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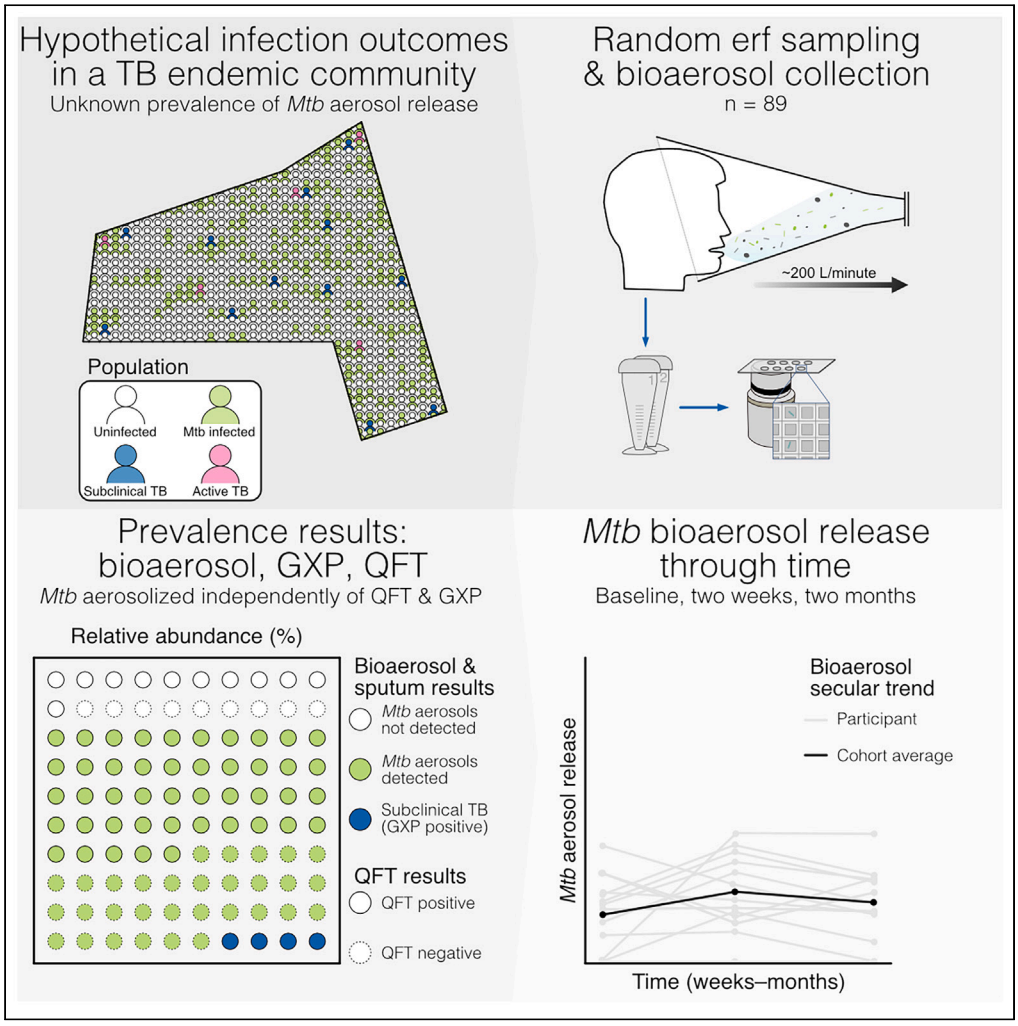

Ryan Dinkele, Sophia Gessner, Benjamin Patterson, ..., Anastasia Koch, Digby F. Warner, Robin Wood

digby.warner@uct.ac.za (D.F.W.)
robin.wood@hiv-research.org.za (R.W.)

Highlights

Pathology is widely considered a prerequisite for *M. tuberculosis* (*Mtb*) transmission

Subclinical TB is common and increasingly recognized as a driver of *Mtb* transmission

The contribution of asymptomatic infection to *Mtb* transmission remains unknown

Aerosolization of *Mtb* is common in high-TB regions and undetectable by standard tests

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Persistent *Mycobacterium tuberculosis* bioaerosol release in a tuberculosis-endemic setting

Ryan Dinkele,[1,2] Sophia Gessner,[1,2] Benjamin Patterson,[3] Andrea McKerry,[4] Zeenat Hoosen,[4] Andiswa Vazi,[4] Ronnett Seldon,[4] Anastasia Koch,[1,2] Digby F. Warner,[1,2,5,*] and Robin Wood[2,4,*]

SUMMARY

Pioneering studies linking symptomatic disease and cough-mediated *Mycobacterium tuberculosis* (*Mtb*) release established the infectious origin of tuberculosis (TB), simultaneously informing the notion that pathology is a prerequisite for *Mtb* transmission. Our recent work has challenged this assumption: by sampling TB clinic attendees, we detected equivalent release of *Mtb*-containing bioaerosols by confirmed TB patients and individuals not receiving a TB diagnosis and observed time-dependent reduction in *Mtb* bioaerosol positivity during 6-month follow-up of both cohorts, irrespective of anti-TB chemotherapy. Now, we report widespread *Mtb* release in our TB-endemic setting: of 89 randomly recruited community members, 79.8% (71/89) produced *Mtb*-containing bioaerosols independently of QuantiFERON status, a standard test for *Mtb* exposure. Moreover, during 2-month longitudinal sampling, only 2% (1/50) were serially *Mtb* bioaerosol negative. These results necessitate a reframing of the prevailing paradigm of *Mtb* transmission and TB etiology, perhaps explaining the historical inability to elucidate *Mtb* transmission networks in TB-endemic regions.

INTRODUCTION

Infection with *Mycobacterium tuberculosis* (*Mtb*) encompasses a spectrum of outcomes.[1–4] At its mildest, the immune system clears or contains infecting *Mtb* bacilli with minimal discernible impact on the host.[5] At its most severe, pulmonary *Mtb* infection results in advanced disease that is associated with a 40%–70% fatality rate in the absence of effective treatment.[6] Despite the rarity of progression to active disease at an individual level and the availability of effective chemotherapy,[5,7] tuberculosis (TB) remains a leading global cause of mortality,[8] with notification rates surpassing those reported in the early 1900s in certain settings.[6]

In endemic regions, most incident TB is thought to arise from the recent (<2 years) transmission of *Mtb*.[9,10] However, with only 1%–30% of new *Mtb* infections traceable to known TB cases,[11–14] it is likely that numerous undetected sources of *Mtb* transmission exist within these communities. Consistent with this possibility, there is growing interest in the notion that early TB states—encompassing *Mtb* infection, subclinical, and clinical TB[4]—might provide a previously overlooked reservoir of transmission.[15,16] Establishing the propensity for *Mtb* release—often equated with "infectiousness"—during early TB is therefore essential but very difficult to accomplish.[17]

Sputum diagnosis is generally considered the gold standard for assessing infectiousness among TB patients.[18] Through this lens, data from extensive community TB screening efforts utilizing sputum-GeneXpert—which have identified large numbers of asymptomatic cases[19]—support the notion of unrecognized transmitters in high-burden communities. And, although these results accommodate the potential for *Mtb* transmission to occur prior to the development of recognizable TB symptoms,[16] they nevertheless rely on sputum production (itself a symptom of disease) for the detection of *Mtb*. This dependency on sputum production imposes a significant constraint on attempts to detect individuals with early TB and is exacerbated by the difficulties encountered in obtaining useable samples from many individuals.[18]

Bioaerosol sampling offers a non-invasive method to collect peripheral lung fluid and/or particulate matter independent of the specific disease state.[20] Moreover, the detection of *Mtb* in bioaerosols signifies the release of bacilli by an individual, tentatively offering a concurrent metric of transmission risk.[21,22] Despite these advantages, the utilization of bioaerosol sampling has predominantly been restricted to individuals with bacteriologically confirmed TB. This owes mainly to the challenges inherent in handling extremely paucibacillary samples as well as the assumed importance of frank pathology for *Mtb* release—in turn reinforcing the primacy of sputum bacterial burden as key predictor of infectiousness. Insights gained from the COVID-19 pandemic, however, established the plausibility of asymptomatic transmission,[23,24] with analogous recent results suggesting the cough-independent aerosolization of *Mtb*.[25,26] It seems possible, therefore, that individuals might

[1]UCT Molecular Mycobacteriology Research Unit, Department of Pathology, Faculty of Health Sciences, University of Cape Town, Cape Town 7925, South Africa
[2]Institute of Infectious Disease and Molecular Medicine, Faculty of Health Sciences, University of Cape Town, Cape Town 7925, South Africa
[3]Amsterdam Institute for Global Health and Development, University of Amsterdam, Amsterdam 1105, the Netherlands
[4]Aerobiology and TB Research Unit, Desmond Tutu Health Foundation, Cape Town 7925, South Africa
[5]Wellcome Centre for Infectious Diseases Research in Africa, Faculty of Health Sciences, University of Cape Town, Cape Town 7925, South Africa
*Correspondence: digby.warner@uct.ac.za (D.F.W.), robin.wood@hiv-research.org.za (R.W.)
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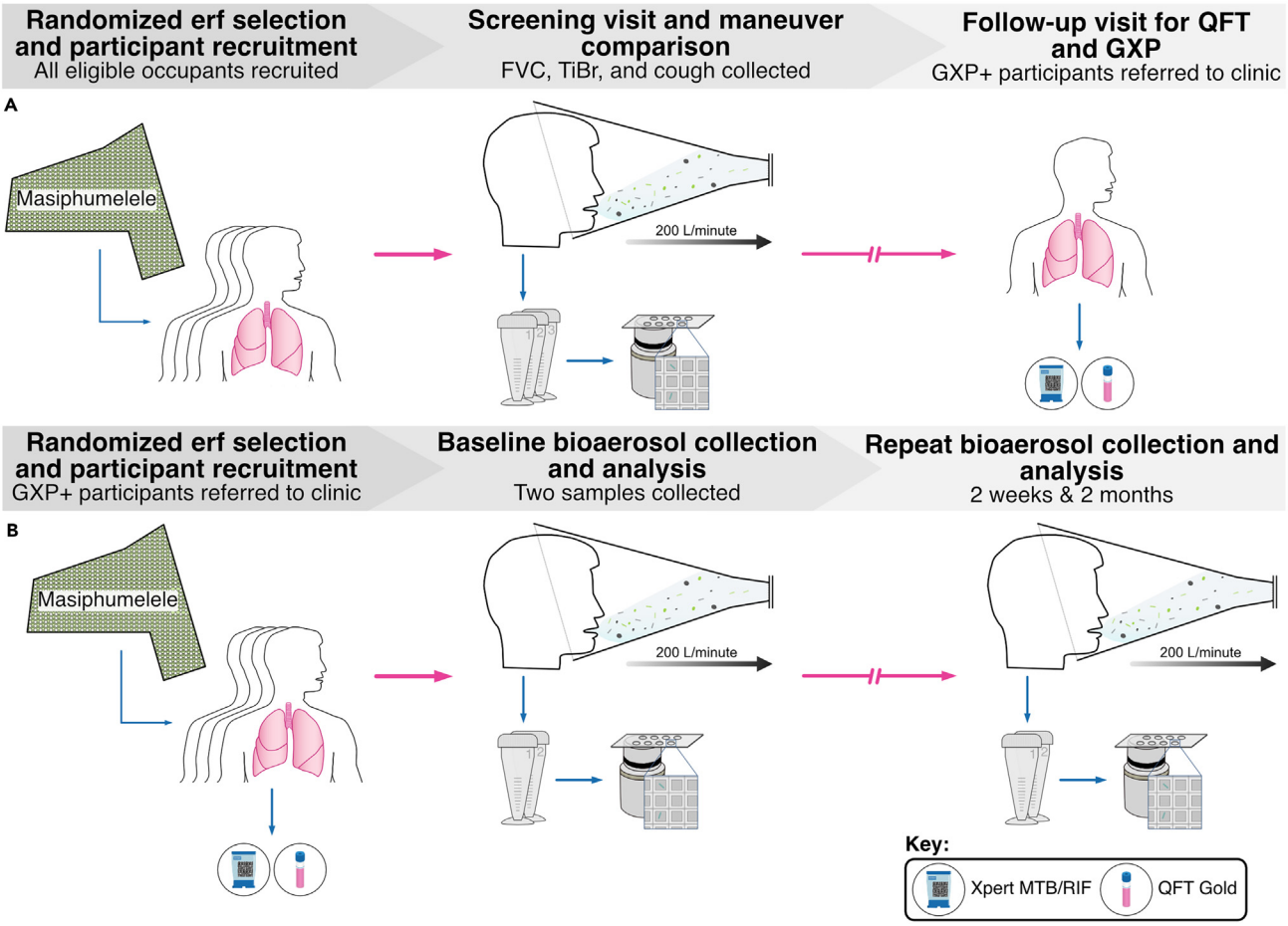


Figure 1. Participant recruitment and bioaerosol sampling algorithms for the two randomized community cohorts

(A) For the initial cross-sectional survey, 39 participants were recruited from randomly selected erfs in Masiphumelele. At a first screening visit, participants produced bioaerosol samples from three respiratory maneuvers: forced vital capacity (FVC), tidal breathing (TiBr), and induced cough. Samples were processed and visualized independently by microscopists blinded to all sample information. Owing to the high prevalence of *Mtb* bioaerosol positivity, all participants were brought back for a follow-up visit during which blood and sputum were collected for QFT and GXP analysis, respectively.

(B) For the longitudinal study of *Mtb* bioaerosol release, 50 participants were recruited. Blood and sputum samples were collected at baseline for QFT and GXP analyses, respectively. Two equivalent bioaerosol samples were collected during 10 min of tidal breathing with deep breaths taken at 30-s intervals. These samples were processed and imaged independently on nanowell-arrayed microscope slides by microscopists blinded to all sample information. This process was repeated at 2 weeks and 2 months after initial recruitment.

progress from *Mtb* infected (often inferred via interferon gamma release assay [IGRA],[27] of which the QuantiFERON-TB Gold [QFT] test is the predominant version) to infectious before producing sputum.

Since 2013, we have studied the spontaneous generation of *Mtb*-containing bioaerosols using the respiratory aerosol sampling chamber (RASC), a highly sensitive, adaptable personal clean room designed to capture all particulate matter (including *Mtb* bacilli) released by individuals.[28] By combining liquid capture of bioaerosols in the RASC with DMN-trehalose-enabled microscopic detection and visualization of *Mtb* bacilli, we recently reported that ~90% of clinic attendees with presumptive TB produced bioaerosols containing viable *Mtb* bacilli.[29] Surprisingly, the high proportion of *Mtb* bioaerosol positivity was independent of final TB diagnosis: *Mtb* bioaerosol release was common to both notified TB patients—who commenced anti-TB chemotherapy—and those not diagnosed with TB (who did not initiate treatment). Moreover, during 6-month follow-up, the rates of decline in symptom severity and *Mtb* bioaerosol release were equivalent in both groups. And, at the 6-month study endpoint, approximately 20% of all participants—including treated TB patients and those who did not receive a TB diagnosis—remained *Mtb* bioaerosol-positive despite clinical resolution, consistent with prior observations from radiological[30] and other assays.[31]

Given the high prevalence of *Mtb* bioaerosol release among clinic attendees not diagnosed with TB, as well as the frequency of *Mtb* bioaerosol release in confirmed TB patients on completion of standard anti-TB treatment, we hypothesized that the production of *Mtb* bioaerosols by individuals living in TB-endemic communities might be more common than previously thought. To investigate this possibility,

Table 1. Summary of participant demographic and clinical information from the two cohorts

Variable	Category	Cohort 1 n (%)	Cohort 2 n (%)	*p* value
Total		39 (100)	50 (100)	–
Gender	Male	12 (30.8)	13 (26)	0.64
Previous TB	Yes	7 (17.9)	4 (8)	0.20
HIV status	Negative	29 (74.4)	38 (76)	**0.02**
	Positive	5 (12.8)	12 (24)	
	Unknown	5 (12.8)	0 (0)	
GeneXpert Ultra	Negative	34 (87.2)	48 (96)	>0.99
	Positive	2 (5.1)	2 (4)	
	NA	3 (7.7)	0 (0)	
QuantiFERON-TB Gold	Negative	17 (43.6)	16 (32)	0.15
	Positive	16 (41)	33 (66)	
	Indeterminate	2 (5.1)	1 (2)	
	NA	4 (10.3)	0 (0)	
Age [mean (sd)]		32.9 (12.6)	34.2 (12.5)	0.636

NA = not available (incomplete or failed assay); Excluded from the analysis.

we utilized our bioaerosol sampling platform[25] to investigate *Mtb* release in 89 randomly selected participants recruited into two consecutive cohorts: the first was a cross-sectional community survey comprising 39 participants, and the second was a longitudinal observational study in which 50 individuals consented to be serially sampled at three separate time points over 2 months. As detailed below, our results suggest that early-stage *Mtb* infection is pervasive and may be an important contributor to the transmission of *Mtb* in high TB-burden communities.

RESULTS

High-prevalence *Mtb* bioaerosol release in a TB-endemic community independent of respiratory maneuver

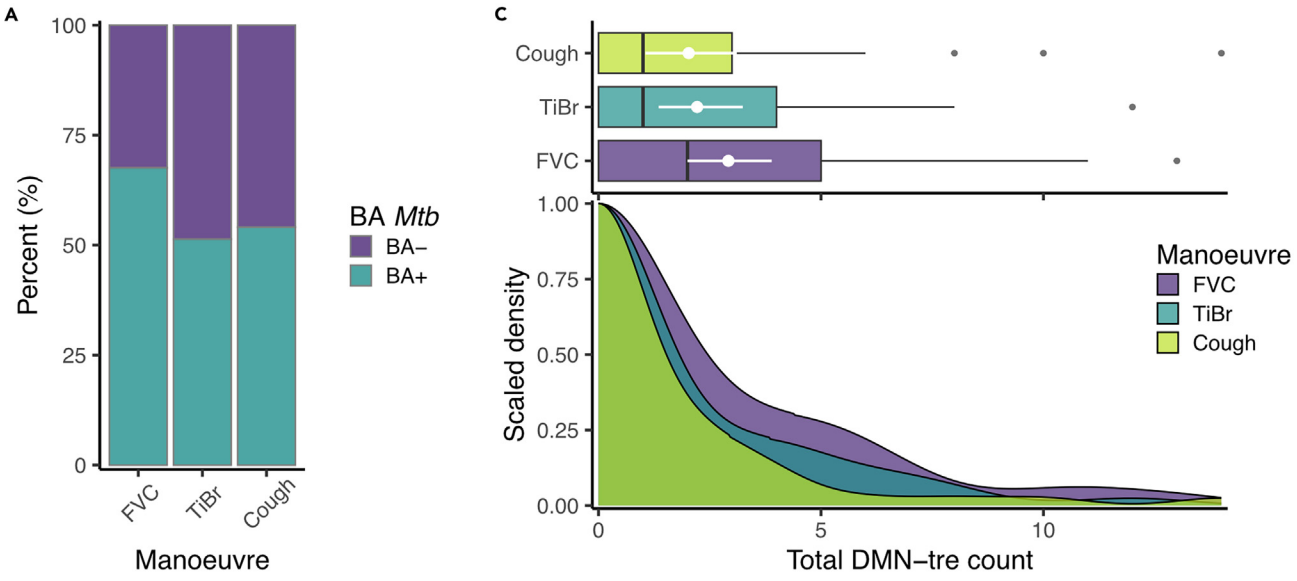
To investigate the prevalence of *Mtb* bioaerosol release in a TB-endemic setting, we initiated a cross-sectional community survey in Masiphumelele, Cape Town, with recruitment randomized geospatially by parcel of land or "erf" (Figure 1). From 10 erfs, we sequentially enrolled 39 individuals (1–12 participants/erf [median = 3]) who were assessed in *Mtb* bioaerosol release, sputum-GeneXpert MTB/RIF (GXP), and QuantiFERON-TB Gold (QFT) assays. The cohort had high rates of human immunodeficiency virus (HIV) infection (13%) and previous TB (18%). Two participants who produced *Mtb* bioaerosols at the screening visit (5%) were GXP-positive at follow-up (one of these participants had completed treatment for TB in 2020, 2 years prior to the study), providing immediate evidence of undiagnosed TB in the community (Table 1).

Advanced lung pathology and chronic cough are often viewed as essential for *Mtb* release.[32] This assumption is, however, incompatible with the notion that *Mtb* may transmit during early-stage infection. Here, we examined the potential for *Mtb* release from a randomly selected community cohort, considering both unrelated cough[33] and tidal breathing[25] as potential mechanisms. This was done by comparing *Mtb* aerosolization from three independently sampled respiratory maneuvers, namely, forced vital capacity (FVC—a deep breathing respiratory maneuver), tidal breathing (TiBr), and cough, according to our previously described methodology.[25]

When comparing the respiratory maneuvers, the percentage of *Mtb*-positive samples ranged from 51.1% to 67.6%, with no significant differences observed in the likelihood of producing *Mtb* between the three respiratory maneuvers (Figures 2A and 2B). Moreover, no differences were observed in the average number of bacteria identified per sample (Figures 2C and 2D). Across FVC, TiBr, and cough, the average counts were 2.9 (95% confidence interval [CI]: 1.9; 4.0), 2.2 (95% CI: 1.4; 3.2), and 2.0 (95% CI: 1.1; 3.1) *Mtb* bacilli per sample, respectively. Overall, we found that 79.5% (31/39) of the participants produced at least one positive bioaerosol sample when considering all three maneuvers. Together, these data suggest that the prevalence of *Mtb* release is high in a TB-endemic setting.

Altering the bioaerosol sampling algorithm did not reduce *Mtb* detection efficiency

The observation that ~80% of a randomly selected community cohort produced *Mtb* bioaerosols was reminiscent of our previous work, which demonstrated the high baseline prevalence (~90%) of *Mtb* release among TB clinic attendees with presumptive disease, regardless of final diagnosis or respiratory maneuver.[29] However, the cross-sectional community survey only provided a snapshot in time of *Mtb* bioaerosol release. To investigate the variability of *Mtb* release through time, we repeated our random community sampling strategy in recruiting a further 50 individuals from 20 erfs (1–7 participants/erf [median = 2]) into a longitudinal observational study of *Mtb* release (Figure 1).



B

	Value	OR	95% CI	P value
Manoeuvre	FVC	3.23	0.89;11.7	*0.0756*
	Cough	1.21	0.36;4.03	0.7591

D

	Value	IRR	95% CI	P value
Manoeuvre	FVC	1.37	0.89;2.11	0.1513
	Cough	0.94	0.59;1.49	0.7903

Figure 2. High-prevalence *Mtb* bioaerosol release in a TB-endemic community independent of respiratory maneuver

(A) The percentage of samples in which putative *Mtb* were detected (turquoise) or absent (purple) from forced vital capacity [FVC (67.6%)], tidal breathing [TiBr (51.4%)], and cough (51.1%).

(B) Results of a logistic regression comparing the odds of a positive bioaerosol result compared to TiBr.

(C) Box and whisker and equivalent density plots comparing the total number of *Mtb* detected between the three respiratory maneuvers. White circle and error bars overlayed onto the box and whisker plots represent the mean ±95% CI.

(D) Results of a negative binomial regression comparing the number of *Mtb* detected between the three respiratory maneuvers. OR = odds ratio, IRR = incident rate ratio, CI = confidence interval, BA = bioaerosol.

Bioaerosols were collected at baseline, and follow-up visits were scheduled at 2 weeks and 2 months postbaseline, in accordance with previously described time intervals.[29] The characteristics of this cohort were not significantly dissimilar to the first 39 participants (Table 1), except for HIV status—where the difference was driven largely by the proportion of individuals reporting their status as "unknown." Given the observation from the initial cohort that FVC and TiBr were sufficient to aerosolize *Mtb*, we reasoned our bioaerosol sampling algorithm could be simplified by removing induced cough. Therefore, rather than collecting three 5-min samples (~15 min), each from a different respiratory maneuver, we implemented a single sampling algorithm, which was repeated twice: 10 min of tidal breathing with deep breaths every 30 s (~20 min sampling in total). This enabled head-to-head comparison of duplicate bioaerosol samples at each visit, allowing for an assessment of variation through time.

We found comparable results between first and second cohorts in both the proportion of participants producing at least one positive sample and the total number of aerosolized *Mtb* (Figures 3A–3C). Average *Mtb* counts at baseline varied from 0 to 16, with 20% (10/50) of the participants producing two negative bioaerosol samples (Figure 4A). Discrepant samples (i.e., where one sample was negative and the other positive) were infrequent [22% (11/50)] with a low maximum sample difference (three bacilli). There was a strong correlation between the first and second bioaerosol sample (Figure 4B) and a high degree of agreement, with 60% of the samples differing by ± 1 (Figures 4C and 4D). Considering the relatively small sample size of each cohort and the comparability of the bioaerosol results, we pooled the data to investigate which covariates were associated with *Mtb* release. No covariates were associated with increased odds of an *Mtb*-positive sample (Table 2). In contrast, biological sex (incident rate ratio [IRR] = 1.88, $p < 0.05$), previous TB (IRR = 2.33, $p < 0.05$), and age ≥45 years (IRR = 0.48, $p < 0.05$) were associated with the number of *Mtb* bacilli detected (Table 3). Surprisingly, neither QFT status—the conventional marker of *Mtb* infection—nor HIV status was associated with *Mtb* release. Overall, these data suggest that individuals who aerosolize *Mtb* do so consistently within short time frames and independently of standard markers of infection.

Persistent *Mtb* release among a randomly selected community cohort is common

Based on the observation from baseline sampling that 94% of matched samples differed by fewer than five *Mtb* bacilli, we reasoned that differences in the average count between two consecutive samples through time of five or greater could be considered significant. According to

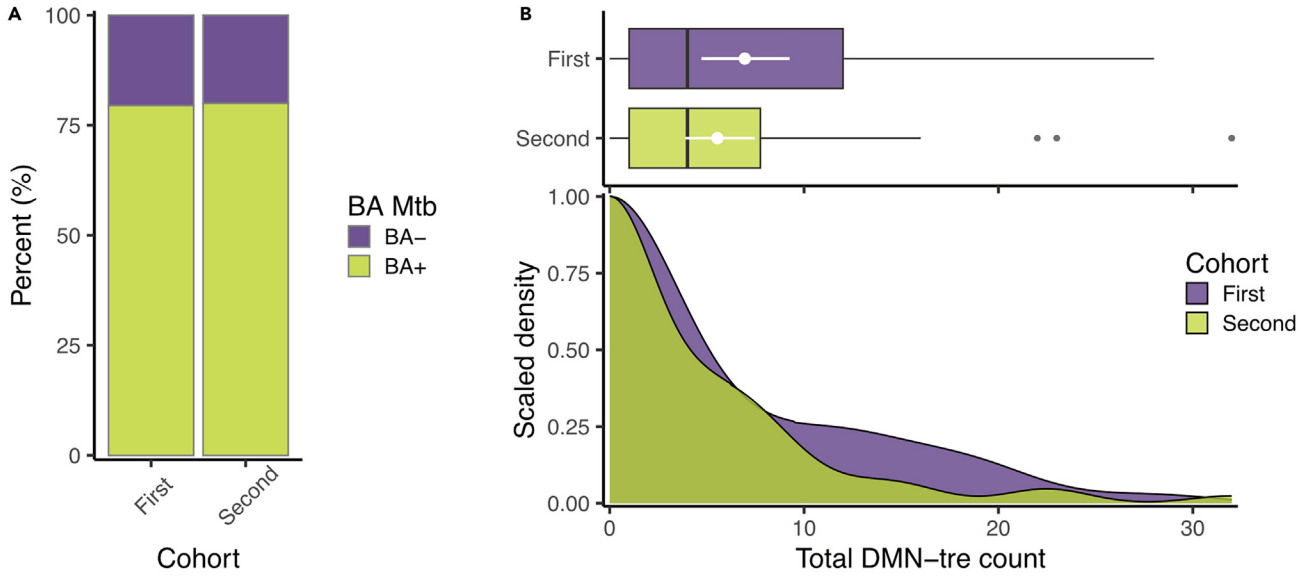


Figure 3. Altering the bioaerosol sampling algorithm did not reduce *Mtb* detection efficiency

(A) The percentage of samples in which putative *Mtb* were detected (green) or absent (purple). The odds of a positive bioaerosol sample were equivalent between the two groups (OR = 1.03, 95% CI = 0.36; 2.92, p = 0.952) (B) Box and whisker and equivalent density plots comparing the total number of *Mtb* detected between the two cohorts. The rates at which *Mtb* were produced during the two samplings were equivalent (IRR = 0.797, 95% CI = 0.47; 1.33, p = 0.386). White circle and error bars overlayed onto the box and whisker plots represent the mean ±95% CI. OR = odds ratio, IRR = incident rate ratio, CI = confidence interval, BA = bioaerosol.

this definition, most (74% [32/43]) participants were constant low-level producers of *Mtb* over 2 months (Figure 5A). The rate of bioaerosol positivity was relatively consistent through time, ranging from 76.7% to 90.7% (Figures 5B and 5C). Most surprisingly, only one individual was negative across all three visits (representing six negative bioaerosol samples in total). Of 43 individuals with complete data for all three time points (baseline, 2 weeks, 2 months), 19% (8/43) and 9% (4/43) were negative at one and two visits, respectively. Although individual participants varied in their bioaerosol count from visit to visit, there was no overall trend in the number of *Mtb* detected per visit (Figures 5D and 5E). This lack of secular trend in *Mtb* bioaerosol release among a predominantly asymptomatic, randomly selected community cohort supports our previous hypothesis that symptomatic *Mtb* bioaerosol clearance is immune driven.[29]

DISCUSSION

The use of new tools in TB research has enabled unexpected insights that question established models of pathogenicity. For *Mtb* transmission, the increasing awareness that TB disease is not required for *Mtb* bioaerosol release interrogates previous assumptions linking pathology to infectiousness,[17] in turn raising important questions about the implications for new diagnostics, drugs, and vaccines.[34]

In work immediately preceding this report, we detected high-prevalence *Mtb* bioaerosol release among TB clinic attendees, irrespective of diagnosis. Moreover, persistent release of *Mtb* bioaerosols was observed post-treatment among bacteriologically confirmed TB cases,[29] a finding consistent with separate work that used positron emission tomography–computed tomography (PET-CT) imaging and *Mtb* mRNA detection to conclude that apparently curative treatment for TB might not eradicate all *Mtb* bacteria.[30] One implication of our previous results was that release of *Mtb* bioaerosols within TB-endemic communities might be considerably more prevalent than previously understood. To address that possibility, we initiated the work reported here, in which we broadened our sampled population to screen a random selection of individuals from the larger of the two communities served in the earlier study. In doing so, we modified the sampling protocol slightly to exclude forced coughing and, importantly, demonstrated high reproducibility in *Mtb* capture numbers when repeated samples were obtained from the same individual during the same visit.

The results reported here were again unexpected: aerosolized *Mtb* was detectable in 80% of participants recruited at random in our high TB-burden community. Moreover, *Mtb* release occurred independently of QFT results, the standard test for current or previous exposure to *Mtb*.[27] From longitudinal sampling of 50 participants over 2 months, it was apparent that most individuals were transiently or persistently releasing *Mtb*: only one participant returned negative *Mtb*-bioaerosol results across all three visits (representing six negative bioaerosol samples). Despite variation in *Mtb* release per participant, no overall trend was observed in *Mtb* release through time for the cohort. This observation contrasts with our previous report of the spontaneous clearance of *Mtb* bioaerosols by predominantly symptomatic clinic attendees with presumptive TB.[29] It may be that, in apparently healthy individuals, the containment of infection is achieved independently of an interferon gamma response or through innate immunity. However, it is tempting to speculate that, in most people, *Mtb* bacilli are able to avoid immune surveillance or are in a relatively immune-privileged site—a possibility strengthened by several recent studies suggesting the ability

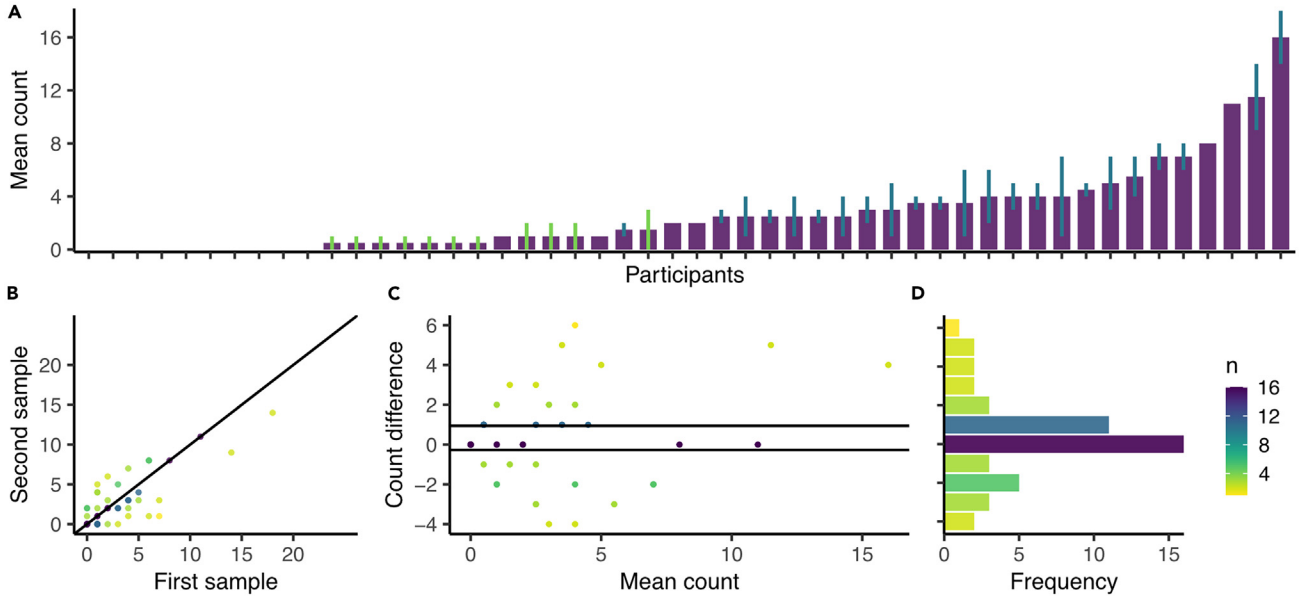


Figure 4. The consistent production of aerosolized *Mtb* during two equivalent respiratory maneuvers

(A) Plot of the mean *Mtb* (DMN-tre positive) count from two samples, with error bars representing the range. No lines indicate equal counts, green lines indicate one count = 0, blue lines indicate two counts >0.

(B) Plot of the *Mtb* (DMN-tre positive) counts of the first and second samples at baseline ($r = 0.810$, $p < 0.0001$) with a fitted line representing a 1:1 correlation.

(C) A Bland-Altman plot indicating the level of agreement between the first and second samples, with a (D) histogram showing the frequency of each count difference. Most samples differed by either 0 or ± 1 (60%) and 94% of the samples differed by four or less.

of *Mtb* to avoid exposure to immune surveillance,[35,36] perhaps through occupation of intercellular niches.[37] Determining the immune correlates of protection and/or the anatomical location of bacilli released in bioaerosols in asymptomatic individuals therefore represents a key research priority.

The inferred potential for *Mtb* colonization (distinguished from "infection" by the absence of a detectable host immunological response[36]) seems an important consideration when designing anti-*Mtb* vaccines and/or deciding who to vaccinate, especially in TB-endemic regions. That is, the persistent bioaerosol release of *Mtb* within this community could indicate a homeostatic interaction between the bacillus and its human host. Although half of the participants were QFT positive, there was no correlation with aerosol *Mtb* exhalation, suggesting that current methods for detecting *Mtb* infection and estimating patient infectiousness are insufficient. Notably, HIV status was not associated with aerosolized *Mtb*, further implying a lack of immune interaction between host and pathogen. Our observations, together with previous studies linking the aerosol release of *Mtb* to infectiousness,[21,22] imply a large reservoir of potential transmitters that remains invisible to national TB programs. However, the applicability of bioaerosol sampling techniques as screening tools for preventing *Mtb* transmission is predicated on the definitive demonstration that incident infections, and importantly, TB cases, arise from this population of relatively well individuals. Further studies are planned to establish the attributable fraction of TB transmission from this population; the immediate implications, though, are that a focus on treatment as the sole intervention to prevent transmission will require reassessing if the attributable fraction is significant in high TB-burden settings.

The observation that *Mtb* bioaerosol release is common in this community and is not detectable by standard measures of *Mtb* infection motivates for an urgent reframing of the prevailing paradigm of *Mtb* transmission and infection, while also emphasizing the importance of bioaerosol sampling in understanding the etiology of TB. The notion that infection with *Mtb* constitutes the primary determinant of TB risk is common in infectious disease research[38] and has dominated thinking about new TB interventions. Our work using advanced breath aerosol collection technology challenges this assumption: in successive studies, we have detected release of *Mtb*-containing bioaerosols in confirmed TB patients and a majority of randomly selected community members, and we have demonstrated the time-dependent reduction (but not elimination) of *Mtb* bioaerosol positivity in TB clinic attendees irrespective of TB chemotherapy. These observations reinforce the axiom that although *Mtb* infection is necessary for TB, it is not sufficient. It appears unlikely that TB arises solely as a consequence of host,[38] bacillary,[39] or environmental[40,41] factors. Instead, the high proportion of *Mtb* bioaerosol-positive individuals detected in this setting in the absence of symptoms suggests that a poorly understood combination of these elements determines disease risk.

Finally, although the significance for TB control and the attributable transmission risk from this population remains to be established, these data provide a plausible explanation for the difficulty in linking transmission chains in high-burden regions. Since the bacilli produced by randomly selected community members appear quantitatively and qualitatively indistinguishable from those released by confirmed TB patients (at least using existing tools to interrogate phenotypic and/or genetic adaptations), it seems plausible that there is some contribution to

Table 2. Results of a logistic regression assessing the odds of a positive bioaerosol sample

Variable	Category	OR	95% CI	*p* value
Age	≥45	1.52	0.34; 10.8	0.622
Biological sex	Male	1.44	0.38; 7.14	0.619
Previous TB	Yes	1.12	0.24; 8.13	0.898
QFT gold[a]	Positive	1.44	0.44; 4.66	0.543
HIV status[b]	Positive	0.63	0.17; 2.66	0.495

OR = odds ratio, CI = confidence interval.
[a]Indeterminate results removed.
[b]Unknown status removed.

ongoing *Mtb* transmission. By extrapolating to the broader community, the implication appears unavoidable that there are far greater numbers of disease- and symptom-free individuals with prolonged shedding of *Mtb* in communal settings than those with TB disease.

Limitations of the study

The results presented earlier must be interpreted in the light of the study's limitations, including sample size and composition, as well as the potential for the false-positive identification of *Mtb*. Participants were recruited from randomly selected erfs during the workday between Monday and Friday. Our sample was therefore biased to individuals both willing and able to participate in the study. However, the average age, QFT status, and HIV prevalence were as expected for the community,[13] suggesting that our observations are internally reliable within Masiphumelele. It seems unlikely, therefore, that the manner of participant recruitment would significantly impact the results of this study.

Our assay for the detection of *Mtb* release is based on the microscopic detection of bacilli matching specific morphological characteristics, which also take up with DMN-tre.[28] This technique is potentially subject to the false-positive identification of *Mtb*, given that other Actinomycetales produce the enzyme required for the probe incorporation.[42] However, we have previously used the RASC for *Mtb* detection by colony formation and droplet-digital PCR.[29,43,44] More recently, we successfully obtained whole-genome sequence data from three bioaerosol samples after 50-day culture, importantly demonstrating the presence of *Mtb*, with no non-tubercular mycobacteria or other Actinobacteria found.[29]

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ryan Dinkele (ryan.dinkele@uct.ac.za).

Materials availability

This study did not generate new unique reagents.

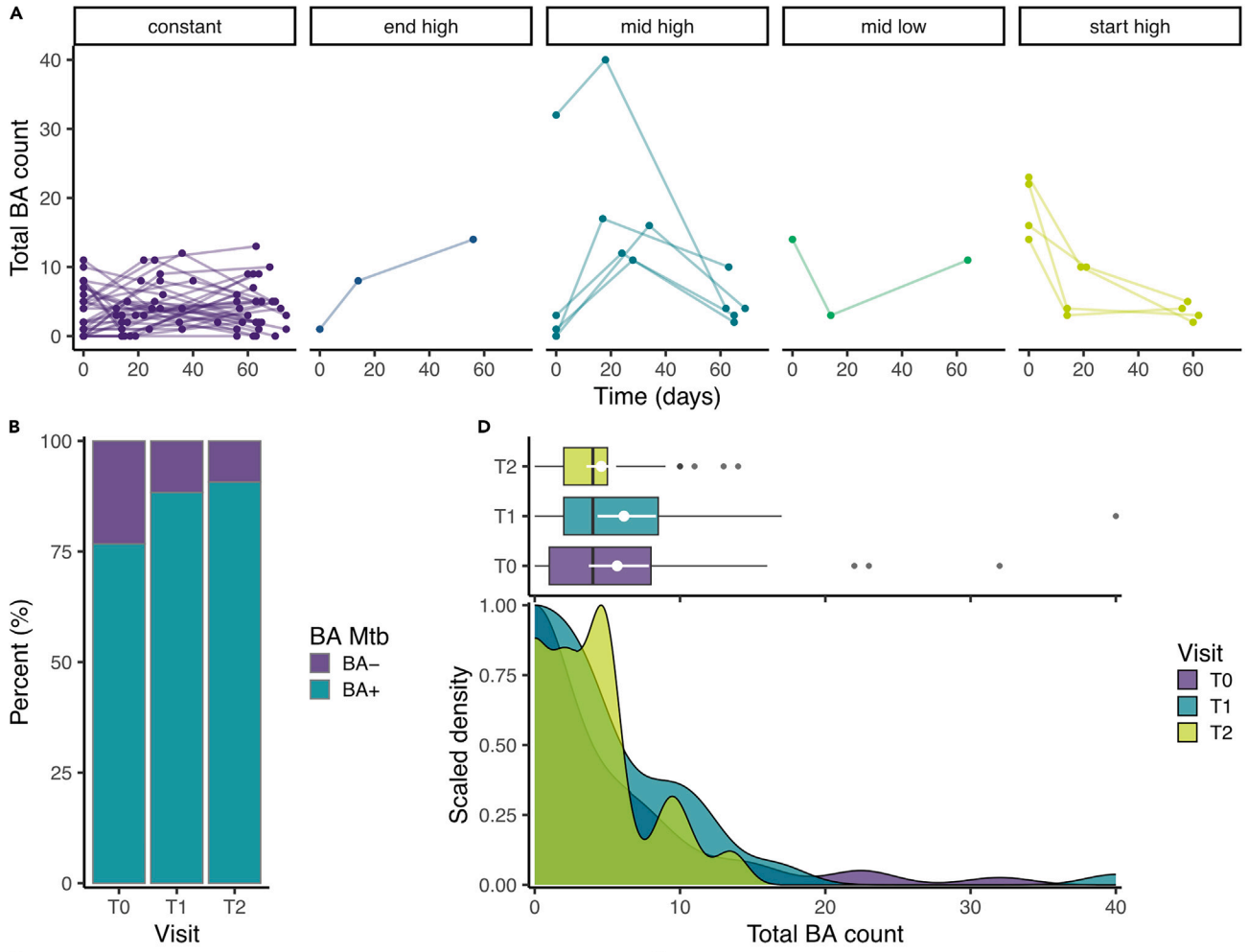
Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Table 3. Results of a negative binomial regression assessing the number of *Mtb* per participant

Variable	Category	IRR	95% CI	*p* value
Age	≥45	0.48	0.24; 1.03	**0.039**
Biological sex	Male	1.88	1.07; 3.42	**0.029**
Previous TB	Yes	2.33	1.18; 5.02	**0.015**
QFT gold[a]	Positive	1.01	0.60; 1.68	0.958
HIV status[b]	Positive	0.86	0.45; 1.70	0.649

IRR = incident rate ratio, CI = confidence interval.
[a]Indeterminate results removed.
[b]Unknown status removed.



C

	Value	OR	95% CI	P value
Visit	T1	0.28	0.06;1.32	0.1072
	T2	0.19	0.04;1.06	0.0582

E

	Value	IRR	95% CI	P value
Visit	T1	1.11	0.74;1.67	0.6186
	T2	0.89	0.58;1.35	0.5818

Figure 5. Persistent *Mtb* release among a randomly selected community cohort is common

(A) Total Mtb (DMN-tre positive) counts (sum of the two samples) through time, stratified by time trend.

(B) The percentage of samples in which putative *Mtb* were detected (turquoise) or absent (purple) at each of the visits.

(C) Results of a logistic regression comparing the odds of a negative BA result compared to T0.

(D) Box and whisker and density plots comparing the total number of *Mtb* bacilli (DMN-tre positive) detected at each visit. White circle and error bars overlayed onto the box and whisker plots represent the mean ±95% CI.

(E) Results of a negative binomial regression comparing the number of *Mtb* bacilli (DMN-tre positive) detected at each visit. OR = odds ratio, IRR = incident rate ratio, CI = confidence interval, BA = bioaerosol.

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AUTHOR CONTRIBUTIONS

Conceptualization and design: R.D., S.G., B.P., A.M., Z.H., A.V., R.S., A.K., D.F.W., and R.W.; acquisition of data: A.M., Z.H., A.V., and R.S.; analysis and interpretation: R.D., D.F.W., and R.W.; first manuscript draft: R.D., D.F.W., and R.W.; funding acquisition: D.F.W. and R.W. All authors critically reviewed and revised the manuscript for intellectual content and approved it prior to submission.

DECLARATION OF INTERESTS

The authors have no conflicting interests to declare.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
DMN-Trehalose	OliLux Biosciences	oliluxbio.com/products
Critical commercial assays		
Xpert® MTB/RIF	Cepheid	GXMTB/RIF-US-10
Software and algorithms		
R (version 4.3.3)	R	www.r-project.org/
lme4	Douglas et al.[45]	cran.r-project.org/web/packages/lme4/index.html

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Study population and participant recruitment

This study was conducted in Masiphumelele, a peri-urban township located south of Cape Town, South Africa. This residential area is divided into demarcated parcels of land, called "erfs" under South African legislation, each with its own unique numeric identifier. Erfs were randomly selected and all consenting participants over 14 years of age were eligible for recruitment into this study. Recruitment was conducted between February–December 2022, with ethical approval from the Human Research Ethics Committee of the University of Cape Town (HREC no. 529/2019).

The Respiratory Aerosol Sampling Chamber

The RASC is a purpose-built personal clean room equipped with a high efficiency bioaerosol collection system, which captures all exhaled particulate matter (bioaerosols) at 100–300 L/min in 15 mL of collection medium (sterilized phosphate-buffered saline supplemented with Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azilocillin (PANTA) [Becton Dickinson]). Bioaerosol samples were concentrated and stained with DMN-trehalose (Olilux Biosciences Inc.) overnight, as previously reported, before transfer to nanowell devices for imaging.[28]

METHOD DETAILS

Bioaerosol generation and sample processing

This study was conducted in two phases (Figure 1). In the first phase, participants 1–39 underwent bioaerosol collection during three respiratory maneuvers: FVC, TiBr, and induced cough, as previously described.[25] During FVC and cough sampling, participants performed the designated maneuver 15 times within a 5-min period, as directed by the study nurse. For tidal breath sampling, participants were instructed to breathe normally into the bioaerosol collection system for 5 min. Each bioaerosol sample underwent independent collection, processing, and enumeration. Enumeration of *Mtb* bacilli was conducted by depositing concentrated and DMN-trehalose-stained bioaerosol samples onto nanowell devices. Bioaerosol positivity was determined by aggregating counts from all three maneuvers. Given the high proportion of bioaerosol positivity within this cohort, all participants were scheduled for a follow-up visit to obtain blood and sputum samples for subsequent QFT and GXP analysis.

The second phase involved the recruitment of participants 40–89 for a longitudinal examination of *Mtb* bioaerosols. This phase comprised 50 individuals sampled on three occasions: baseline, two weeks, and two months. At each visit, two equivalent bioaerosol samples were collected, with each involving 10 min of tidal breathing interspersed with deep breaths every 30 s. Subsequently, these samples were concentrated and arrayed on nanowell devices. Blood and sputum specimens were obtained at baseline for QFT and GXP analysis, respectively.

In all cases, microscopists were blinded to the origin of the sample (which included an empty booth negative control) and enumerated putative *Mtb* bacilli per sample.

QuantiFERON-TB gold assay

In this study, Bio Analytical Research Corporation South Africa (BARC SA) were contracted to perform and interpret QFT assays according to the manufacturer's instructions (Qiagen).

Sputum collection and the GeneXpert assay

For all participants, sputum sampling was attempted. In those capable of producing sputum, a GXP assay was performed and interpreted according to the manufacturer's instructions (Cepheid). Individuals unable to produce sputum were considered sputum-GXP negative.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical methods

For the descriptive statistics, a Fisher's Exact Test was used for the categorical variables given the relatively small sample size. To assess age, the normality and variance of the data were assessed, and a Student's T Test was used for the comparison. Generalized linear regression was used to assess the bioaerosol results, either as a dichotomous outcome (logistic regression) or a count outcome (negative binomial regression). These outcomes were regressed against age, biological sex, previous TB, QFT results, and HIV status. For the respiratory maneuver comparison, we used the equivalent mixed effects regression models using lme4[45] in to account for the fact that each participant produced three samples. All statistical analyses were conducted in R version 4.3.3.[46]