (15.5%)	36 (16.4%)	0.76 [c]

[a] Among study participants with available hemoglobin, age, and sex data (n=834). Covariates with missing observations included: monthly household income (n=178), cigarette smoking (n=2), HbA1c (n=598), ATBD (n=471), coughing (n=27), sputum production with cough (n=145), blood with cough (n=151), fever (n=31), night sweats (n=26).
[b] Hodges Lehmann estimator (two-sided normal approximation based on Wilcoxon sign rank test).
[c] Likelihood ratio test
[d] Sample cell size < 5
[e] Including completed and some primary education
[f] Cut-off value of 5000 INR rounded from the cut-off of 4860 INR, which is based on monthly consumption expenditure for a family of five residing in a rural area (2011-12 prices), per the India national poverty line (Government of India, Planning Commission, 2014 Report) (12)
[g] Based on AFB sputum smear microscopy.
[h] According to self-report
[i] Among those with cough

Table 6. Selected biochemical indicators of study participants [a]

	Total n=834	Men n=610 (73.1%)	Women n=224 (26.9%)	p [b]
Median (IQR) or n (%)				
25(OH)D (nmol/L)	51.5 (36.6, 69.6)	53.5 (40.0, 74.2)	49.2 (31.7, 64.2)	0.09 [i]
<25 nmol/L [c]	13 (9.6%)	8 (9.1%)	5 (10.4%)	0.80 [j]
< 40 nmol/L [d]	41 (30.2%)	22 (25.0%)	19 (39.6%)	0.08 [j]
<50 nmol/L [d, e]	64 (47.1%)	39 (44.3%)	25 (52.1%)	0.39 [j]
<75 nmol/L [e]	110 (80.9%)	67 (76.1%)	43 (89.6%)	<0.05 [j]
Hemoglobin (g/L) [f]	115.0 (99.0, 129.7)	120.0 (103.0, 132.7)	102.0 (90.0, 115.0)	<0.01 [i]
Anemia [g]	600 (71.9%)	416 (68.2%)	184 (82.1%)	<0.01 [j]
Severe anemia [g]	53 (6.4%)	32 (5.3%)	21 (9.4%)	0.04 [j]
Hypochromic microcytic anemia [g, h]	175 (23.0%)	108 (19.2%)	67 (34.2%)	<0.01 [j]
HbA1c (%)	5.4 (5.0, 5.8)	5.4 (5.1, 5.8)	5.3 (4.9, 5.7)	0.07 [i]
≥ 6.5%	29 (12.3%)	21 (13.0%)	8 (10.7%)	0.20 [j]
< 6.5% and ≥ 5.7%	48 (20.3%)	36 (22.4%)	12 (16.0%)	
< 5.7%	159 (67.4%)	104 (64.6%)	55 (73.3%)	
ESR (mm/hr)	30 (15, 55)	30 (12, 60)	25 (15, 45)	0.31 [i]
Elevated ESR [g]	492 (59.0%)	375 (61.5%)	117 (52.2%)	0.02 [j]
White blood cell count (cells/cmm)	9,500 (7,740, 12,300)	9,700 (7,900, 12,380)	9,200 (7,300, 12,100)	0.03 [i]
Elevated WBC (>11,000)	274 (34.4%)	213 (36.4%)	61 (28.9%)	<0.05 [j]
Differential count (%)				
Neutrophils	68.0 (60.0, 76.0)	70.0 (61.0, 77.0)	64.0 (57.0, 72.5)	<0.01 [i]
Lymphocytes	23 (16, 31)	22 (15, 30)	27.5 (20.0, 34.0)	<0.01 [i]

Monocytes	4 (3, 5)	4 (3, 5)	4 (3, 5)	0.73 [i]
Eosinophils	4 (3, 5)	4 (3, 5)	4 (3, 5)	0.56 [i]

[a] Among study participants with available anemia (hemoglobin) data (n=834). Covariates with missing observations included: 25(OH)D (n=698), hypochromic microcytic anemia (n=74), white blood cell count (n=38), lymphocytes (n=1).
[b] Comparison by sex
[c] Scientific Advisory Council of Nutrition cut-off value recommended to prevent rickets
[d] Institute of Medicine cut-off values for 25(OH)D deficiency and insufficiency among healthy populations
[e] Endocrine Society cut-off values for 25(OH)D deficiency and insufficiency among populations at risk of vitamin D deficiency
[f] Hemoglobin adjusted for smoking, based on WHO recommendations. 0.3 g/L hemoglobin was subtracted among any individuals who self-reported as currently smoking (19).
[g] See definitions in Supplementary Table 1
[h] Among those with anemia
[i] Hodges Lehmann estimator (two-sided normal approximation based on Wilcoxon sign rank test). Normality assumptions not met based on Shapiro-Wilk test statistic.
[j] Likelihood ratio test

Anthropometric indicators

Based on the standard WHO criteria for BMI, 46.8% of men and 40.2% of women were considered underweight (BMI < 18.5 kg/m^2; ***Table 7***). However, only 9.0% of men and 19.6% of women were considered overweight or obese (BMI ≥ 25.0; ***Table 7***). Considering alternative WHO BMI cut-offs recommended for Asian populations (23), 83.1% of men and 69.2% of women were underweight or normal weight (BMI < 23.0 kg/m^2; ***Table 7***). Median WC was 70.3 cm (IQR 65.0, 80.1) among men; and 66.9 cm (IQR 60.7, 77.5) among women (p<0.01; ***Table 7***). Overall, 79.3% of study participants were below the IDF WC cut-off (men 74.8%, women 91.9%; p<0.01; ***Table 7***).

Correlates of anemia and severe anemia

In multivariate regressions analyses, ATBD (adjusted odds ratio [aOR] 2.51 [95% confidence interval [CI]: 1.01, 6.25]), elevated ESR (aOR 3.51 [95% CI: 2.48, 4.97]), and BMI (aOR 0.92 [95% CI: 0.88, 0.95]) were significantly associated with anemia (all p<0.05), adjusting for age and sex (***Table 8***).

Table 7. Anthropometric indicators among men and women [a]

	Total n=834	Men n=610 (73.1%)	Women n=224 (26.9%)	p [b]
		Median (IQR) or n (%)		
BMI (kg/m^2)	18.8 (16.8, 22.2)	18.6 (16.8, 21.5)	19.6 (16.9, 23.8)	<0.01 [c]
WHO standard categories				
<18.5	357 (45.0%)	271 (46.8%)	86 (40.2%)	< 0.01 [d]
≥ 18.5 to < 25.0	342 (43.1%)	256 (44.2%)	86 (40.2%)	
≥ 25.0 to < 30.0	72 (9.1%)	45 (7.8%)	27 (12.6%)	
≥ 30.0	22 (2.8%)	7 (1.2%)	15 (7.0%)	
WHO alternative categories				
<18.5	357 (45.0%)	271 (46.8%)	86 (40.2%)	< 0.01 [d]
≥ 18.5 to < 23.0	272 (34.3%)	210 (36.3%)	62 (29.0%)	
≥ 23.0 to < 27.5	121 (15.3%)	82 (14.2%)	39 (18.2%)	
≥ 27.5	43 (5.4%)	16 (2.8%)	27 (12.6%)	
WC (cm)	69.5 (63.4, 79.6)	70.3 (65.0, 80.1)	66.9 (60.7, 77.5)	<0.01 [c]
< IDF cut-off [e]	634 (79.3%)	442 (74.8%)	192 (91.9%)	< 0.01 [d]
MUAC (cm)	23.7 (21.5, 26.4)	23.6 (21.5, 26.2)	24.0 (21.5, 27.0)	0.24 [c]
Low MUAC (<22.0 cm women, <23.0 cm men) [f]	306 (38.2%)	246 (41.7%)	60 (28.4%)	<0.01 [d]
Low MUAC (<19.0 cm women, <20.0 cm men) [f, g]	103 (12.9%)	86 (14.6%)	17 (8.1%)	0.01 [d]
Triceps skinfold thickness (mm)	9.3 (5.3, 15.3)	13.3 (8.3, 20.0)	12.0 (7.0, 18.5)	<0.01 [c]
Total body fat (kg)	18.3 (12.7, 25.0)	15.7 (11.0, 21.0)	28.0 (22.2, 35.1)	<0.01 [c]
Trunk fat (kg)	18.4 (11.6, 25.4)	16.0 (10.3, 22.7)	26.0 (18.7, 35.1)	<0.01 [c]

[a] Among study participants with available anemia (hemoglobin) data (n=834). Covariates with missing observations included: BMI (n=41), WC (n=34), MUAC (n=33), skinfold thickness (n=91), total body fat (n=41), trunk fat (n=41).
[b] Comparison between men and women
[c] Kruskal Wallis test. Normality assumptions not met based on Shapiro-Wilk test statistic.
[d] Likelihood ratio test
[e] International Diabetes Federation WC cut-off values among South Asian populations (men <80 cm, women <90 cm)
[f] MUAC cut-off values from previously suggested values (76)
[g] Severe wasting, grade 4 malnutrition cut-off values (10)

Table 8. Biochemical, clinical, and sociodemographic correlates of anemia [a]

		Univariate regressions		Multivariate regression [b]	
				n=793	
	n	*OR*	*95% CI*	*aOR*	*95% CI*
ATBD [c]	834				
Yes		4.23	1.77, 10.14	2.51	1.01, 6.25
No		---	---	---	---
Not assessed		0.59	0.42, 0.82	0.51	0.35, 0.74
Elevated ESR [a]	834	3.70	2.69, 5.07	3.51	2.48, 4.97
BMI (kg/m^2)	793	0.91	0.88, 0.95	0.92	0.88, 0.95
Age (years)	834	1.00	0.99, 1.01	1.01	1.00, 1.02
Sex	834				
Female		---	---	---	---
Male		0.47	0.32, 0.68	0.27	0.18, 0.42
Highest educational attainment	834				
Illiterate (versus any formal education)		1.59	1.17, 2.16		
Any formal education (primary, secondary, graduate, diploma, post-graduate)		---	---		
Monthly household income (INR) [d]	656				
< 5000		1.62	1.15, 2.27		
≥ 5000		---	---		

[a] See definitions in Table 11
[b] Based on complete-case analysis
[c] Based on AFB sputum smear microscopy
[d] Cut-off value of 5000 INR rounded from the cut-off of 4860 INR, which is based on monthly consumption expenditure for a family of five residing in a rural area (2011-12 prices), per the India national poverty line (Government of India, Planning Commission, 2014 Report) (12)

Hemoglobin concentrations were significantly lower (102.0 g/L [IQR 88.0, 116.0]) in individuals with ATBD, compared to those without ATBD (114.0 g/L [IQR 100.0, 128.0]) or individuals who were not assessed for ATBD (118.0 g/L [IQR 100.0, 132.0]; p<0.01). Median hematocrit also differed across ATBD status (ATBD, no ATBD, no ATBD assessment; p<0.01).

Predictive performance of anthropometric indicators for elevated HbA1c

Sensitivity, specificity, PPV and NPV of anthropometric indicators (BMI [standard and alternative WHO categorizations], and WC [IDF cut-offs]) of HbA1c ≥ 6.5% are presented in ***Table 9***. Considering BMI (standard WHO categories) as an indicator for HbA1c ≥ 6.5%, the AUC was 0.58 among all study participants (p=0.25), sensitivity was 0.21 (95% CI: 0.06, 0.35), and specificity was 0.92 (95% CI: 0.88, 0.96; ***Table 9***).

We also considered the predictive performance of anthropometric indicators for elevated HbA1c, using a cut-off of ≥ 5.7% as the outcome of interest (***Table 10***). BMI (standard WHO categories) had similar AUCs (overall 0.49 [p=0.77], men 0.54 [p=0.44], women 0.57 [p=0.42]), relative to diabetes as the outcome (HbA1c ≥ 6.5%). The AUC for WC were 0.57 (p=0.10) among all study participants, 0.53 (p=0.56) among men, and 0.61 (p=0.19) among women.

Associations between elevated HbA1c and anthropometry

WC (aRR 1.03 [95% CI 1.01, 1.06]; continuous variable) was associated with HbA1c ≥ 6.5%, adjusting for age and sex. Similarly, individuals in the highest tertile of WC were

Table 9. Comparison of anthropometric (BMI, WC) screening cut-offs for HbA1c ≥ 6.5% [a, b]

		HbA1c ≥6.5%	HbA1c <6.5%		Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
BMI [c] (kg/m^2)								
	≥ 25.0	6	16	22	0.21 (0.06, 0.35)	0.92 (0.88, 0.96)	0.27 (0.09, 0.46)	0.89 (0.84, 0.93)
	< 25.0	23	178	201				
		29	194	223				
	≥ 23.0	9	29	38	0.31 (0.14, 0.48)	0.85 (0.80, 0.90)	0.24 (0.10, 0.37)	0.89 (0.85, 0.94)
	< 23.0	20	165	185				
		29	194	223				
WC [d] (cm)								
	≥ IDF cut-off	14	33	47	0.50 (0.31, 0.69)	0.84 (0.78, 0.89)	0.30 (0.17, 0.43)	0.92 (0.88, 0.96)
	< IDF cut-off	14	168	182				
		28	201	229				

[a] Among study participants with available data (hemoglobin, HbA1c, as well as either BMI [categorical; n=223] or WC [n=229]).
[b] American Diabetes Association cut-points of HbA1c ≥ 6.5%.
[c] WHO classifications (standard and alternative categorization for Asian populations)
[d] IDF WC cut-off values among South Asian populations (men <80 cm, women <90 cm)

Table 10. Comparison of anthropometric (BMI, WC) screening cut-offs for HbA1c ≥ 5.7% [a, b]

		HbA1c ≥5.7%	HbA1c <5.7%		Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
BMI [c] (kg/m^2)								
	≥ 25.0	10	12	22	0.14 (0.06, 0.21)	0.92 (0.88, 0.96)	0.45 (0.25, 0.66)	0.68 (0.62, 0.75)
	< 25.0	64	137	201				
		74	149	223				
	≥ 23.0	16	22	38	0.22 (0.12, 0.31)	0.85 (0.80, 0.91)	0.42 (0.26, 0.58)	0.69 (0.62, 0.75)
	< 23.0	58	127	185				
		74	149	223				
WC [d] (cm)								
	≥ IDF cut-off	23	24	47	0.31 (0.20, 0.41)	0.84 (0.79, 0.90)	0.49 (0.35, 0.63)	0.71 (0.65, 0.78)
	< IDF cut-off	52	130	182				
		75	154	229				

[a] Among study participants with available data (hemoglobin, HbA1c, as well as either BMI [n=223] or WC [n=229]).
[b] American Diabetes Association cut-points of HbA1c ≥ 5.7%.
[c] WHO classifications (standard and alternative categorization for Asian populations)
[d] IDF WC cut-off values among South Asian populations (men <80 cm, women <90 cm)

positively associated with HbA1c ≥ 6.5% (Tertile 3 vs 1: adjusted risk ratio [aRR] 2.87 [95% CI 1.19, 6.92]), compared to those in the lowest WC tertile, adjusting for age and sex.

DISCUSSION

In summary, we conducted a "phase 0" nutritional assessment among a clinical source population from which we will recruit study participants for a RCT. Our results indicated a high prevalence of malnutrition, based on suboptimal biochemical (25[OH]D, hemoglobin) as well as anthropometric indicators (BMI, WC). More than 80% of study participants had 25(OH)D < 75 nmol/L; over 70% had anemia, including most men (68.2%) and women (82.1%). Among those with anemia, only 29.2% had hypochromia and microcytosis, which suggests that the etiology of anemia is multifactorial and cannot be largely attributed to iron deficiency in this population. Separately, anemia was associated with ATBD, adjusting for covariates. Common cut-off values for anthropometric screening indicators had suboptimal predictive performance in detecting elevated HbA1c among an adult outpatient population with lower adiposity. Findings suggest the need for population-specific cut-offs for BMI and WC, given that each remained respectively associated with HbA1c.

Malnutrition remains widely prevalent

We found that 30-80% of this source population had low vitamin D (based on cut-off values of <40, <50, or <75 nmol/L). These estimates were higher than a prior global estimate, which suggested approximately one billion people had deficient or insufficient

vitamin D (28), although the consistent use of standard reference materials and assay standardization is necessary for improved global estimates. In conjunction with the limited availability of vitamin D rich foods (natural, fortified) at the study site location, the high prevalence of low vitamin D suggests the potential to benefit the source population, in the context of a RCT of vitamin D supplementation.

Based on the WHO classification, the public health significance of anemia in our source population (82.1% women; 68.2% men) is considered severe (19). Anemia is estimated to affect 1.6 billion individuals globally (29). Over 20 million women of reproductive age have severe anemia, which is associated with increased mortality and adverse health outcomes, including poor cognitive function and lowered work productivity (30). In the second global nutrition target, the World Health Assembly established a goal of reducing anemia by 50% among women of reproductive age by 2025 (4, 5).

A previous global estimate of anemia prevalence is 24.8% (29), which is less than half the proportion observed in our source population. Moreover, in contrast to the global estimate of anemia among women (29.4% (95% CI: 24.5, 35.0) (30), four of every five females had anemia in our study. The global mean hemoglobin among non-pregnant women (126.0 g/L [95% credibility interval 124.0-128.0]; 15-49 years) (31) was higher than the median (102.0 g/L) among women in our source population. In South Asia, the mean hemoglobin among non-pregnant women was 119.0 g/L (95% credibility interval 115.0-124.0) (31), which was higher than our findings. Additionally, one study in India found similarly high prevalence of anemia among pregnant women (84.9%) and

adolescent girls (90.1%) (32).

Multifactorial etiology of anemia, including tuberculosis

Iron deficiency has been estimated to account for approximately 50% of anemia globally (30, 33); this is reflected in global recommendations that target iron deficiency as a strategy to improve iron deficiency anemia (34, 35). However, in our source population, 70.8% of study participants with anemia did not hypochromia and microcytosis, which are commonly considered hematological indicators of iron deficiency anemia (20). Previous studies have also corroborated the substantial heterogeneity and multifactorial etiology of anemia across diverse populations (30, 36). Aside from iron deficiency (30, 33), numerous other causes of anemia include: genetic hemoglobin disorders, other nutritional deficiencies (e.g. vitamin B12), and infectious and inflammatory diseases (30, 37-39). Critically, the effective prevention and management of anemia at the population level requires further delineation of the distribution, specific causes, and risk factors of anemia in diverse populations (40).

Additionally, our finding of the association between anemia and ATBD was consistent with a previous study in Tanzania, which showed a 4-fold increased risk of ATBD recurrence associated with anemia without iron deficiency ($p<0.001$) (39). Among adult patients with ATBD in South Korea, 31.9% had anemia (41).

Population-specific BMI and WC cut-off values for diabetes screening

Globally, nearly one of every ten (8.5%) adults has diabetes (42), which is similar to the

prevalence of diabetes (12.0%) among our study participants. Worldwide, the number of people with diabetes has more than tripled in the past four decades (42); and is projected to increase to 552 million by 2030 (43). Low- and middle-income countries have disproportionate deaths caused by high blood glucose and diabetes currently, and incident diabetes cases in future estimates (42, 43).

Higher BMI (overweight and obesity) and WC are well-established modifiable risk factors of type 2 diabetes mellitus (T2DM) (15, 24, 42, 44, 45). International and national public health entities (including the WHO (23, 42, 46, 47) and IDF (24)) recommend common cut-off values of elevated BMI and WC that identify individuals at risk for T2DM.

However consistent with our findings, a growing body of evidence suggests research gaps and limitations in the predictive performance of commonly used anthropometric indicator cut-offs for T2DM screening. First, the heterogeneity of body fat distribution is hypothesized to affect T2DM risk (48), which could cause common cut-points of anthropometric screening indicators for diabetes to perform worse (more false negatives or positives) in some populations. Central and android (upper body) obesity as well as visceral fat (49) have been more strongly associated with insulin resistance and T2DM, relative to overall obesity (50, 51). Studies have demonstrated that individuals with similar BMI sometimes differ substantially in body fat distribution and percentage (52-55), metabolic syndrome (56), T2DM (57, 58) and insulin resistance (59). As an example, the predisposition for central fat accumulation among Asian populations has

been observed to differ from Caucasians (60), which could explain differential T2DM risk among individuals with the same BMI (23).

Second, studies have begun elucidating the biological basis for these observed patterns. At the cellular level, functional metabolic differences between adipocytes (brown, white, and beige [brown in white]) have been characterized (61-63). Critically, studies have shown metabolically active brown adipocytes associated with improved T2DM indicators and lower BMI (62, 64); in contrast, white adipocytes were associated with visceral fat, which has been linked to insulin resistance (65).

Successfully addressing the diabetes epidemic requires considering effective screening among populations with different body composition patterns. Overall in our study, common cut-off values for anthropometric screening indicators had suboptimal predictive performance in detecting diabetes among an adult outpatient population with lower adiposity. Findings suggest the need for population-specific cut-offs for BMI and WC, given that: 1) each remained respectively associated with diabetes status; and 2) standard cut-offs misclassify the diabetes and pre-diabetes status of a number of study participants.

Similar to our results, the respective associations between BMI and WC with elevated HbA1c have been confirmed in several studies among populations in the US and Europe (45, 66, 67), India (68, 69) and China (70). These studies included a US study among Mexican Americans, which found an 11 times greater risk of non-insulin dependent

diabetes mellitus among those in the highest quartile of WC, compared to the lowest quartile (66). Separately, previous studies have also shown a wide heterogeneity of predictive performance of anthropometric indicators in diabetes and pre-diabetes screening. As an example, one US study among 12,814 adults (45-64 years; African American and white), areas under the ROC curves were similar for BMI (African American men 0.69, White men 0.70; African American women 0.66, White women 0.72) and WC (African American men 0.70, White men 0.70; African American women 0.69, White women 0.73) in predicting diabetes (71).

Although our results reveal several research gaps, the importance and challenges of determining appropriate population-specific cut-off values of anthropometric indicators have been acknowledged in previous literature (72-74) and by the WHO (23, 54). Future research questions include: What are the appropriate cut-offs across racial and ethnic subgroups, based on representative samples with external validity? How do different fat distributions (including differing body fat percentage and adipocyte type) affect the risk of T2DM incidence and severity? What are the cellular mechanisms involving different adipocyte types that contribute to T2DM development and progression?

In our study, there were several strengths, including the: sample size, assessment of multiple BMI and WC categories (based on widely used cut-off values and population distribution [quantiles]), use of a 25(OH)D assay instrument that was participating in DEQAS, serological indicators to define microcytosis and hypochromia, and hemoglobin adjustment for smoking (19).

This study had several limitations. These included the cross-sectional study design (with a single timepoint assessment, which precludes etiological inference); potential residual confounding; external validity (generalizability of findings, especially among healthy populations); assessment of additional causes of low hemoglobin; biological samples obtained per standard of care (only from participants with a clinical indication and not collected at random or from all participants); and limited biomarker data, such as diagnostics of human immunodeficiency virus (HIV) and other clinical morbidities. Despite this, according to the National Acquired Immunodeficiency Syndrome Control Organisation (Government of India), the HIV prevalence was 0.66% among adults (15-49 years) in Andhra Pradesh and Telangana (75).

In conclusion, our results highlight the importance of nutritional profiling among underlying source populations, as preparation for RCTs. Among a clinical population in rural South India, we found: 1) a high prevalence of adverse nutritional indicators (suboptimal vitamin D, anemia, and underweight); 2) the co-occurrence of malnutrition, infectious diseases and chronic ailments (including associations between: anemia and ATBD, diabetes and anthropometry); and 3) the need for improved population-specific screening for diabetes and pre-diabetes. Effective malnutrition prevention, treatment, and research efforts require comprehensive nutritional assessments, in order to inform study designs and intervention strategies in target populations.

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Author contributions

S.M., W.B., and E.A.Y. designed research; E.A.Y. conducted research; E.A.Y., J.F., and S.M. analyzed data; and all authors contributed to drafting and critically revising the paper. S.M. had primary responsibility for final content. All authors read and approved the final manuscript.

Conflicts of interest

E.A.Y., J.L.F., P.M.B., W.B., D.G.R., and M.J.G. have no conflicts of interest. S.M. is an unpaid board member of a diagnostic start-up focused on developing assays for low-cost and point-of-care measurement of certain nutrients from a drop of blood using results from his research as a faculty member at Cornell University.

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Table 11. Supplemental - Definitions of anemia and related red blood cell indices

		Hematological indicator	Age		Male	Female	Ref
Anemia		Hemoglobin (g/L) [b, c]	≥ 18 years	Non-pregnant	<130	<120	(19)
				Pregnant [c]	---	<110	
Severe anemia		Hemoglobin (g/L) [a, b]	≥ 18 years	Non-pregnant	<80	<80	(19)
				Pregnant [c]	---	<70	
Red blood cell size	Microcytic	MCV (femtoliters/cell)	---		<80		(20)
	Normocytic		---		≥ 80 and ≤ 100		
	Macrocytic		---		>100		
Erythrocyte color (hemoglobin content)	Hypochromic	MCH (picograms/cell)	---		<27		(20)
	Normochromic				≥ 27 and ≤ 33		
	Hyperchromic				>33		
Inflammation		ESR (mm/hr)	<50 years		>15	>20	(21, 22)
			≥50 years and <85		>20	>30	
			≥85 years		>30	>42	

[a] Data collection site is <1000 meters above sea level, and therefore there were no hemoglobin adjustments for altitude (19). Among current smokers, 0.3 g/L was subtracted from hemoglobin.
[b] Biologically unlikely hemoglobin values (<25, >200 g/L) considered missing
[c] No study participants self-reported as pregnant

Table 12. Supplemental - STROBE checklist for cross-sectional studies [1]

	Item No	Recommendation	Page number(s)
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	37
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	37-38
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	39-40
Objectives	3	State specific objectives, including any pre-specified hypotheses	39-40
Methods			
Study design	4	Present key elements of study design early in the paper	40-41
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	40-47
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants	40-47
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	40-47
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	40-47
Bias	9	Describe any efforts to address potential sources of bias	58-64
Study size	10	Explain how the study size was arrived at	40-41
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	40-47
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	40-47
		(*b*) Describe any methods used to examine subgroups and interactions	40-47
		(*c*) Explain how missing data were addressed	40-47
		(*d*) If applicable, describe analytical methods taking account of sampling strategy	N/A
		(*e*) Describe any sensitivity analyses	40-47
Results			

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analyzed	41-42
		(b) Give reasons for non-participation at each stage	42
		(c) Consider use of a flow diagram	42
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	47-53
		(b) Indicate number of participants with missing data for each variable of interest	47-53
Outcome data	15*	Report numbers of outcome events or summary measures	47-58
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	47-58
		(*b*) Report category boundaries when continuous variables were categorized	47-58
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	N/A
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	47-58
Discussion			
Key results	18	Summarize key results with reference to study objectives	58-64
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	58-64
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	58-64
Generalizability	21	Discuss the generalizability (external validity) of the study results	58-64
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	65

[1] Available at: http://strobe-statement.org/. Accessed February 10, 2016.

CHAPTER III: Vitamin D status and metabolic risk factors among a patient population with a high prevalence of low body mass index

Elaine A. Yu,[1] Patsy M. Brannon,[1] Marshall J. Glesby,[2] Julia L. Finkelstein,[1] Wesley Bonam,[3] David G. Russell,[4] Saurabh Mehta[1,5*]

[1]Division of Nutritional Sciences, Cornell University, Ithaca, New York, United States

[2]Weill Cornell Medical College, New York, New York, United States

[3]Arogyavaram Medical Centre, Madanapalle, Andhra Pradesh, India

[4]Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, New York, United States

[5]Institute for Nutritional Sciences, Global Health, and Technology, Cornell University, Ithaca, New York, United States

* Draft manuscript formatted according to journal requirements, in preparation for submission.

Abbreviations

1,25(OH)2D: 1,25-dihydroxyvitamin D

25(OH)D: 25-hydroxyvitamin D

AFB: acid-fast bacilli

aRR: adjusted risk ratio

BMI: body mass index

DBP: vitamin D binding protein

HbA1c: glycated hemoglobin

IDF: International Diabetes Federation

INR: Indian rupees

RR: risk ratio

TB: tuberculosis

US: United States

VDR: vitamin D receptor

WC: waist circumference

WHO: World Health Organization

ABSTRACT

Background: Vitamin D is a potential modifiable risk factor for metabolic abnormalities, however this association remains unclear among populations with heterogeneous body distributions. Our objective was to evaluate associations between 25-hydroxyvitamin D (25[OH]D) and metabolic risk factors (glycated hemoglobin [HbA1c], waist circumference [WC], blood pressure) among a population with a high prevalence of low body mass index (BMI).

Methods: In this cross-sectional study, adult outpatients (n=156) in India provided blood samples, which were assayed for serum 25(OH)D and HbA1c. Anthropometry and blood pressure were measured.

Results: Median 25(OH)D was 51.8 nmol/L (IQR 36.0, 70.0). Forty-four percent of participants had BMI <18.5 kg/m^2; median WC was 72.8 cm (IQR 66.0, 82.0) among men and 67.0 cm (IQR 60.0, 76.0) among women. 31.5% of patients had HbA1c ≥ 5.7%. Individuals in the lowest 25(OH)D quintile had an increased risk of HbA1c ≥ 5.7% (aRR 1.61; 95% CI: 1.02, 2.56), compared to other quintiles. 25(OH)D was inversely associated with WC ($p<0.01$), although not hypertension ($p>0.05$).

Conclusion: Elevated HbA1c ≥ 5.7% and WC were associated with low 25(OH)D. These findings indicate the need to elucidate further mechanisms and roles of vitamin D in preventing and managing metabolic risk factors among populations with low BMI.

Key words: Vitamin D, metabolism, glycated hemoglobin, waist circumference

INTRODUCTION

Metabolic risk factors affect 20-25% of adults globally and increase the risk for Type 2 *diabetes mellitus* and cardiovascular disease [1-3]. In 2015, metabolic risk factors (including high body mass index [BMI] and fasting plasma glucose) contributed the most to disability-adjusted-life-years worldwide [4]. The global mean fasting plasma glucose has risen over the past four decades [5]. Successfully countering the burden of disease from metabolic abnormalities requires more effective prevention, early diagnosis and treatment [2]. Current mitigation efforts are substantially hindered by a limited understanding of the pathophysiology and etiology, as well as modifiable risk factors (*e.g.* vitamin D) of metabolic abnormalities.

One major challenge has been understanding metabolic risk factors among populations with heterogeneous body composition. First, *do metabolic processes (including physiological and genetic differences) differ among people with low BMI?* Although high BMI (overweight, obese) is considered a risk factor for metabolic disorders [1], metabolic abnormalities are also commonly observed among some populations with low and normal BMI [6-10]. The prevalence of metabolic risk factors and abnormalities have increased in the past few decades among Asian countries (including India) [11,12], which have lower mean BMI than other countries in Europe and North America [13]. Globally, more than 60% of people with diabetes reside in Asia; approximate half of these individuals live in India and China [14].

There are several hypothesized explanations of the differential metabolic risk among

those with low BMI, including: 1) physiological (adipose tissue and cell type distribution [15-17], total body fat percentage [18]); and 2) genetic differences [19,20]. Subsequently, if metabolic processes differ among people with heterogeneous body composition, *are there differences in clinical recommendations (including prevention, detection, and treatment of metabolic risk factors) and modifiable risk factors (e.g. vitamin D) against metabolic risk factors and disorders?*

Vitamin D is a potential host-directed adjuvant for metabolic risk factors because it affects energy homeostasis (including glycemic control, central adiposity, lipid metabolism) and blood pressure [21-24]. At the cellular level, 1,25-dihydroxyvitamin D ($1,25[OH]_2D$) regulates adipocyte metabolism [25,26], including inhibiting adipogenesis [27-29]. From genetic studies, vitamin D receptor (VDR) and binding protein (DBP) have been associated with adipocyte metabolism, glucose tolerance, and insulin secretion [26,30]. Animal models have corroborated that $1,25(OH)_2D$ inhibits renin gene expression, which regulates blood pressure [22]. Despite the evidence of links between vitamin D and metabolic risk factors, there are major research gaps in understanding this association, including among individuals with heterogeneous body compositions.

Therefore, our study objective was to assess the associations between serum 25-hydroxyvitamin D (25[OH]D) and metabolic indicators (glycated hemoglobin [HbA1c], waist circumference [WC], blood pressure) among an adult patient population with a high prevalence of low BMI. The secondary objective was to assess the association

between 25(OH)D and active tuberculosis (TB) disease, given the high prevalence of patients with suspected or confirmed active TB disease at the data collection site.

MATERIALS AND METHODS

Ethical conduct of research

The Cornell University Institutional Review Board, and Arogyavaram Medical Centre Institutional Ethics Committee approved of this study. Study participants provided informed consent to participate.

Study population

The study design has been previously detailed. Briefly, this cross-sectional study recruited and enrolled study participants (n=156) during their clinical visits between September 2014 and May 2016. The study site was in a rural region of Andhra Pradesh, India at a rural hospital. This hospital has inpatient and outpatient facilities, which regularly provide services for patients with suspected or confirmed active TB disease. Hospital physicians referred outpatients to study staff. Exclusion criteria included: age <18 years; unavailable data for key covariates (serum 25[OH]D, total body fat, BMI); and declining to participate or an incomplete visit for any other reason (*e.g.* severe illness or complications).

Data collection

Trained research assistants collected sociodemographic and clinical information from study participants through structured interviews. A study physician conducted a

complete examination, including blood pressure measurements. Anthropometric measurements were assessed, based on World Health Organization (WHO) and other common recommendations [31,32]. Height and WC were measured to the nearest 0.1 centimeter (cm); weight was assessed to the nearest 0.1 kilogram (kg). Total body and trunk fat (kg) were assessed by bioelectrical impedance analysis (BC-418 MA; Tanita Corporation, Tokyo, Japan; 8 electrode).

Subgroups of study participants provided blood and sputum samples, based on clinical assessments by their hospital physicians and standard of care. Hospital phlebotomists collected blood samples, per the standard of care procedures. For active TB disease assessment, patients were requested to provide one sputum samples at the time of his or her initial hospital visit, and a second sputum sample the subsequent morning.

Laboratory assays

Serum 25(OH)D (nmol/L) was assayed by chemiluminescence immunoassay (LIAISON; DiaSorin Inc., Stillwater, Minnesota, United States [US]). We also participated in the D External Quality Assurance Scheme (DEQAS; www.deqas.org) program; our results were within +/- 20% of the National Institute of Standards and Technologies (NIST) target values. Blood samples were assayed for HbA1c (%) by high-performance liquid chromatography (D-10; Bio-Rad Laboratories, Hercules, California, US). Each sputum sample was assessed for active TB disease by the detection of standard acid-fast bacilli (AFB) with Ziehl-Neelsen staining and conventional light microscopy. Additionally, complete blood counts were assessed by

an automated hematology analyzer (BC-2800; Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, People's Republic of China).

Outcomes

WC was considered as continuous and categorical variables (International Diabetes Federation [IDF] cutoffs, tertiles). The IDF cutoffs were the sex-specific values (males <90 cm, females <80 cm), which are recommended for individuals in South Asia [33]. Biologically unlikely values were considered missing. HbA1c was categorized by common cut-off values: ≥ 6.5%, ≥ 5.7% to < 6.5%, <5.7% [34,35]. High systolic blood pressure was considered ≥ 140 mm Hg; and high diastolic blood pressure was ≥ 90 mm Hg. Additionally, we categorized individuals as having abnormal blood pressure if: systolic blood pressure ≥ 140 mm Hg, diastolic blood pressure ≥ 90 mm Hg, or both were elevated.

Active TB disease status was defined as at least one positive AFB sputum smear result. This included patients with one positive AFB result (regardless of first or second sputum sample), as well as two positive AFB results, according to the standard active TB disease diagnostic guidelines in India [36].

Exposure

Vitamin D status was defined with several cut-off values (25[OH]D <25.0 nmol/L [37], <40.0 nmol/L [38], <50.0 nmol/L [38,39], <75.0 nmol/L [39]). Additionally, we categorized 25(OH)D (nmol/L) by quintiles.

Covariates

Sociodemographic and clinical characteristics

Low monthly household income was considered < 5000 Indian rupees (INR). This cut-off value was rounded up from 4860 INR, which is based on the estimated monthly consumption expenditures for a family of five residing in a rural area (2011-2012 prices) and national poverty line (Government of India, Planning Commission, 2014 Report) [40]. Self-reported educational attainment was categorized as illiterate, primary (grades 1-5), secondary or middle (grades 6-12), and higher (including graduate, diploma, post-graduate). We defined anemia and severe anemia by the WHO recommendations for hemoglobin cut-off values [41]. Among individuals who currently smoked, per self-report, 0.3 g/L was subtracted from the measured hemoglobin concentration [41].

Anthropometry and body composition

Biologically unlikely values were considered missing. BMI was categorized per standard WHO cut-offs (underweight < 18.5 kg/m^2; normal weight ≥ 18.5 and < 25.0 kg/m^2; overweight ≥ 25.0 and < 30.0 kg/m^2; and obese ≥ 30.0 kg/m^2) [32]. We included additional BMI categorizations, based on a WHO expert consultation regarding BMI cutpoints for Asian populations (≥ 18.5 to < 23.0 kg/m^2; ≥ 23.0 to < 27.5 kg/m^2; ≥ 27.5 kg/m^2) [10]. We defined limb fat as the sum of body fat (kg) of all four limbs.

Statistical analysis

Normality was assessed with the Shapiro Wilk test statistic. For descriptive statistics,

we reported medians and interquartile ranges (IQRs) for continuous variables, and n (%) for categorical variables. Differences between subgroups were compared by Wilcoxon sign rank or likelihood ratio tests.

Per the study objectives, we assessed associations with multivariate linear and binomial regressions. For each outcome of interest, the selection of potential confounders was based on approaches suggested by Rothman and Greenland [42]. In brief, we identified known or suspected risk factors based on *a priori* literature review [22,43]. For each association of interest, we included the confounders in the final adjusted model based on a 10% change-in-estimate criterion [42]. For example, considering the association between vitamin D and WC, this set of covariates (including age, sex, active TB disease, anemia, monthly household income) were then adjusted for in each final model. For binary outcomes, binomial regressions were utilized when models converged. Modified Poisson estimates were utilized if binomial models failed to converge [44].

SAS statistical software (version 9.4; SAS Institute Incorporated, Cary, North Carolina, US) was utilized for all analyses. Statistical significance was assessed by two-tailed tests and an alpha value of 0.05. Descriptive statistics were reported in ***Table 13***. In multivariate regressions, analytical subgroups were based on complete case analysis for each association (vitamin D and respectively, WC [n=150; ***Table 14***], HbA1c [n=149; ***Table 15***], blood pressure [n=99; ***Table 16***], and active TB disease [n=81; ***Table 17***]); the missing-data indicator method was utilized for covariates with missingness [42].

RESULTS

25-hydroxyvitamin D

Median serum 25(OH)D was 51.8 nmol/L (IQR 36.0, 70.0; ***Table 13***). Median 25(OH)D concentration was 24.9 nmol/L (IQR 19.1, 28.7), 38.8 nmol/L (IQR 35.4, 42.3), 51.7 nmol/L (IQR 49.0, 55.2), 67.5 nmol/L (IQR 63.1, 70.0), and 82.9 nmol/L (IQR 76.9, 102.1) in quintiles 1 through 5.

Metabolic risk factors

Among all study participants, median HbA1c was 5.3% (IQR 5.0, 5.8; ***Table 13***). 17 (11.4%) patients had HbA1c ≥ 6.5%, and 30 (20.1%) had HbA1c ≥ 5.7%. Median WC was 70.0 cm (IQR 63.0, 80.2); this differed across BMI category ($p<0.01$). Overall, median systolic blood pressure was 110 mmHg (IQR 100, 120); 11.1% of study participants had systolic blood pressure ≥ 140 mmHg. Median diastolic blood pressure was 70 mmHg (IQR 60, 80); 13.1% of patients had elevated diastolic blood pressure (≥ 90 mmHg). 8.1% of patients had abnormal blood pressure (either elevated systolic, diastolic, or both).

Active tuberculosis disease and other study participant characteristics

Among patients assessed for active TB disease (n=81), 19 (23.5%) had a positive AFB sputum smear microscopy result (***Table 13***).

In terms of sociodemographic characteristics, the median age was 45.0 years (IQR 30.0, 60.0). The majority of the study population was male (n=100 [64.1%]). 39.1% of study

Table 13. Sociodemographic and clinical characteristics, stratified by BMI, among patients in rural South India [a,b]

	N=156	BMI				p [c]
		Under weight (<18.5)	Normal weight (≥ 18.5 to < 23.0)	Over weight (≥ 23.0 to < 27.5)	Obese (≥ 27.5)	
		64 (43.8%)	59 (40.4%)	16 (11.0%)	7 (4.8%)	
Sociodemographic						
Age (years)	45.0 (30.0, 60.0)	44.0 (29.5, 60.0)	40.0 (29.0, 53.0)	53.5 (44.5, 62.5)	40.0 (19.0, 50.0)	0.10 [d]
Sex (male)	100 (64.1%)	42 (65.6%)	38 (64.4%)	9 (56.3%)	2 (28.6%)	0.27 [e]
Education (completed level/class)						0.04 [e]
Illiterate	61 (39.1%)	26 (40.6%)	17 (28.8%)	11 (68.8%)	3 (42.9%)	
Primary [f]	40 (25.6%)	17 (26.6%)	18 (30.5%)	2 (12.5%)	0 (0.0%)	
Secondary, middle	35 (22.4%)	15 (23.4%)	13 (22.0%)	3 (18.8%)	3 (42.9%)	
Graduate, diploma, post-graduate	20 (12.8%)	6 (9.4%)	11 (18.6%)	0 (0.0%)	1 (14.3%)	
Monthly household income (INR)						
< 5000 [g]	60 (47.2%)	29 (53.7%)	21 (42.9%)	6 (60.0%)	2 (28.6%)	0.41 [e]
Clinical						
25(OH)D (nmol/L)	51.8 (36.0, 70.0)	53.5 (37.9, 75.9)	51.7 (35.3, 70.3)	47.4 (29.1, 56.6)	35.4 (16.2, 39.4)	0.05 [d]
<25 [i]	17 (10.9%)	7 (10.9%)	6 (10.2%)	2 (12.5%)	2 (28.6%)	0.65 [e]
< 40 [j]	49 (31.4%)	19 (29.7%)	18 (30.5%)	6 (37.5%)	6 (85.7%)	0.03 [e]
<50 [j,k]	73 (46.8%)	29 (45.3%)	27 (45.8%)	9 (56.3%)	6 (85.7%)	0.17 [e]
<75 [k]	126 (80.8%)	47 (73.4%)	48 (81.4%)	16 (100.0%)	7 (100.0%)	<0.01 [e]
Waist circumference (cm)	70.0 (63.0, 80.2)	63.1 (59.0, 67.5)	75.0 (70.0, 81.5)	91.0 (83.0, 95.3)	87.0 (84.0, 103.2)	<0.01 [d]
Body composition						
Total body fat (%)	18.1 (12.2, 24.0)	12.2 (7.8, 17.7)	20.7 (15.7, 27.9)	28.3 (23.1, 37.3)	40.0 (32.1, 43.1)	<0.01 [d]
Trunk fat (kg)	4.3 (2.2, 7.3)	2.2 (1.3, 3.7)	6.0 (4.1, 8.0)	10.0 (8.4, 12.5)	17.2 (13.2, 17.7)	<0.01 [d]
Limb fat (kg) [l]	3.9 (2.6,	2.6 (1.6,	4.3 (3.7,	7.1 (5.9,	11.5 (9.8,	<0.01 [d]

	5.6)	3.4)	5.7)	8.9)	12.4)	
Fat free mass (kg)	38.1 (33.1, 43.2)	35.7 (30.9, 39.1)	41.2 (34.1, 44.3)	43.2 (34.4, 51.7)	41.9 (39.1, 58.8)	<0.01 [d]
HbA1c (%)	5.3 (5.0, 5.8)	5.3 (5.0, 5.7)	5.4 (5.0, 5.9)	5.5 (5.1, 6.1)	5.7 (4.5, 6.3)	0.55 [d]
≥ 6.5	17 (11.4%)	5 (8.2%)	8 (14.3%)	3 (20.0%)	1 (14.3%)	0.59 [e]
< 6.5 and ≥ 5.7	30 (20.1%)	11 (18.0%)	11 (19.6%)	2 (13.3%)	3 (42.9%)	
< 5.7	102 (68.5%)	45 (73.8%)	37 (66.1%)	10 (66.7%)	3 (42.9%)	
Active TB disease [m]	19 (23.5%)	10 (23.3%)	6 (27.3%)	1 (16.7%)	2 (14.3%)	0.70 [e]
Hemoglobin (g/dL)	107.0 (96.5, 125.0)	100.0 (90.0, 118.0)	115.5 (100.0, 130.0)	113.0 (101.0, 120.9)	115.0 (110.0, 117.0)	0.04 [d]
Anemia	105 (77.2%)	49 (87.5%)	33 (66.0%)	12 (75.0%)	5 (83.3%)	0.06 [e]
Severe anemia	11 (8.1%)	6 (10.7%)	3 (6.0%)	0 (0.0%)	0 (0.0%)	0.23 [e]
Blood Pressure [n]						
Systolic (mmHg; continuous)	110 (100, 120)	110 (100, 120)	110 (110, 120)	120 (110, 130)	120 (110, 130)	0.09 [d]
Systolic ≥ 140 mmHg	11 (11.1%)	4 (8.9%)	4 (12.9%)	2 (18.2%)	1 (20.0%)	0.78 [e]
Diastolic (mmHg; continuous)	70 (60, 80)	70 (60, 80)	70 (70, 80)	70 (70, 90)	80 (80, 80)	0.36 [d]
Diastolic ≥ 90 mmHg	13 (13.1%)	4 (8.9%)	5 (16.1%)	3 (27.3%)	1 (20.0%)	0.45 [e]
Abnormal blood pressure	8 (8.1%)	2 (4.4%)	3 (9.7%)	2 (18.2%)	1 (20.0%)	0.42 [e]

Footnotes

[a] Abbreviations: body mass index (BMI), glycated hemoglobin (HbA1c), Indian rupees (INR), interquartile range (IQR), tuberculosis (TB)

[b] Among study participants with available 25(OH)D data (n=156). Covariates with missing observations included: monthly household income (n=183), BMI (n=10), waist circumference (n=6), total body fat (n=10), trunk fat (n=10), limb fat (n=10), HbA1c (n=7), active tuberculosis disease (n=75), hemoglobin (n=20), systolic or diastolic blood pressure (n=57).

[c] Comparison by BMI categories

[d] Wilcoxon sign rank test. Normality assumptions not met based on Shapiro-Wilk test statistic.

[e] Likelihood ratio test

[f] Including completed and some primary education

[g] Cut-off value of 5000 INR rounded from the cut-off of 4860 INR, which is based on monthly consumption expenditure for a family of five residing in a rural area (2011-12 prices), and the India national poverty line (Government of India, Planning Commission, 2014 Report) (10)

[h] Any current self-reported tobacco use, including cigarettes, chewing tobacco, pan, gutkha, bidi

[i] Scientific Advisory Council of Nutrition cut-off value recommended to prevent rickets

[j] Institute of Medicine cut-off values for 25(OH)D deficiency and insufficiency among healthy populations

[k] Endocrine Society cut-off values for 25(OH)D deficiency and insufficiency among populations at risk of vitamin D deficiency

[l] Sum of body fat (kg) in four limbs (arms, legs)

[m] Based on acid-fast bacilli sputum smear microscopy

[n] Elevated blood pressure cut-off values from the National Institutes of Health (NHLBI): https://www.nhlbi.nih.gov/health/health-topics/topics/hbp

participants were illiterate (n=61); and similarly, approximately half had a monthly household income < 5000 INR (n=60 [47.2%]).

Approximately half of study participants (n=64 [43.8%]) were considered underweight (BMI < 18.5 kg/m^2; ***Table 13***); nearly one-fifth were either overweight (23.0 kg/m^2 ≤ BMI < 27.5 kg/m^2; 11.0%) or obese (BMI ≥ 27.5 kg/m^2; 4.8%). Median values of body composition indicators included: total body fat 18.1% (IQR 12.2, 24.0), trunk fat 4.3 kg (IQR 2.2, 7.3), limb fat 3.9 kg (IQR, 2.6, 5.6), and fat free mass 38.1 kg (IQR 33.1, 43.2). All body composition indicators differed across BMI categories (all p<0.01).

Association between 25(OH)D and metabolic risk factors

Serum 25(OH)D (nmol/L) was associated with WC (cm; p<0.05), adjusting for age, sex, active TB disease, and tobacco use (***Table 14***). Similarly, 25(OH)D < 50 nmol/L was associated with WC (cm; p=0.05), adjusting for the same covariates (***Table 14***). However in contrast, 25(OH)D was not associated with elevated WC (above the IDF cut-off values) in other multivariate linear and binomial (or Poisson) regression models (p>0.05; ***Table 14***).

Vitamin D (25[OH]D) < 50 nmol/L was associated with HbA1c (%; p=0.04), adjusting for age and fat free mass (***Table 15***). The lowest quintile of serum 25(OH)D was associated with an increased risk of HbA1c ≥ 5.7% (adjusted risk ratio [aRR] 1.61 [95% CI 1.02, 2.56]) compared to the other quintiles, adjusting for age and trunk fat (***Table 15***). However, the other associations that were assessed between 25(OH)D (continuous,

Table 14. Serum 25-hydroxyvitamin D and waist circumference (n=150)

Vitamin D (25[OH]D)	Waist circumference (continuous [cm]; linear regression)				Waist circumference (categorical; binomial regression) [a, c]			
	Unadjusted		Adjusted [b]		Unadjusted		Adjusted [b]	
	β (SE)	p	β (SE)	p	RR	95% CI	aRR	95% CI
Continuous (nmol/L)	-0.08 (0.04)	0.03	-0.10 (0.04)	<0.01	1.00 [d]	1.00, 1.01	1.00 [d]	1.00, 1.01
< 50 nmol/L (Endocrine Society)	3.15 (1.93)	0.10	3.63 (1.84)	0.05	0.85	0.72, 1.01	0.82 [d]	0.56, 1.19
Quintiles (low 1 vs 2-5)	0.24 (2.53)	0.92	0.24 (2.45)	0.92	0.98	0.78, 1.22	0.95 [d]	0.58, 1.54

Footnotes
[a] High vs low WC. Cut-offs based on IDF WC cutoffs among South Asian populations.
[b] We considered known or suspected risk factors for waist circumference as potential confounders. These potential confounders were included if p<0.25 from univariate regressions (linear or binomial regression model beta coefficients; likelihood ratio tests). Based on a change in estimate approach, covariates were included in the final adjusted model if they changed the estimate by ≥10%. The final covariates for the association of waist circumference (categorical) and vitamin D (quintiles 1 vs 2-5) were utilized in final models in this table; these included: age, sex, active TB disease, anemia, monthly household income.
[c] Binomial regression unless otherwise stated
[d] Poisson regression due to no model convergence

<50 nmol/L) were not associated with HbA1c (continuous, ≥ 6.5% or ≥ 5.7%; p>0.05; ***Table 15***). For example, 25(OH)D (nmol/L) was not associated with HbA1c (%; p=0.27), adjusting for covariates.

The considered associations between 25(OH)D (continuous, <50 nmol/L) were not associated with blood pressure, including systolic (continuous, elevated), diastolic (continuous, elevated), and abnormally high blood pressure (p>0.05; ***Table 16***).

Association between 25(OH)D and active tuberculosis disease

The lowest quintile of 25(OH)D was not associated with active TB disease (aRR 1.93 [95% CI 0.53, 7.08]), accounting for age, sex, anemia, total body fat, and limb fat (***Table 17***).

Table 15. Serum 25-hydroxyvitamin D and glycated hemoglobin (n=149)

Vitamin D (25[OH]D)	HbA1c (continuous; linear Regression)				HbA1c (categorical ≥ 6.5%; binomial regression) [a, c]				HbA1c (categorical ≥ 5.7%; binomial regression) [a, c]			
	Unadjusted		Adjusted [b]		Unadjusted		Adjusted [b]		Unadjusted		Adjusted [b]	
	β (SE)	p	β (SE)	p	RR	95% CI	RR	95% CI	RR	95% CI	RR	95% CI
Continuous (nmol/L)	<-0.01 (<0.01)	0.48	<-0.01 (<0.01)	0.27	1.00	0.98, 1.01	0.99 [d]	0.97, 1.01	0.99	0.98, 1.01	0.99	0.98, 1.00
< 50 nmol/L (Endocrine Society)	0.40 (0.27)	0.13	0.54 (0.27)	0.04	1.57	0.63, 3.90	1.80 [d]	0.67, 4.84	1.36	0.84, 2.19	1.54	0.98, 2.41
Quintiles (low 1 vs 2-5)	0.09 (0.33)	0.79	0.21 (0.33)	0.52	0.82	0.25, 2.66	0.92 [d]	0.26, 3.34	1.46	0.88, 2.40	1.61	1.02, 2.56

Footnotes

[a] HbA1c cut-offs based on WHO and IDF recommendations

[b] We considered known or suspected risk factors for HbA1c as potential confounders. These potential confounders were included if p<0.25 from univariate regressions (linear or binomial regression model beta coefficients; likelihood ratio tests). Based on a change in estimate approach, covariates were included in the final adjusted model if they changed the estimate by ≥10%. The final covariates for the association of HbA1c (categorical, >=5.7%) and vitamin D (quintiles 1 vs 2-5) were utilized in final models in this table; these included: age, trunk fat.

[c] Binomial regression unless otherwise stated

[d] Poisson regression due to no model convergence

DISCUSSION

In summary, our findings showed associations between vitamin D (25[OH]D) and HbA1c (≥ 5.7%), as well as WC among an adult outpatient population in rural India. Neither blood pressure nor active TB disease was associated with serum 25(OH)D concentrations.

Table 16. Serum 25-hydroxyvitamin D and blood pressure (n= 99)

Vitamin D (25[OH]D)	Systolic (continuous; linear regression) [b]		Elevated systolic (categorical ≥140 mmHg; binomial regression) [a, b, c]		Diastolic (continuous; linear regression) [b]		Elevated diastolic (categorical ≥90 mmHg; binomial regression) [a, b, c]		Abnormal (categorical either systolic ≥140 mmHg or diastolic ≥90 mmHg; Poisson regression) [a, b, c]	
	Adjusted									
	β (SE)	p	RR	95% CI	β (SE)	p	RR	95% CI	RR	95% CI
Continuous (nmol/L)	-0.07 (0.07)	0.35	0.97 [d]	0.94, 1.01	-0.04 (0.05)	0.48	1.00 [d]	0.97, 1.03	0.97 [d]	0.92, 1.02
< 50 nmol/L (Endocrine Society)	5.45 (3.28)	0.10	4.00 [d]	0.96, 16.56	3.49 (2.41)	0.15	0.93 [d]	0.27, 3.22	4.07 [d]	0.62, 26.60
Quintiles (low 1 vs 2-5)	0.19 (4.61)	0.97	1.14 [d]	0.18, 7.17	-0.19 (3.37)	0.96	0.55 [d]	0.08, 3.66	2.55 [d]	0.19, 33.45

Footnotes

[a] Elevated blood pressure cut-off values from the National Institutes of Health (NHLBI): https://www.nhlbi.nih.gov/health/health-topics/topics/hbp

[b] We considered known or suspected risk factors for blood pressure as potential confounders. These potential confounders were included if $p<0.25$ from univariate regressions (linear or binomial regression model beta coefficients; likelihood ratio tests). Based on a change in estimate approach, covariates were included in the final adjusted model if they changed the estimate by ≥10%. The final covariates for the associations between hypertension (elevated systolic, diastolic, or both) and vitamin D (quintiles 1 vs 2-5) were utilized in final models in this table; these included: age, active TB disease, body fat, limb fat, trunk fat, fat free mass, illiterate, household income.

[c] Binomial regression unless otherwise stated

[d] Poisson regression due to no model convergence

Table 17. Serum 25-hydroxyvitamin D and active TB disease (n=81)

Vitamin D (25[OH]D)	Active TB disease (categorical; binomial regression) [a, c]			
	Unadjusted		Adjusted [b]	
	RR	95% CI	RR	95% CI
Continuous (nmol/L)	0.99	0.98, 1.01	0.99 [d]	0.97, 1.01
< 50 nmol/L (Endocrine Society)	0.91	0.41, 2.02	0.98 [d]	0.38, 2.52
Quintiles (low 1 vs 2-5)	1.39	0.55, 3.53	1.93 [d]	0.53, 7.08

Footnotes

[a] Active TB disease assessed by AFB sputum smear microscopy

[b] We considered known or suspected risk factors for active TB disease as potential confounders. These potential confounders were included if $p<0.25$ from univariate regressions (linear or binomial regression model beta coefficients; likelihood ratio tests). Based on a change in estimate approach, covariates were included in the final adjusted model if they changed the estimate by ≥10%. The final covariates for the association of active TB disease (categorical) and vitamin D (quintiles 1 vs 2-5) were utilized in final models in this table; these included: age, sex, anemia, body fat, limb fat, household income.

[c] Binomial regression unless otherwise stated

[d] Poisson regression due to no model convergence

Vitamin D as a modifiable risk factor of metabolic indicators

Previous systematic reviews have found associations between vitamin D supplementation and HbA1c [45,46], which is consistent with our result. In a systematic review of vitamin D (or vitamin D analogues) supplementation during pre-diabetes, vitamin D supplementation was associated with a mean HbA1c difference of -1 mmol/mol (95% CI -2, 0; p=0.008) [45]. Another systematic review found vitamin D supplementation (versus placebo) was associated HbA1c (mean difference -0.13%; p=0.04) [46]. Other systematic reviews have observed null results [47-49], however intervention studies have included inconsistent methodology, including vitamin D dosage (duration, frequency, dosage).

Our finding that serum 25(OH)D was inversely associated with WC, which has been corroborated by other studies focusing on visceral adiposity [22]. At the cellular level, other key findings have supported epidemiological findings, including the: a) isolation of VDR as well as hydroxylating enzymes of vitamin D in adipose tissues; and b)

storage and release of vitamin D in adipocytes [25-29,50]. Many questions remain, in order to elucidate the etiology and mechanisms of 25(OH)D in the context of adiposity and energy homeostasis [21], including: How does VDR signaling occur in adipocytes, particularly during abnormal metabolism? How does the vitamin D activation:inactivation ratio in adipocytes differ during metabolic dysfunction as well as in different types of adipose tissue and cells? Are there other lipid-mediated regulatory processes that affect or interact with vitamin D metabolism in adipocytes (*e.g.* PPAR-gamma)?

Despite our result of a null association between low 25(OH)D and high systolic blood pressure, prior literature has supported an inverse association between 25(OH)D and the renin-angiotensin-aldosterone-system (RAAS), which regulates hypertension [51-53]. One hypothesized mechanism is that elevated vitamin D inhibits renin gene expression, which dampens the RAAS activity and subsequently decreases blood pressure [52-55]. One study corroborated that cardiac renin mRNA expression increased in VDR knockout mice [56]. In a randomized controlled trial among patients with arterial hypertension and 25(OH)D <30 ng/mL at baseline, vitamin D3 supplementation (2800 IU) resulted in an increased mean difference of plasma aldosterone concentration ($p=0.04$), relative to placebo [55]. Additionally, a systematic review of vitamin D supplementation randomized controlled trials found that vitamin D supplements were associated with lowered blood pressure in two of nine meta-analyses [57].

Vitamin D in active tuberculosis disease

Other studies have found evidence of the link between vitamin D and the human host defense against *Mycobacterium tuberculosis* [58,59], in contrast to our finding. One systematic review showed lower vitamin D status among patients with active TB disease, compared to controls; and vitamin D deficiency was associated with an increased risk of incident active TB disease among those with latent TB infection [60]. Other clinical studies have also found null results, however additional longitudinal studies are needed, particularly in order to account for active TB disease severity, anti-TB treatment (*e.g.* duration, drug combinations [1st versus 2nd line], adherence), and other comorbidities (*e.g.* HIV).

Strengths and limitations

In this study, strengths included: the assessment of several metabolic risk factors, and a study population with a high prevalence of low BMI, which allowed for consideration of these associations among individuals with heterogeneous body composition.

However, there were several limitations. First, causal inferences were precluded by the cross-sectional study design. Second, findings cannot be generalized to other populations, given the small sample size and non-random selection of study participants who provided blood samples. Third, lipid data were not available. Low high-density lipoprotein cholesterol (men <40 mg/dL, women <50 mg/dL) and elevated triglycerides (≥ 150 mg/dL) are considered one criteria in the definition of metabolic syndrome [2]. Additionally, BIA was utilized for body composition assessments instead of other methods (*e.g.* dual-energy x-ray absorptiometry or underwater weighing) [61,62]; and

there was no clinical diagnoses data of diabetes.

Conclusion

Our results corroborated the associations of low vitamin D with both elevated HbA1c and WC among a population with a high prevalence of low BMI. While further mechanistic and longitudinal studies are needed to better understand the roles of vitamin D, these findings support the potential role of vitamin D as a modifiable risk factor in preventing and managing metabolic risk factors in populations with low BMI.

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Author contributions

S.M., W.B., and E.A.Y. designed research; E.A.Y. conducted research; E.A.Y., J.F., and S.M. analyzed data; and all authors contributed to drafting and critically revising the paper. S.M. had primary responsibility for final content. All authors read and approved the final manuscript.

Conflict of interest

E.A.Y., P.M.B., M.J.G. J.L.F., W.B., and D.G.R. have no conflicts of interest. S.M. is an unpaid board member of a diagnostic start-up focused on developing assays for low-cost and point-of-care measurement of certain nutrients from a drop of blood using results from his research as a faculty member at Cornell University.

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Table 18. Supplemental - STROBE checklist for cross-sectional studies [1]

	Item No	Recommendation	Page number(s)
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	80
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	80
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	81-83
Objectives	3	State specific objectives, including any pre-specified hypotheses	81-83
Methods			
Study design	4	Present key elements of study design early in the paper	83-86
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	83-86
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants	83-86
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	83-87
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	83-87
Bias	9	Describe any efforts to address potential sources of bias	93-98
Study size	10	Explain how the study size was arrived at	N/A
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	83-96
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	83-96
		(*b*) Describe any methods used to examine subgroups and interactions	83-96
		(*c*) Explain how missing data were addressed	83-96
		(*d*) If applicable, describe analytical methods taking account of sampling strategy	N/A
		(*e*) Describe any sensitivity analyses	N/A
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analyzed	83
		(b) Give reasons for non-participation at each stage	83

		(c) Consider use of a flow diagram	N/A
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	88-96
		(b) Indicate number of participants with missing data for each variable of interest	88-96
Outcome data	15*	Report numbers of outcome events or summary measures	88-96
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	88-96
		(*b*) Report category boundaries when continuous variables were categorized	88-96
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	N/A
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	88-96
Discussion			
Key results	18	Summarize key results with reference to study objectives	93-98
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	93-98
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	93-98
Generalizability	21	Discuss the generalizability (external validity) of the study results	93-98
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	99

[1] Available at: http://strobe-statement.org/. Accessed February 10, 2016.

CHAPTER IV: The composition of the gut microbiome among outpatients with active tuberculosis disease in South India and its association with vitamin D status

Elaine A. Yu,[1] Julia L. Finkelstein,[1] Patsy M. Brannon,[1] Marshall J. Glesby,[2] Wesley Bonam,[3] David G. Russell,[4] Saurabh Mehta[1,5*]

[1]Division of Nutritional Sciences, Cornell University, Ithaca, New York, United States

[2]Weill Cornell Medical College, New York, New York, United States

[3]Arogyavaram Medical Centre, Madanapalle, Andhra Pradesh, India

[4]Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, New York, United States

[5]Institute for Nutritional Sciences, Global Health, and Technology, Cornell University, Ithaca, New York, United States

* Draft manuscript formatted according to journal requirements, in preparation for submission.

Abbreviations

$1,25(OH)_2D$: 1,25-dihydroxyvitamin D

25(OH)D: 25-hydroxyvitamin D

AMC: Arogyavaram Medical Centre

ATBD: active tuberculosis disease

CRP: C-reactive protein

CYP: cytochrome P450

DNA: deoxyribonucleic acid

GWAS: genome-wide association study

LC-MS/MS: liquid chromatography tandem-mass spectrometry

NMF: nonnegative matrix factorization

OTUs: operational taxonomic units

PCR: polymerase chain reaction

PCoA: principal coordinates analysis

QIIME: Quantitative Insights into Microbial Ecology platform

RCT: randomized controlled trial

rRNA: ribosomal ribonucleic acid

TB: tuberculosis

T_H: T helper

T_{reg}: T regulatory

VDR: vitamin D receptor

ABSTRACT

Background

Globally, active tuberculosis is the ninth leading cause of death. Prior studies indicate that inflammation has a dual role in mediating both the pathogenesis of and the human host immune response against *Mycobacterium tuberculosis*. Therefore, understanding key human host factors (including vitamin D, the gut microbiome) with bi-directional effects on inflammation is important in mitigating tuberculosis (TB) transmission and the burden from active TB disease. As an initial step towards understanding these complex associations, we investigated the gut microbiome composition among patients with ATBD at ATT initiation and assessed its association with vitamin D status in a rural setting in India.

Methods

In this cross-sectional study, outpatients (n=32) with ATBD (Xpert MTB/RIF-confirmed) were enrolled at initiation of ATT at a hospital. Rectal swab samples were assayed by 16S rRNA sequencing (Illumina MiSeq; V3-V4 hypervariable regions). Serum 25-hydroxyvitamin D (25[OH]D) concentrations were assessed by liquid chromatography mass spectrometry and categorized by cut-off values (<25, <40, <50, <75 nmol/L) and quintiles.

Results

Median serum 25(OH)D concentration was 46.0 nmol/L (IQR 36.1, 60.7). Overall, the majority of identified sequences were from four phyla: *Firmicutes* (median 46.1% [IQR

36.5, 51.6]), *Bacteroidetes* (32.4% [24.5, 40.4]), *Proteobacteria* (9.3% [6.1, 55.3]), and *Actinobacteria* (2.1% [1.3, 4.2]). The median *Firmicutes:Bacteroidetes* ratio was 1.3 (IQR 1.0, 1.8). Mean alpha diversity indices were: Shannon (5.1 [SD 1.1]), and Chao1 (1023.6 [350.5]); the median Simpson index was 0.9 (IQR 0.9, 1.0). Alpha diversity indices did not differ by vitamin D status (cut-off values and quintiles; all $p>0.05$).

Conclusions

Among this population of adult outpatients with ATBD, the relative abundance of identified operational taxonomic units were predominantly in the *Bacteroidetes* and *Firmicutes* phyla, which is consistent with previous literature among healthy populations. Further longitudinal and mechanistic studies are needed to assess the roles of vitamin D and the human microbiome in the context of inflammation and ATBD, particularly in resource-limited environments.

Keywords

Microbiome, vitamin D, micronutrients, tuberculosis

INTRODUCTION

Globally, active tuberculosis (TB) disease (ATBD) is the ninth leading cause of death, and caused 53 million deaths from 2000 to 2016 [1]. Inflammation mediates the success of the human host versus Mycobacterium tuberculosis [2, 3], and is regulated by both the human host and M. tuberculosis [2]. In the human host, an adequate pro-inflammatory response prevents ATBD, however subsequent balance is required to avoid deleterious consequences [4]. As part of its survival mechanism, M. tuberculosis interferes with the inflammatory response, in order to restrict host immunity and support caseating necrotic granulomas [2, 3]. Therefore, our goal was to elucidate human host factors (including vitamin D, gut microbiome) that have bi-directional effects on the tightly regulated inflammatory response during ATBD.

Vitamin D modulates the innate and adaptive immune responses D [5-15], including inflammation that influences the host response to M. tuberculosis [16] and microbial communities [14]. Hypothesized mechanisms include: 1) T cell development and function (decreasing T helper [TH]1, TH2, TH17; increasing Treg); and 2) upregulating antimicrobial peptides (e.g. cathelicidin) [9-12, 14, 15].

Separately, there have been complex interactions observed between gut microbiome, inflammation, ATBD and anti-TB treatment (ATT) [17-19]. ATT causes dysbiosis of the human gut microbiome, which was shown in a human study (with dysbiosis lasting

at least 1.2 years) [17], and a longitudinal murine study that assessed dysbiosis throughout the duration of ATT [18]. Among available studies, ATBD was associated with lower bacterial diversity and number of species [19].

Despite previous evidence (including hypothesized mechanisms), there is a limited understanding of the linkages between vitamin D and the gut microbiome during disease states [20-25], including ATBD [22, 23]. Therefore, our study objectives were to: 1) characterize the gut microbiome composition and diversity; and 2) compare differences between those with differing serum micronutrient concentrations of 25-hydroxyvitamin D (25[OH]D), among adult patients with ATBD.

METHODS

Patients

This study protocol was approved by ethics review committees at Cornell University (Institutional Review Board, United States) and Arogyavaram Medical Centre (AMC; Institutional Ethics Committee, India). All study participants voluntarily provided written informed consent, prior to any data collection. This cross-sectional study included adult patients (n=32). Study participants were recruited and enrolled at a hospital (AMC) in a rural region of South India. Inclusion criteria were: any outpatient; ATBD based on Xpert MTB/RIF. The exclusion criteria were: age (<18 years); receiving ATT (>4 weeks in past 5 years); other severe illnesses or conditions requiring

hospitalization (aside from preventative TB hospitalization); and pregnancy or lactation.

Data collection

Blood and sputum samples were collected from study participants, per standard of care. Rectal swabs were collected and inserted in tubes with modified Cary-Blair medium (2 mL), according to the manufacturer instructions (Copan FecalSwabTM Regular Flocked Collection Kit; Thermofisher Scientific [Copan Italia], Brescia, Italy). The rectal swab samples were initially stored at 4ºC and subsequently at -20ºC or -80ºC until deoxyribonucleic acid (DNA) extraction. Trained research assistants and a study physician collected data regarding current and past morbidities (e.g. diarrhea, respiratory illness) and medications (including antibiotics), anthropometry, dietary recall, and sociodemographic information through structured interviews and clinical examinations.

25-hydroxyvitamin D

Serum samples were evaluated for 25(OH)D (ng/mL) by liquid chromatography tandem-mass spectrometry (LC-MS/MS; Waters AcquityTM ultra-performance liquid chromatography with triple quadrupole detector; Waters Corporation, Milford, Massachusetts, United States [US]). The coefficients of variation (CVs) for the LC-MS/MS instrument were 7.6%, 7.4%, and 7.6% (Internal Quality Control Kit for total hydroxyvitamin D; Bio-Rad Laboratories, Irvine, CA, US). Results were multiplied by

a conversion factor of 2.496, and reported in nmol/L.

Active TB disease and CRP

Sputum samples were assayed by Xpert MTB/RIF (Cepheid, Sunnyvale, California, US) to evaluate ATBD status [26]. C-reactive protein (CRP) was assessed by latex enhanced immunoturbidimetry (mg/dL; Advia 1800 Chemistry Analyzer; Siemens Healthcare Diagnostics Inc., Washington, DC, US).

Gut microbiome: 16S rRNA sequencing and analysis

Overall, the workflow (DNA extraction, amplification, 16S ribosomal ribonucleic acid (rRNA) sequencing, and taxonomic classification) was based on the Earth Microbiome Project protocols [27].

DNA extraction and real-time quantitative PCR

DNA was extracted from rectal swabs with QIAmp DNA Mini kits (Qiagen Inc., Valencia, CA, US), based on the manufacturer instructions. Genomic DNA concentration was quantified (NanoDrop spectrophotometer; NanoDrop Technologies, Wilmington, DE, US). 25 ng of each sample was utilized for polymerase chain reaction (PCR) amplification (KAPA HiFi HotStart PCR Kit; KAPA Biosystems, Boston, MA, US), which was optimized for V3-V4 primers (26 cycles, 0.2 μM concentrations of

forward and reverse primers).

Library preparation

V3 and V4 hypervariable regions in the 16S rRNA gene of bacteria were targeted by primers (proprietary; Genotypic Technology Pvt. Ltd.; Bangalore, Karnataka, India). Sequencing adaptors (Nextera XT v2 Index Kit, Illumina, San Diego, CA, US) were added to barcode the amplicon libraries (***Table 24***). PCR validation steps included: positive and negative control samples, and amplicon confirmation with 1.2% agarose gel.

Sequencing and taxonomic classification

Amplicons (16S rRNA gene, V3 and V4 regions) were sequenced by the MiSeq platform (Illumina, San Diego, CA, US). The initial processing of sequencing reads included: demultiplexing the raw paired end reads from Miseq (bcl2fastq software; Illumina, San Diego, CA, US), quality filtering (e.g. fragment length, primer sequence presence [FastQC2] [28]), and stitching from the EA-Utils NGS suite (fastq-join, https://expressionanalysis.github.io/ea-utils/). Reads with Phred quality score <30 were excluded.

Taxonomic assignment of sequences was based on closed-reference operational

taxonomic units (OTUs) with the Quantitative Insights into Microbial Ecology platform (QIIME, version 1.9.0) [29]. The uclust software package (version 1.2.22) was utilized to compare sequences with the Greengenes reference database sequences (version 13.8) [30, 31]. Reads failing to match the reference database with <97% sequence identity were discarded, based on other protocols [27, 32].

Visualization

R packages were utilized for data visualization, including heat maps with nonnegative matrix factorization (NMF; R package) [33]. Weighted and unweighted UniFrac distances [34] were visualized by principal coordinates analysis (PCoA).

Definitions

Prior to alpha diversity calculations, data were rarified with a depth of 100,000 reads. Shannon, Simpson, and Chao diversity indices were calculated through QIIME.

25(OH)D (nmol/L) was categorized by several cutpoints: D <25.0 [35], <40.0 [36], <50.0 [36, 37] and <75.0 nmol/L [37]. Serum 25(OH)D was also categorized in tertiles

and quintiles.

Statistical analysis

Preliminary analysis considered assumptions for normality (by Shapiro-Wilk test statistic). For descriptive analysis of study participant characteristics, means (with standard deviations [SD]), medians (with interquartile ranges), and n [%] were reported. Differences between subgroups of study participants (based on outcomes and patient characteristics of interest) were compared by parametric (ANOVA) or non-parametric (Kruskal-Wallis, Fisher's exact) tests.

The associations of interest (serum 25[OH]D concentrations) were considered in linear regression models. Confounding was accounted for, based on the approach proposed by Rothman and Greenland [38] and a priori literature search of potential confounders [39]. Aside from microbiome analysis software (e.g. QIIME), other software included R and SAS (SAS Institute Inc., Cary, North Carolina, US).

All statistical tests were 2-sided, and $p<0.05$ were considered statistically significant. After statistical tests, we utilized false discovery rates (FDR) per the Benjamini and

Hochberg approach [40], as post-hoc multiple testing corrections.

RESULTS

Sociodemographic and clinical characteristics

Among the study population, the median age was 55.0 years (IQR 42.0, 60.0), and ranged between 19.0 and 70.0 years (***Table 19***). Over three-quarters of the study participants were male (78.1%; ***Table 19***). All participants reported coughing during the month prior to the study visit; 60.0% had sputum in their cough (***Table 19***). Also, 6.7% had hemoptysis in the previous four weeks (***Table 19***). Eighty percent of study participants had fever and loss of appetite (***Table 19***). None had reported having night sweats (***Table 19***). These clinical signs did not differ by 25(OH)D status (<50 nmol/L; p>0.05; ***Table 19***).

Serum biomarkers

Median serum 25(OH)D was 46.0 nmol/L (IQR 36.1, 60.7; total of D2 and D3); stratified across tertiles, the median values were: 27.0 nmol/L (IQR 20.6, 35.4), 45.5 nmol/L (IQR 42.3, 49.2), and 75.8 nmol/L (IQR 60.6, 90.4; ***Table 20***). The median values of 25(OH)D2 was 2.8 nmol/L (IQR 2.5, 4.1); and D3 was 42.2 nmol/L (IQR 33.5, 58.1; ***Table 20***). Considering several cut-off values of low vitamin D status, 81.3% of study participants had 25(OH)D <75 nmol/L, 62.5% were <50 nmol/L, 34.4% were

<40 nmol/L, and 12.5% were <25 nmol/L (***Table 20***). The median serum concentration of CRP was 7.2 mg/dL (IQR 3.6, 11.2; ***Table 20***). CRP was inversely correlated (ß -2.57 [SE 1.10]; $p<0.05$) with 25(OH)D, adjusting for age and sex.

Gut microbiome

In this clinical adult study population, the mean number of identified sequences was 165,323.9 (SD 92,958.9). Sequences predominantly belonged to four phyla: Firmicutes (median 46.1% [IQR 36.5, 51.6]), Bacteroidetes (median 32.4% [IQR 24.5, 40.4]), Proteobacteria (median 9.3% [IQR 6.1, 55.3]), and Actinobacteria (median 2.1% [IQR 1.3, 4.2]; ***Table 21***). Bar plot and heat map visualizations of the relative bacterial abundance of OTUs at the phyla level also confirmed this observation (***Figures 3A*** and ***4A***). The median ratio between Firmicutes and Bacteroidetes was 1.3 (IQR 1.0, 1.8; ***Table 21***), and ranged from 0.1 to 14.2. The Firmicutes:Bacteroidetes ratio differed significantly by age ($p<0.05$).

In terms of the family level, many OTUs were categorized as Prevotellaceae, Ruminococcaceae, and Lachnospiraceae (***Figure 3B***). At the genus level, sequences primarily were in the following categories: Prevotella (median 11.7% [IQR 4.0, 24.3]), Bacteroides (median 5.0% [IQR 1.2, 14.3]), Faecalibacterium (median 4.8% [IQR 1.5,

Table 19. Sociodemographic and clinical characteristics of study participants

Median (IQR) [a]*; n (%)*		25(OH)D [b]		
	Overall n=32 [c]	<50 nmol/L n=20	≥50 nmol/L n=12	p
Age (years)	55.0 (42.0, 60.0)	52.0 (38.0, 59.0)	56.0 (55.0, 60.0)	0.21 [d]
Sex (male)	25 (78.1%)	14 (70.0%)	11 (92.7%)	0.21 [e]
Coughing [g]	15 (100.0%)	8 (100.0%)	7 (100.0%)	--- [f]
With sputum [g]	9 (60.0%)	5 (62.5%)	4 (57.1%)	1.00 [e]
With blood [g]	1 (6.7%)	0 (0.0%)	1 (14.3%)	--- [f]
Fever [g]	12 (80.0%)	6 (75.0%)	6 (85.7%)	1.00 [e]
Night sweats [g]	0 (0.0%)	0 (0.0%)	0 (0.0%)	--- [f]
Loss of appetite [g]	12 (80.0%)	6 (75.0%)	6 (85.7%)	1.00 [e]

[a] Based on the Shapiro-Wilk test, the median (and IQR) values were reported since the null hypothesis was rejected
[b] 25(OH)D (nmol/L) assessed by LCMS
[c] Covariates with missing observations included: age (n=14), clinical signs and symptoms (n=17).
[d] Comparison between subgroups by Kruskal-Wallis test statistic
[e] Fisher's exact test
[f] Sample cell sizes including zero values
[g] Clinical signs and symptoms were reported from the month prior to the study visit, and based on self-report

Table 20. Serum concentrations of vitamin D and CRP [a]

Median (IQR); n (%)	
	Overall n=32
25(OH)D (nmol/L)	
Total (D_2 and D_3)	46.0 (36.1, 60.7)
D_2	2.8 (2.5, 4.1)
D_3	42.2 (33.5, 58.1)
Cut-off values	
<25 [b]	4 (12.5%)
< 40 [c]	11 (34.4%)
<50 [c, d]	20 (62.5%)
<75 [d]	26 (81.3%)
Tertiles	
1 (low)	27.0 (20.6, 35.4)
2	45.5 (42.3, 49.2)
3 (high)	75.8 (60.6, 90.4)
Quintiles	
1 (low)	21.7 (16.9, 26.0)
2	38.5 (35.4, 41.8)
3	46.0 (44.7, 49.0)
4	58.9 (54.7, 60.8)
5 (high)	87.7 (77.7, 90.7)
CRP (mg/dL)	7.2 (3.6, 11.2)
Tertiles	
1 (low)	1.4 (1.1, 3.5)
2	7.1 (4.7, 7.9)
3 (high)	12.5 (11.0, 14,1)

[a] Plasma concentrations reported for two study participants had inadequate serum aliquot volume
[b] Scientific Advisory Council of Nutrition cut-off value recommended to prevent rickets
[c] Institute of Medicine cut-off values for 25(OH)D deficiency and insufficiency among healthy populations
[d] Endocrine Society cut-off values for 25(OH)D deficiency and insufficiency among populations at risk of vitamin D deficiency

Table 21. Relative abundance of OTUs (%) at phyla level, among total and comparisons by vitamin D status [a]

	Total	25(OH)D (nmol/L)												
		Cut-off											Tertiles	Quintiles
	Median (IQR) [b]	< 75	≥ 75	p [c]	< 50	≥ 50	p [c]	< 40	≥ 40	p [c]	< 25	≥ 25	p [c]	p [c]	p [c]
Firmicutes	46.1 (36.5, 51.6)	46.3 (38.4, 51.8)	38.0 (18.1, 48.7)	0.45	46.3 (32.5, 50.7)	41.0 (36.7, 52.5)	0.94	44.5 (24.4, 46.7)	46.5 (38.8, 52.4)	0.45	32.5 (16.7, 42.2)	46.5 (38.6, 52.1)	0.30	0.59	0.30
Bacteroidetes	32.4 (24.5, 40.4)	32.6 (26.2, 40.6)	27.3 (5.9, 34.6)	0.81	32.5 (21.8, 40.4)	30.4 (27.3, 42.6)	0.88	35.9 (21.0, 40.7)	30.5 (26.8, 39.1)	0.88	35.6 (27.5, 39.8)	31.5 (23.6, 40.5)	0.88	0.88	0.81
Proteobacteria	9.3 (6.1, 28.1)	9.1 (6.0, 21.0)	35.3 (10.4, 55.3)	0.44	9.1 (6.1, 24.8)	9.9 (4.5, 38.9)	0.91	12.7 (2.9, 29.9)	9.2 (6.8, 21.0)	0.91	21.3 (10.8, 46.6)	9.2 (5.9, 24.3)	0.44	0.89	0.44
Fusobacteria	0.3 (<0.1, 3.8)	0.5 (<0.1, 5.4)	0.1 (<0.1, 0.4)	0.92	0.5 (<0.1, 7.5)	0.1 (<0.1, 0.8)	0.92	<0.1 (<0.1, 6.6)	0.3 (<0.1, 3.4)	0.92	3.3 (<0.1, 7.5)	0.3 (<0.1, 3.1)	0.92	0.92	0.92
Actinobacteria	2.1 (1.3, 4.2)	2.4 (1.5, 4.4)	1.3 (0.8, 3.3)	0.66	2.5 (1.4, 4.8)	1.7 (1.0, 2.8)	0.66	2.6 (1.5, 5.3)	1.8 (1.2, 3.3)	0.66	2.0 (1.2, 6.6)	2.1 (1.3, 4.2)	0.91	0.66	0.76
Firmicutes: Bacteroidetes ratio	1.3 (1.0, 1.8)	1.3 (1.0, 1.7)	1.6 (0.9, 2.7)	0.70	1.2 (1.0, 1.8)	1.5 (0.9, 2.1)	0.70	1.2 (1.0, 1.4)	1.5 (1.1, 1.9)	0.62	0.8 (0.5, 1.2)	1.4 (1.1, 2.2)	0.30	0.70	0.62

[a] Included five phyla with the highest mean relative abundance of OTUs
[b] Based on the Shapiro-Wilk test, the median (and IQR) values were reported since the null hypothesis was rejected.
[c] Comparison between subgroups by Kruskal-Wallis test statistic

6.5]), as well as several unidentified genera (***Table 22***; ***Figure 4B***).

Overall, mean number of OTUs was 915.8 (SD 340.0), and ranged between 381 to 1737 among the study population (***Table 23***). The mean values of the following diversity indices were: Shannon (5.1 [SD 1.1]), Chao1 (1023.6 [SD 350.5]); the median value of Simpson was 0.9 (IQR 0.9, 1.0; ***Table 23***).

Gut microbiome and vitamin D

The relative abundance of OTUs at the phyla and genus levels did not differ significantly, based on any of the considered subgroups based on 25(OH)D concentration (<25, <40, <50, <75 nmol/L; tertiles and quintiles; all p>0.05; ***Tables 21 and 22***). The ratio of *Firmicutes*:*Bacteroidetes* were similar across categories of vitamin D status (p>0.05; ***Tables 21 and 22***). Diversity indices (Shannon, Simpson, Chao1) also did not differ by vitamin D status (p>0.05; ***Table 23***).

DISCUSSION

Among this population of adult outpatients with ATBD, the relative abundance of identified OTUs were predominantly in the *Bacteroides* and *Firmicutes* phyla, which is similar to other studies among healthy adults.

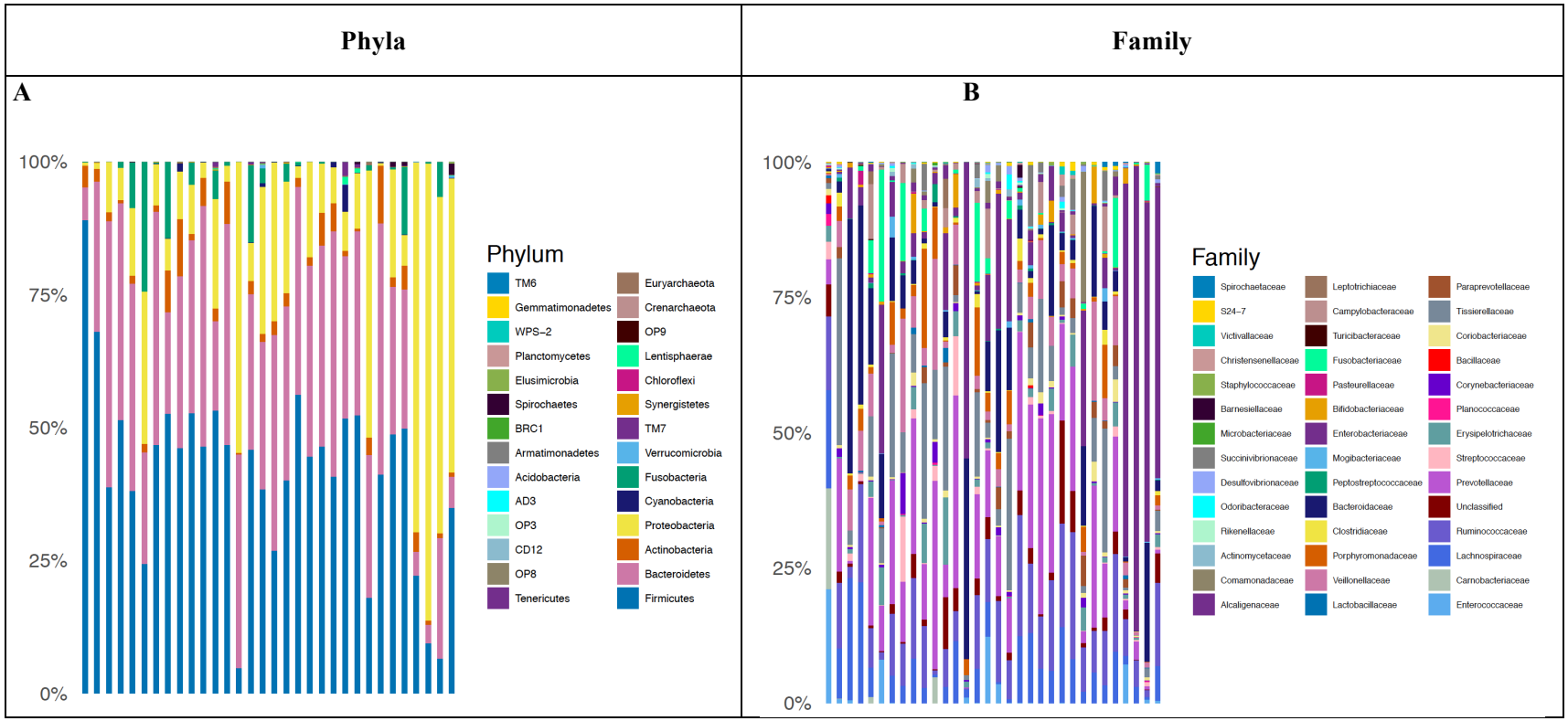

Figure 3: Bar plots of relative bacterial abundance in the gut microbiome of adult outpatients

Bar plots indicate the proportion of bacterial OTUs among the total number of OTUs identified in each of the samples (columns) at the phyla (**Fig. 1A**) and family levels (**Fig. 1B**). The identified taxa with the highest proportions were included in these plots.

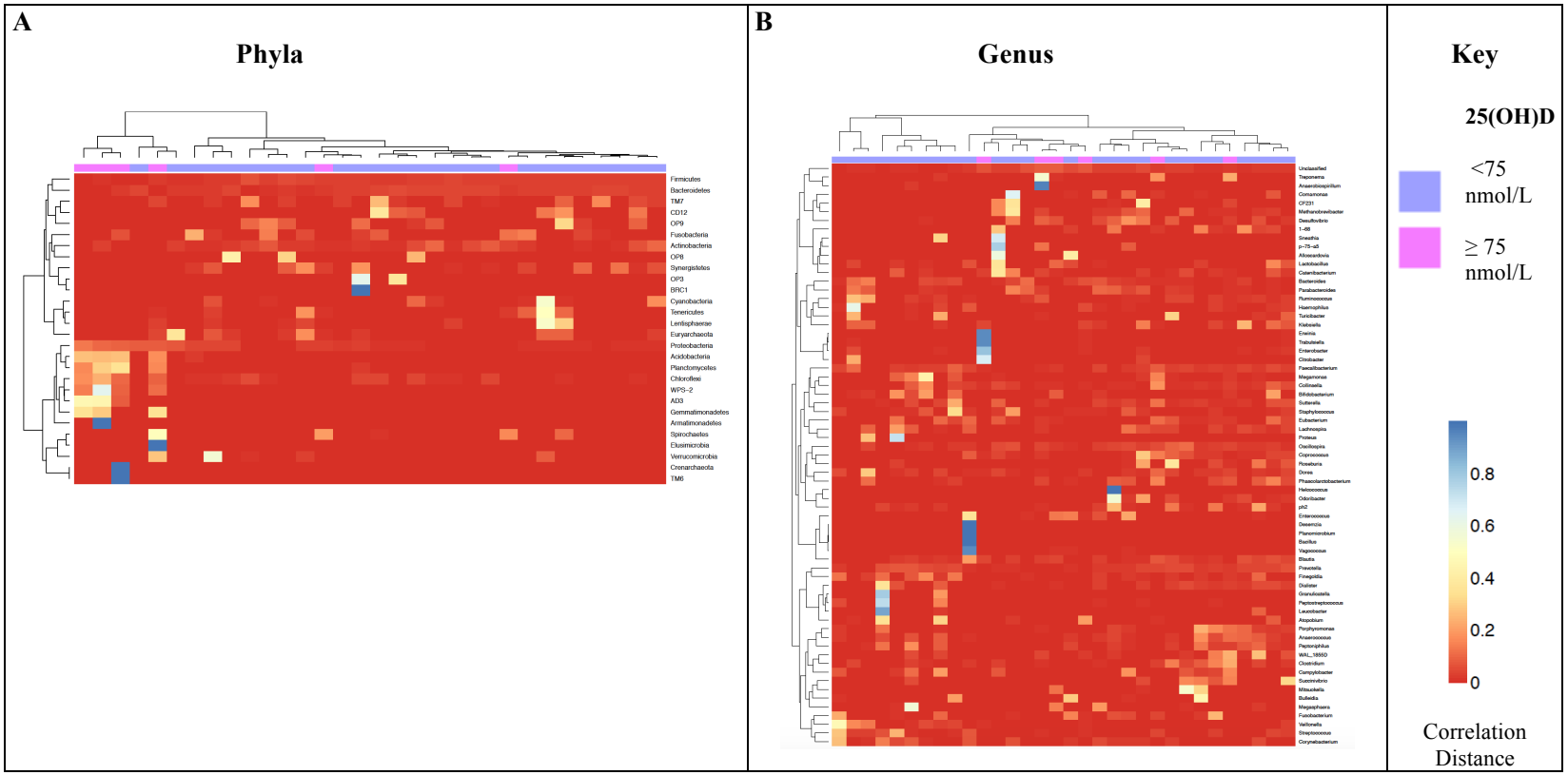

Figure 4: Heat map representation of the relative abundance of bacterial OTUs, stratified by vitamin D status

*Relative bacterial abundance at the **A**) phyla and **B**) genus levels, with vitamin D status indicated by purple bars (see key above). The identified taxa with the highest proportions were considered. The correlation distance (see color scale in key) was considered with NMF and row clustering.*

Table 22. Relative abundance of OTUs (%) at genus level, among total and comparisons by vitamin D status [a]

	Total	25(OH)D (nmol/L)												
		Cut-off											Tertiles	Quintiles
	Median (IQR) [b]	< 75	≥ 75	p [c]	< 50	≥ 50	p [c]	< 40	≥ 40	p [c]	< 25	≥ 25	p [c]	p [c]	p [c]
Prevotella	11.7 (4.0, 24.3)	13.8 (6.0, 25.0)	6.6 (2.2, 22.7)	0.95	13.3 (5.3, 24.3)	11.2 (2.8, 24.5)	0.95	11.1 (1.7, 26.5)	12.2 (6.0, 22.7)	0.95	17.3 (5.9, 24.3)	11.7 (4.0, 24.6)	0.95	0.95	0.95
Bacteroides	5.0 (1.2, 14.3)	5.0 (1.0, 14.0)	4.4 (1.9, 14.5)	0.92	4.5 (0.9, 12.9)	5.3 (2.0, 15.7)	0.92	4.4 (0.7, 21.9)	2.1 (0.9, 5.2)	0.92	18.0 (9.2, 22.2)	4.2 (0.9, 11.8)	0.54	1.00	0.92
Faecalibacterium	4.8 (1.5, 6.5)	4.9 (1.2, 7.3)	3.2 (1.8, 5.8)	0.47	5.0 (1.2, 7.4)	3.3 (2.4, 5.5)	0.44	5.0 (1.2, 7.6)	4.4 (1.8, 5.8)	0.44	6.8 (3.6, 8.1)	4.5 (1.5, 6.1)	0.44	0.44	0.44
Porphyromonas	0.4 (<0.1, 2.2)	0.4 (<0.1, 2.4)	0.2 (<0.1, 0.8)	0.91	0.4 (<0.1, 2.2)	0.4 (<0.1, 2.2)	0.91	0.4 (<0.1, 1.7)	0.3 (<0.1, 2.4)	0.91	0.5 (0.1, 1.3)	0.4 (<0.1, 2.4)	0.91	0.91	0.91
1-68	0.2 (<0.1, 2.0)	0.2 (<0.1, 3.1)	0.4 (<0.1, 0.5)	0.86	0.2 (<0.1, 2.7)	0.4 (<0.1, 0.8)	0.86	0.2 (<0.1, 0.3)	0.4 (<0.1, 2.3)	0.86	0.2 (0.1, 2.1)	0.3 (<0.1, 2.0)	0.86	0.86	0.86
WAL_1855D	0.2 (<0.1, 1.7)	0.2 (<0.1, 1.3)	0.8 (<0.1, 2.8)	0.98	0.2 (<0.1, 1.5)	0.1 (<0.1, 2.2)	0.98	0.2 (<0.1, 1.3)	0.2 (<0.1, 1.8)	0.98	0.7 (0.2, 2.1)	0.1 (<0.1, 1.7)	0.98	0.98	0.98

[a] Included five bacterial genus with the highest mean relative abundance of OTUs, and that were categorized when compared to the reference database

[b] Based on the Shapiro-Wilk test, the median (and IQR) values were reported since the null hypothesis was rejected.

[c] Comparison between subgroups by Kruskal-Wallis test statistic

Table 23. Diversity indices and OTUs, stratified by vitamin D status

		Total	25(OH)D (nmol/L)													
			Cut-off												Tertiles	Quintiles
		Mean (SD) [a]	< 75	≥ 75	p [b]	< 50	≥ 50	p [b]	< 40	≥ 40	p [b]	< 25	≥ 25	p [b]	p [b]	p [b]
Diversity index	**Shannon**	5.1 (1.1)	5.1 (1.1)	4.8 (1.2)	0.73	5.0 (1.2)	5.1 (1.0)	0.73	4.8 (1.1)	5.2 (1.1)	0.73	4.9 (1.6)	5.1 (1.1)	0.73	0.73	0.73
	Simpson [a]	0.9 (0.9, 1.0)	0.9 (0.9, 1.0)	0.9 (0.8, 1.0)	0.86	0.9 (0.9, 1.0)	0.9 (0.9, 1.0)	1.00	0.9 (0.9, 1.0)	0.9 (0.9, 1.0)	0.86	0.9 (0.8, 1.0)	0.9 (0.9, 1.0)	1.00	0.90	0.86
	Chao1	1023.6 (350.5)	1019.1 (350.3)	1042.8 (315.3)	0.92	1000.2 (347.3)	1062.5 (367.8)	0.92	992.7 (358.4)	1039.7 (354.1)	0.92	962.0 (364.5)	1032.4 (354.5)	0.92	0.92	0.92
OTUs		915.8 (340.0)	919.6 (351.3)	899.2 (315.3)	0.90	884.0 (346.8)	968.8 (336.6)	0.90	848.7 (356.4)	950.9 (334.6)	0.90	875.8 (456.3)	921.5 (330.7)	0.90	0.90	0.90
Species (#)		647.5 (219.3)	659.2 (226.8)	596.8 (192.7)	0.97	646.5 (223.0)	649.3 (222.9)	0.97	653.7 (229.3)	644.2 (219.6)	0.97	748.5 (182.1)	633.1 (223.2)	0.97	0.97	0.97

[a] Mean (SD) reported for all values except for Simpson (due to normality assumptions assessment, per Shapiro-Wilk test)
[b] Comparisons between subgroups by ANOVA (parametric) or Kruskal-Wallis (non-parametric) test statistics

Gut microbiome composition and abundance in active TB disease

Our findings were consistent with previous literature, which also found that the relative OTU abundance were predominantly in the Bacteroides and Firmicutes phyla [41-44]. Other studies have found higher proportions of Bacteroides and Firmicutes (> 90.0% [41, 44]), compared to 78.5% in our results.

Other studies have noted the potential relevance of the ratio of Firmicutes:Bacteriodetes as a microbiota indicator associated with aging and obesity [44, 45]. Our median Firmicutes:Bacteroidetes ratio (1.3) was either similar [46] or much lower than other studies among adults (e.g. 10.9 [45]), although our population included patients with ATBD. Prior studies have also found that the Firmicutes:Bacteriodetes ratio is affected by obesity [44, 47]. Based on another study at the same medical center, our source population nearly all had BMI considered underweight or normal, which could account for the lower ratio observed.

Vitamin D during active TB disease

The median vitamin D status (46.0 nmol/L) of our study participants was within a wide range of 25(OH)D concentrations observed among other studies of individuals with ATBD [48-50]. Previous studies have observed mean or median 25(OH)D concentrations in plasma or serum from 9.8 nmol/L to 78.3 nmol/L among patients with ATBD [48-50].

The evidence from vitamin D supplementation trials among patients with ATBD on its

impact on TB-related treatment outcomes has been inconsistent [51-55]. Hypothesized explanations include that it is necessary to first determine the appropriate intervention (including dosage, frequency, duration) [56].

Gut microbiome and vitamin D

While our results showed no association between vitamin D and gut microbiota, previous evidence has shown links between the gut microbiome and vitamin D [57]. Bacterial products (secondary bile acids, digested foods [e.g. butyrate, short-chain fatty acids]) have been shown to induce antimicrobial peptides (cathelicidin, LL-37) that interact with vitamin D and vitamin D receptor (VDR) [57-59]. Two murine gene deletion studies demonstrated that VDR and cytochrome P450 (CYP27B1) knockout mice had altered intestinal microbiota, including increased Bacteroidetes [60, 61]. Additionally, 1,25-dihydroxyvitamin D (1,25[OH]2D) treatment in CYP27B1 knockout mice was protective against colitis severity and decreased Helicobacteraceae in fecal samples [61].

In a study among 150 young adults in Brazil, the relative bacterial abundances of Coprococcus and Bifidobacterium were inversely correlated with 25(OH)D concentration [43]. In a genome-wide association study (GWAS) among 1,812 individuals in Germany, allelic variations of the VDR gene were associated with differing gut microbial characteristics [62].

C-reactive protein and vitamin D

In our study, serum CRP was inversely associated with vitamin D, which was similar to other studies [63, 64]. From a systematic review and meta-analysis of vitamin D supplementation randomized controlled trials (RCTs), higher dosages of cholecalciferol (≥ 1,000 IU per day) were associated with lower CRP ($p<0.05$) [64]. Additionally, previous evidence corroborated the complex dynamics between inflammation, vitamin D, gut microbiome, and ATBD [16, 63, 65]. As a general inflammatory indicator, CRP may function as an effect modifier (such as in modulating the association between vitamin D and ATBD or anti-TB treatment-related outcomes [16, 63]) or exposure (e.g. an indicator of inflammation which alters gut microbial communities [65]).

Strengths and limitations

To our knowledge, this is the first study to examine vitamin D and the gut microbiome among patients with ATBD at ATT initiation. Given the persistence of major challenges in addressing ATBD [1], evaluating key human host factors (including vitamin D and the gut microbiome) that affect inflammation may represent a means to improve ATBD treatment and prevent ATBD-related adverse outcomes. Additionally, there are a limited number of studies that have evaluated intestinal microbiome among individuals residing in resource-limited settings, including India. Given this context, 16S rRNA sequencing provided the appropriate resolution for our study objective.

However, this study also had several limitations, including the small sample size, cross-sectional study design, and laboratory assays. Further longitudinal studies with multiple timepoints as well as mechanistic studies (including functional analysis) are necessary,

in order to better understand causal relationships [66]. Additionally, 16S rRNA sequencing has a number of technological limitations, including short read length and error rates (e.g. detecting some variations) [67].

One major challenge is appropriately accounting for confounding factors that might affect the association between vitamin D and the gut microbiome, particularly in light of the relatively recent technological advances allowing for high throughput 16S rRNA sequencing [66-68]. Moreover, the symbiotic relationship between the gut microbiome and human hosts (including micronutrient status such as vitamin D) encompasses bi-directional and complex interactions, which span from mutualistic, commensal, to parasitic relationships [57, 69-73]. Gastrointestinal tract microbiota are involved in the digestion and de novo synthesis of micronutrients [71-75]. Conversely, micronutrients (and related factors) have been linked to altered gut microbiota composition and diversity, as well as bacterial fitness [71-74, 76, 77].

Broadly, there are major challenges in understanding the gut microbiome and vitamin D among patients with ATBD. Many stem from research regarding the intra- and inter-individual variability of the gut microbiome in health as well as disease [78]. However, the Human Microbiome Project recently found microbiota variability can be accounted for by blood biomarkers (7.6%), clinical characteristics (5.2%), as well as diet (5.7%) [78]. Additionally, other microbiomes (such as airway microbiome) need to be considered in future studies, including prior to and during ATBD and with differing vitamin D status.

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Author contributions

S.M., W.B., and E.A.Y. designed research; E.A.Y. conducted research; E.A.Y., J.F., and S.M. analyzed data; and all authors contributed to drafting and critically revising the paper. S.M. had primary responsibility for final content. All authors read and approved the final manuscript.

Conflicts of interest

E.A.Y., P.M.B., M.J.G. J.L.F., W.B., and D.G.R. have no conflicts of interest. S.M. is an unpaid board member of a diagnostic start-up focused on developing assays for low-cost and point-of-care measurement of certain nutrients from a drop of blood using results from his research as a faculty member at Cornell University.

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Table 24. Supplemental - Illumina adapter sequences

5’ AATGATACGGCGACCACCGAGATCTACAC [i5] TCGTCGGCAGCGTC 3’
5’ CAAGCAGAAGACGGCATACGAGAT [i7] GTCTCGTGGGCTCGG 3’
[i5, i7]: Unique dual index sequence to identify sample-specific sequencing data

CHAPTER V: Conclusions

In summary, metabolic abnormalities affect a large proportion of the global population, and increase the risk of other morbidities (Type 2 *diabetes mellitus,* cardiovascular diseases). Active TB disease is the ninth leading cause of death globally. Some geographic regions have the dual burden of metabolic abnormalities and active TB disease, which have observed synergistic interactions at the population level. Our overall goal was to assess key human host factors, including vitamin D and the gut microbiome, which affect this dual burden and have bi-directional effects on inflammation.

Therefore, our primary objectives were to:

2) assess the predictive performance of common anthropometric cut-offs utilized in diabetes population screening;

3) examine the association between vitamin D and metabolic indicators;

4) assess gut microbiota differences based on serum 25-hydroxyvitamin D status;

among a population with a high prevalence of suspected or confirmed active TB disease, and low or normal body mass index (BMI). Our main findings are summarized below.

Population-screening (anthropometric cut-offs) for elevated HbA1c

Our findings indicate that one-third of study participants had elevated HbA1c (≥ 5.7%), despite the fact that nearly nine of every ten individuals had low or normal BMI (<25 kg/m^2; ***Chapter II***). BMI and WC were respectively associated with HbA1c (≥ 6.5%), however had low sensitivity for identifying those with elevated HbA1c. These results

suggest the need for population-specific cut-offs for BMI and WC, as screening tools for diabetes.

Vitamin D and metabolic abnormalities

Individuals in the lowest 25(OH)D quintile had an increased risk of HbA1c ≥ 5.7% (aRR 1.61; 95% CI: 1.02, 2.56), compared to other quintiles. 25(OH)D was inversely associated with WC ($p<0.01$), although not hypertension ($p>0.05$; ***Chapter III***).

Vitamin D and the gut microbiome

Among this population of adult outpatients with ATBD, the relative abundance of identified OTUs were predominantly in the Bacteroidetes and Firmicutes phyla, which is consistent with previous literature among healthy populations (***Chapter IV***).

Overall, further studies are needed to elucidate the potential roles and mechanistic pathways of vitamin D and the gut microbiome in preventing and managing the dual burden of disease from metabolic abnormalities and active TB disease among populations with low BMI in resource-limited settings.

APPENDIX

The human microbiome in the fight against tuberculosis *

Authors: Madeleine R. Wood[1✧], Elaine A. Yu[1✧], Saurabh Mehta[1,2]

[✧] M.R. Wood and E.A. Yu are joint first authors

Affiliations:

[1] Division of Nutritional Sciences, College of Human Ecology, Cornell University, Ithaca, New York, United States

[2] Institute for Nutritional Sciences, Global Health, and Technology, Cornell University, Ithaca, New York, United States

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Abbreviations

CD – cluster of differentiation

H. pylori – Helicobacter pylori

M. tb – Mycobacterium tuberculosis

OTU – operational taxonomic unit

PCoA – Principal Coordinate Analysis

rRNA – ribosomal ribonucleic acid

SCFA – short chain fatty acid

TB – tuberculosis

T_h – T helper

T_{reg} – regulatory T

Abstract

The human microbiome is an intriguing potentially modifiable risk factor in our arsenal against *Mycobacterium tuberculosis*, the leading infectious disease killer globally. Previous studies have shown associations between the human microbiome and pulmonary disease states, however etiological links between the microbiome and tuberculosis (TB) infection or disease remain unclear. Immunomodulatory roles of the microbiome may prove to be a critical asset in the host response against TB, including in preventing TB infection, reducing progression from latency, mitigating disease severity, and lowering the incidence of drug resistance and co-infections.

This review examined the associations between TB and the gut and lung microbiome. Eight studies were identified through a PubMed database search, including one animal study (n = 1), case report (n = 1), and case-control studies (n = 6). TB infection and disease were associated with reduced gastrointestinal microbial diversity in a murine model and human case report. Sputum microbial diversity differed by TB status in case-control studies, although some reported heterogeneous findings. Current evidence suggests that the gut and lung microbiome are associated with TB infection and disease. However as studies are limited, etiological and longitudinal research is needed to determine clinical relevance.

INTRODUCTION

Mycobacterium tuberculosis caused 1.5 million deaths in 2014,[1] and one-third of the global population has latent tuberculosis (TB).[2] The scourge of TB infection and disease has been documented since early human history, and highlights the substantial remaining challenges of TB control and eradication efforts. In addition to current strategies, successfully addressing TB infection and disease may require targeting other modifiable risk factors, including host microbiota.

The number of host cells comprising the human body is vastly outnumbered by the number of symbiotic microorganisms,[3] and therefore interactions between host cells and microbes occur constantly. The microbiome refers to "the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space."[4] Previous studies have highlighted potential roles of the gastrointestinal and lower respiratory tract's microbiome in the immunological response against TB infection and disease. Generally, bi-directional linkages between the gut microbiome and immune system have been well-documented.[5, 6] Gut microbiota have been associated with a number of disease states, including asthma, autoimmune disorders, cardiovascular diseases, non-alcoholic fatty liver disease.[5, 6, 7]

This review assessed the association between TB infection and disease and the gut and lung microbiome. The microbiome may have important implications for addressing major challenges to effective TB control, including through immunomodulation, in order to reduce and address TB transmission, progression from latency, disease severity,

and drug resistance.

Tuberculosis Pathology and Immune Response

M. tuberculosis bacilli are transmitted from patients with active TB through airborne droplets.[2] In lungs, the innate host response includes the detection (by Toll-like receptors) and elimination (via alveolar macrophages and dendritic cells inducing antimicrobial peptides [cathelicidin] and autophagy) of *M. tuberculosis*.[8] Gamma-interferon and tumor necrosis factor-alpha activate the antimycobacterial capacity of macrophages, through producing nitric oxide, reactive oxygen and nitrogen intermediates.[8] Immune cells (macrophages, fibroblasts, T and B cells) accumulate to form granulomas, surrounding *M. tuberculosis* and restricting growth.[8] Despite these host defenses, survival mechanisms of *M. tuberculosis* (including inhibiting phagolysosome fusion) allow some bacteria to persist within granulomas.[8]

In terms of adaptive immunity, cell-mediated responses by cluster of differentiation (CD)4+ and CD8+ T cells are critical to successfully address *M. tuberculosis*.[8] Primary effector functions of CD4+ and CD8+ T cells include: producing gamma-interferon and cytokines to activate macrophages, and lysing infected macrophages.[8] The T helper (Th_1) cytokine expression pattern is important to eliminate *M. tuberculosis*;[9] conversely, Th_2 and regulatory T (T_{reg}) cell responses support *M. tuberculosis* survival.[8, 10] However, an enhanced Th_1 response simultaneously causes inflammation and host tissue damage,[11] which suggests a balanced Th_1/Th_2 immune response is ideal for patient health outcomes. In summary, the dynamic interplay between host immune response

and *M. tuberculosis* survival mechanisms (such as T cell homeostasis) is modulated through numerous factors, potentially including the microbiome.

The Gut and Lung Microbiome

Gut bacteria play important roles in nutrient metabolism, intestinal homeostasis (through preventing overgrowth of intestinal pathogens), and immunity.[5, 12, 13] Several studies have examined the associations between altered gut microbiota and clinical pathologies including inflammatory bowel disease, diabetes, and obesity.[5, 6, 14]

Historically, the lower respiratory tract has been considered sterile in healthy individuals, but recent culture-independent studies have shown evidence to the contrary.[15, 16] Given the limited available studies focusing on the lung microbiome, one key research gap is determining whether the observed microbiota are simply a continuation of the upper respiratory tract or separate. Preliminary findings corroborate that microbial populations present in the lung are distinct from the upper respiratory tract,[17] and show intra-lung heterogeneity.[16]

Immunomodulation of Microbiota

Murine studies involving gut microbiota have provided evidence of the modulation of microbiota by the immune system. In one study, transgenic mice with expression of human defensin 5, antimicrobial polypeptides secreted by Paneth cells in the small intestine, received a virulent *Salmonella typhimurium* challenge.[18] The transgenic mice had lower bacterial burden in their terminal ilea, compared to wild-type controls.[18]

Additionally, mice lacking specific proteins involved in innate immunity (Toll-like receptor 5, nucleotide-binding and oligomerization domain-like receptor pyrin domain-containing 6) had intestinal dysbiosis and associated pathologies, including colitis or insulin sensitivity.[19,20] Subsequently, the transfer of altered microbiota to wild-type mice was observed to lead to disease.[20] Based on preliminary data, hypotheses include that: immune responses (including against *M. tuberculosis*) facilitate cross-talk between microbial populations (e.g. between the lung and gut microbiome); and dysbiotic microbiota may adversely influence some clinical pathologies.

Microbial diversity is hypothesized to affect the growth of particular opportunistic pathogens, due to resource competition. Krishna et al. (2016) observed that opportunistic pathogens (*Rothia mucilaginosa*) were associated with increased complexity and diversity of sputum microbiota among patients with active TB.[21] Iwai et al. (2014) reported that lower bacterial burden was associated with increased community richness (taxa per sample) and phylogenetic diversity among Ugandans with human immunodeficiency virus and acute pneumonia.[22]

Influence of the Microbiome on Immunity

Conversely, specific commensal gut microorganism species have been observed to *modulate* the immune system.[23,24,25,26] Gut bacteria species (*Bacteroides fragilis* species, Clostridium genus) were associated with altered Treg cell counts, function, and development.[23,24] Previous studies have reported the influence of bacteria on respiratory health, including: 1) airway inflammation, and 2) lung damage (in the context of

influenza and probiotic supplementation). In germ-free mice, increased airway inflammation was rescued by the administration of certain bacterial species (*Faecalibacterium, Lachnospira, Rothia*, and *Veillonella*).[26] Similarly, greater abundance of bacteria species (*Veillonella, Prevotella*) in the supraglottic-characteristic taxa were associated with higher indicators of airway inflammation.[27]

Moreover, two studies showed links between gut bacteria and lung damage. Disrupted gut microbiota (decreased *Bifidobacterium* and *Lactobacillus*) was associated with altered immune response to influenza A infection and increased lung damage in a murine model.[28] Separately, probiotic supplementation of *Bifidobacterium* was associated with improved Toll-like receptor 7 response and reduced lung damage.[28] One hypothesis is that probiotics are protective against pneumococcal disease through modulating the upper respiratory tract microbiome.[29]

In summary, given these dynamic bi-directional interactions between the microbiome and host immunity, commensal bacteria could have a role in the immunological response to pulmonary TB infection and disease. Specific commensal microorganisms may facilitate the cell-mediated immune response to TB infection and disease, and modulate inflammation and lung damage through functional changes of the metagenome and metabolite production.[12, 13]

TUBERCULOSIS AND THE MICROBIOME

In this review, 8 studies examined the association between pulmonary TB infection and

disease and the microbiome, including: an animal study,[30] a case report,[31] and 6 case-control studies (***Table 25***).[21, 32, 33, 34, 35, 36] Key study findings are highlighted in the following two sections (regarding TB and the gut or lung microbiome, respectively), and study methodology is compared in the third section.

Tuberculosis and the Gut Microbiome

Low bacterial species richness and abundance were found in gastrointestinal tract samples from mice and a patient with active TB (***Table 25***).[30, 31] Five female BALB/c mice were infected with aerosolized *M. tuberculosis* (CDC1551 strain).[30] Pre- and post-infection samples differed, in terms of bacterial abundance (among 88 operational taxonomic units [OTUs]; $q<0.01$) and composition (beta-diversity indices of weighted and unweighted UniFrac distances by Principal Coordinate Analysis [PCoA]; both $p\leq0.005$).[30] In a second experiment, mice were infected with a different strain of *M. tuberculosis* (H37Rv; n=5), and compared to 1:1 age-matched controls.[30] Fecal samples from a single time point post-infection similarly showed differential clustering and bacterial abundance (among 73 OTUs; q<0•01), compared to uninfected samples.[30] In the case report, Dubourg et al. (2013) obtained stool samples from a patient with multi-drug resistant active TB who previously received multiple oral antibiotic regimens.[31] Gut microbiota were severely depleted (39 bacterial species, 18 phylotypes, and 19 OTUs).[31]

Other studies have corroborated the potential role of intestinal microbiota in TB. Perry et al. (2010) reported that individuals with latent TB and *Helicobacter pylori (H. pylori)*

Table 25. Studies assessing the association between tuberculosis and the lung and gut microbiome [a]

Study Design	Sample Size		Location	Exposure	Outcome				Key Findings		Ref
					Assessment	Biological Specimen	Microbial Assessment Method(s)	Hypervariable Regions [b]			
	Cases	Controls									
Murine Model	5 female BALB/C mice	---	Maryland, United States	TB infection (aerosolized *M. tuberculosis* CDC1551 strain)	Bacterial diversity [c]	Stool	16S rRNA sequencing	V1-V2	<u>*Pre- vs post-infection*</u> • Alpha diversity (Shannon diversity index): Post-infection decrease (in all mice) • Beta diversity (PCoA; UniFrac): $p < 0.001$ • 88 OTUs more abundant pre-infection: $q < 0.01$	• Majority of differential OTUs: Firmicutes phyla • Relative species abundance of Lachnospiraceae and Ruminococcaceae and Bacteriodales order greater pre-infection and among controls	30
	5 female BALB/C mice	5 mice (1:1 age-matched)		TB infection (aerosolized *M. tuberculosis* H37Rv strain)					<u>*TB vs Control*</u> • Differential clustering (PCoA; UniFrac distances): $p < 0.001$ • 73 different OTUs: $q < 0.01$		
Case Study	Patient with multi-drug resistant TB	---	Marseille, France	---	Microbial diversity	Stool	16S and 18S rRNA sequencing; culture	V6	• Depleted gut microbiota: 39 bacterial species, 18 phylotypes, 19 OTUs • Most phyla (from pyrosequencing) confirmed with culture (Firmicutes, Actinobacteria, Proteobacteria), except Fusobacteria species (only culture) and Cyanobacteria/Chloroplast (only pyrosequencing) • New species detected by culture (*Candidatus "Paenibacillus antibioticophila"*) and 18S rRNA sequencing (3 fungal species)		31
Case-Control	31 patients with pulmonary TB	24 controls	Shanghai, China	TB status	Bacterial diversity	Sputum (cases); saliva / pharyngeal secretions (controls)	16S rRNA sequencing	V3	<u>*TB vs Control*</u> • Greater phyla and genera richness among patients with TB (24 phyla; 564 genera) vs controls (17 phyla; 235 genera) • Most prevalent phyla: Firmicutes, Proteobacteria, Bacteriodetes, Crenarchaeota, and Actinobacteria • Lower Bacteriodetes and higher Actinobacteria prevalence		32

									• Certain genera (*Stenotrophomonas, Cupriavidus, Pseudomonas, Thermus*) and phyla (Aquificae, Planctomycetes) only in TB samples • Differential clustering (PCoA; UniFrac distances)	
	32 patients with unilateral pulmonary TB	24 healthy controls	Jiangsu, China	TB status	Bacterial diversity	Bronchoalveolar lavage (cases); saliva, pharyngeal secretions (controls)	16S rRNA sequencing	V3	<u>*TB vs Control*</u> • Clustering (PCoA; UniFrac distances): Distinct • Genus: Most abundant genus differed <u>*TB (Uninfected vs Infected Lungs)*</u> • Clustering (PCoA; UniFrac distances): Similar • Genus: *Mycobacteria* and *Porphyromonas* abundances greater inside TB lesions • Shannon diversity index: Higher in bronchoalveolar lavage fluid from lungs with lesions, compared to controls	36
	22 patients with TB	14 controls with TB-like coughing	Hong Kong SAR, China	TB status	Bacterial diversity	Sputum	16S rRNA sequencing	V1-V2	<u>*TB vs Control*</u> • Phyla: Lower Firmicutes, and higher Proteobacteria and Bacteriodetes abundance • Genra: Lower *Streptococcus*, and higher *Neisseria* and *Prevotella* • OTUs: 8 more prevalent, 2 less prevalent • No differences in clustering (PCoA; UniFrac distances) <u>*Overall*</u> • 98% of identified sequences accounted for 5 phyla (Proteobacteria, Firmicutes, Bacteriodetes, Fusobacteria, Actinobacteria) • Most prevalent bacterial genra: *Neisseria, Prevotella, Streptococcus*	33
	Group 1: Newly diagnosed pulmonary	Group 4: Healthy controls (n = 20)	Shanghai, China	TB status	Bacterial diversity	Sputum (cases); throat swabs (controls)	16S rRNA sequencing	V1-V2	• Clustering (PCoA; UniFrac distances): Differed between Groups 1-3 vs 4; similar between Groups 1-3 • Relative species abundance (phyla, genera): Heterogeneous between groups • Some genera (*Bergeyella, Sharpea*) only in Groups 1-3	34

	TB (n = 25) Group 2: Recurrent TB (n = 30) Group 3: Treatment failure (n = 20)									
	6 patients with TB	6 controls without TB	Medellin, Colombia	TB status	Bacterial and fungi diversity	Sputum, oropharyngeal, nasal samples	16S rRNA sequencing (bacteria; fungi [fungal nuclear ribosomal internal transcribe spacer ITS1 regions])	V1-V2	<u>*Bacteria: TB vs Control*</u> • Shannon diversity index: Bacterial diversity (in nasal samples) less diverse • Clustering (PCoA; UniFrac distances): Sputum and oropharyngeal samples clustered together, distinct from nasal samples • Oropharyngeal: *Streptococcaceae* more abundant <u>*Bacteria: Overall*</u> • Phyla: Relative abundance differed across sample type (bacterial diversity higher in sputum and lower in nasal samples) <u>*Fungi: TB vs Control*</u> • Genus: *Cryptococcus* (in oropharyngeal samples) significantly lower <u>*Fungi: Overall*</u> • Shannon diversity index: Fungal diversity greatest in nasal samples • Phyla: 90% sequences in Ascomycota and Basidiomycota; Ascomycota significantly differed between study participants	[35]
	25 patients	16 controls	Madhya	TB status	Bacterial diversity	Sputum	16S rRNA	V6-V7	<u>*TB vs Control*</u>	[21]

	with pulmonary TB		Pradesh, India				sequencing		• Clustering (PCoA): Distinct • Phyla: Higher Firmicutes and Actinobacteria, lower Proteobacteria and Fusobateria • Most prevalent genera (cases: *Streptococcus, Neisseria, Veillonella*; controls: *Gammaproteobacteria, Streptococcus, Neisseria, Haemophilus)* • Species: *Veillonella dispar* and *Rothia mucilaginosa* greater • Shannon diversity index: Lower for TB (3.88; 602 OTUs) than controls (4.13; 490 OTUs)	

[a] PubMed search strategy was based on the following terms: "((microbiota[mesh] OR microbiota[tw] OR microbiome*[tw] OR virome*[tw] OR gut bacteria[tw]) AND (tuberculosis[mesh] OR tuberculosis[tw] OR TB[tw]))," and restricted by publication date (prior to June 6, 2016). Exclusion criteria included: 1) not primary data source (including reviews, editorials); 2) no whole microbiome sequencing techniques; 3) not among patients or animals with TB or exposure to TB (including assessing vaccine response among healthy volunteers, microbiome among patients with HIV or reproductive tract inflammation). Among the 44 studies initially identified, 8 studies were within the scope of this review and included.

[b] Assessed by 16S rRNA sequencing

[c] First experiment: Baseline (pre-infection) to 179 days (post-infection). Second experiment: 0-46 days.

had more interferon gamma and Th_1-like cytokines, compared to those without *H. pylori*.[37] *Actinobacteria* in infant stool samples was associated with increased T cell responses to vaccination, including Bacille Calmette Guérin vaccination for TB.25 Furthermore, cynomolgus macaques with *H. pylori infection* were less likely to develop active TB if inoculated with *M. tuberculosis*.[37] Another murine study demonstrated how specific enteric bacteria (*Helicobacter hepaticus*) modulated the immune system to alter susceptibility to *M. tuberculosis* and vaccine response in a mouse model.[38] One hypothesis was that activation of the innate immune system facilitates an enhanced response to other pathogens, such as *M. tuberculosis*.

Several recent studies have provided evidence of the immunomodulatory mechanism of the gut microbiome, which produces short chain fatty acid (SCFA) metabolites that may affect the host response against *M. tuberculosis*.[39] Intestinal microbiota produce SCFAs through the fermentation of resistant starches and dietary fiber.[40] SCFAs have roles in host metabolism (as substrate for de novo lipid and glucose synthesis) and immunomodulation (through downregulating pro-inflammatory cytokines and T_{reg} cells).[41] In a study involving *M. tuberculosis* stimulation of human peripheral blood mononuclear cells, physiological concentrations of the SCFA butyrate significantly decreased pro-inflammatory cytokine production.[39] Given the importance of the cell-mediated response (including T_{reg} cells [42]) against TB infection, SCFA production is one hypothesized mechanism of the link between the microbiome and TB infection and disease.

Overall, the gut microbiome of TB samples had lower bacterial abundance and composition, relative to controls. However, given the limited data and heterogeneous study designs, preliminary evidence suggests the need for further studies to confirm etiology through mechanistic and clinical studies. Potential next steps include improving our understanding of the role of metabolites in mediating the association between the gut microbiome and TB disease and infection.

Tuberculosis and the Lung Microbiome

Several case-control studies in this review showed that bacterial diversity (richness, abundance, OTU clustering, Shannon index) in the respiratory tract differs between individuals with active TB, compared to controls (***Table 25***).[21, 32, 33, 34, 36] Based on PCoA, four studies found distinct OTU clustering of sputum samples from study participants with TB disease, compared to controls.[21, 32, 34, 36] However, one study observed no differences in OTU clustering.[33] All case-control studies identified differences in bacterial richness or relative abundance, based on taxonomic categorizations (phyla, genus, species), in sputum from individuals with and without active TB. However, the specific differential taxonomic groups were inconsistent and not replicated across studies, which may reflect the relatively small and diverse sample populations.

In 5 case-control studies, sputum was considered an indicator for the microbiome of the lung and lower respiratory tract among study participants with active TB. Given that sputum is likely to be contaminated by the upper respiratory tract during expectoration, the continued use of sputum reflects the challenge of directly obtaining samples from

the lung.

Sample collection methods for referent groups varied, including samples from deep coughing of healthy individuals,[32, 36] throat swabs,[34] bronchoalveolar lavages,[36] and sputum from individuals with TB-like coughing.[33] Botero et al. (2014) reported that the microbial compositions of sputum and oropharyngeal samples were similar, which supports the use of throat swabs from controls.[35] Limitations include: deep cough, throat swab, and sputum samples may represent the upper respiratory tract (instead of the lung and lower respiratory tract microbial composition); TB-like coughing could be caused by other diseases that affect the microbiome; and the invasiveness of bronchoalveolar lavages. Thus, interpretations of study findings need to account for potential sample contamination and the appropriate selection of controls.

As a brief summary, several studies showed distinct bacterial richness or relative abundance of the lung microbiome among patients with active TB, compared to controls. Further studies are needed, particularly in light of differences in study designs and discrepant observations.

Comparison of Study Methods

Strengths of studies in this review included the consistent use of next-generation platforms for 16S ribosomal ribonucleic acid (rRNA) sequencing to assess the bacterial microbiome in all eight studies. Seven studies utilized 454 instruments (GS FLX, GS FLX-Titanium [Roche]), and one study used an Ion Torrent PGM (Thermo Fisher

Scientific). Two studies additionally evaluated fungal species,[31, 35] and one study compared 16S rRNA results with culture and 18S rRNA sequencing.[31] In all studies, sequence analyses were conducted through software (including Quantitative Insights Into Microbial Ecology, Mothur), and OTUs were identified through aligning sequences with reference databases (Ribosomal Database Project, Greengenes), in order to eliminate chimeras. Some studies considered differences (including sociodemographic characteristics) of sample populations, which are supported by previous literature. Additionally the eight studies included study participants from several geographic locations, which allowed for comparison and confirmation of similar findings across diverse populations.

Available studies had several limitations, in addition to aforementioned challenges. Compared to Sanger sequencing (such as with ABI 3730 Genetic Analyzer [Applied Biosystems]), 454 sequencing has been reported to have higher error rates due to greater insertion and deletion rates.[43] Importantly, methodological heterogeneity in data analyses rendered comparisons across studies difficult; these included differences in hypervariable regions (V1-V3, V6, V7), diversity indices (including alpha- [Shannon index, Chao1] and beta-diversity [unweighted and weighted UniFrac, Jaccard]), and additional analyses (PCoA, hierarchical clustering heatmap).

Separately in the case-control studies, the sample sizes (12-95) and geographic locations (Asia [China, Hong Kong, India], South America [Colombia]) ranged widely. Therefore, results were difficult to generalize to other countries. Additionally, numerous

other potential confounding factors (antibiotic use [including anti-TB treatment], comorbidities, environmental factors, diet) were either unaccounted for or considered differently across study populations. Standard anti-TB medications are a combination of antibiotics, which are expected to alter the microbiome, and therefore need to be considered while interpreting study results. Four of the six case-control studies excluded participants with recent antibiotic use (one or three months prior to baseline).[32, 33, 35, 36] However, two studies did not report the exact length of time that participants did not receive antibiotics before sample collection,[21] or prior antibiotic use.[34] In the case report, the patient was receiving anti-TB treatment for multi-drug resistant TB at the time of sample collection.[31]

DISCUSSION

In this review, there is limited evidence regarding the key question of the bi-directional associations between TB infection and disease and the lung and gut microbiome. Potential mechanistic pathways need to be considered through epidemiological and mechanistic studies, including immunological, nutritional, metabolic and endocrine factors (***Figure 5***). Furthermore, there are a number of other research gaps and related questions that have not been directly addressed by studies to date. One of these overarching knowledge gaps involves inter-individual variability, which is a key focus area of the Human Microbiome Project.[44] What is the extent that a core set of human microbiome genes or species is shared between individuals?[44] Additionally, what are

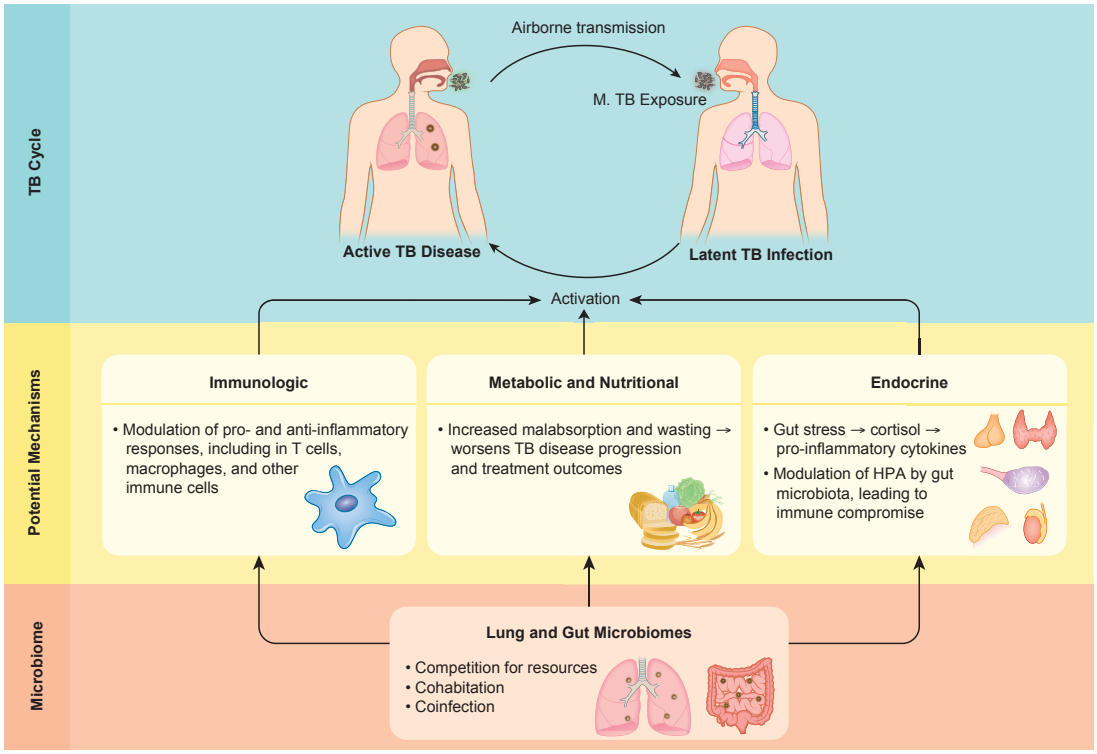

Figure 5. Potential mechanistic pathways between the microbiome and TB

the temporal changes of lung and gut microbiota (including throughout TB disease progression and treatment)? Separately, to what extent does crosstalk occur between the lung and gut microbiome, particularly in response to TB infection and disease-related perturbances? Followingly, do these interactions between the respiratory and gastrointestinal microbiome modulate immune responses to TB infection and disease?

CONCLUSION

Based on preliminary evidence from studies in this review, the lung and gut microbiome were associated with TB infection and disease. The microbiome is a potential modifiable risk factor for TB infection and disease, however the number of available studies is limited. Most studies have focused on characterizing the microbial

profile among individuals with and without active TB disease. Future studies are necessary to further elucidate etiology, key mechanisms, and potential clinical significance. Specifically, it is important to assess: 1) the sputum and gut microbiome as risk factors for TB infection and disease susceptibility, disease progression, and treatment outcomes; and 2) the effects of TB infection and disease on the sputum and gut microbiome, which can subsequently impact health via alteration of immune responses.

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