



Environment pollutants exposure affects the endogenous activation of within-host *Mycobacterium tuberculosis*

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ABSTRACT

Objective: Epidemiological studies have linked ambient pollutants with tuberculosis (TB) risk, but the association has not been fully understood. Here, for the first time, we applied whole-genome sequencing (WGS) to assess the reproductive state of *Mycobacterium tuberculosis* (MTB) by profiling the mutation rate of MTB (MTBMR) during within-host endogenous reactivated progression, intending to dissect the actual effects of ambient pollutants on the endogenous reactivation.

Methods: We conducted a retrospective cohort study on bacteriologically confirmed TB patients and followed them for relapse in Jiangsu and Sichuan Province, China. Endogenous and exogenous activation were distinguished by WGS of the pathogen. The average concentration of air pollution was estimated by considering a lag of 0–1 to 0–12 months. We applied a generalized additive model with a Poisson function to evaluate the relationships between ambient pollutants exposure and MTBMR.

Results: In the single-pollutant adjusted models, the maximum effect for PM₁₀ (MTBMR increase: 81.87%, 95% CI: 38.38, 139.03) and PM_{2.5} (MTBMR increase: 73.91%, 95% CI: 22.17, 147.55) was observed at a lag of 0–12 months for every 10 µg/m³ increase. For SO₂, the maximum effect was observed at lag 0–8 months, with MTBMR increasing by 128.06% (95% CI: 45.92, 256.44); and for NO₂, the maximum effect was observed at lag 0–9 months, with MTBMR increasing by 124.02% (95% CI: 34.5, 273.14). In contrast, the O₃ concentration was inversely associated with MTBMR, and the maximum reduction of MTBMR was 6.18% (95% CI: -9.24, -3.02) at a lag of 0–9 months. Similar results were observed for multi-pollutant models.

Conclusions: Increased exposure to ambient pollutants (PM₁₀, PM_{2.5}, SO₂, and NO₂) contributed to a faster MTBMR, indicating that MTB exhibits increased reproductive activity, thus accelerating within-host endogenous reactivation. O₃ exposure could decrease the MTBMR, suggesting that MTB exerts low reproductive activity by inhibiting within-host endogenous activation.

1. Introduction

Active tuberculosis (TB) is a chronic contagious disease due to viable

Mycobacterium tuberculosis (MTB) that causes clinical symptoms with abnormal radiographic findings or microbiologic confirms. The natural disease spectrum of TB includes (i) latency (the most common course,

Abbreviations: TB, tuberculosis; MTB, *Mycobacterium tuberculosis*; LTBI, latent tuberculosis infection; WGS, whole-genome sequencing; SNPs, single-nucleotide polymorphisms; MNPs, multi-nucleotide polymorphisms; MTBMR, mutation rate of *Mycobacterium tuberculosis*; IQR, interquartile range; GAM, generalized additive model; CIs, confidence intervals.

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which encompasses persistent or eliminated disease burden), (ii) slow or (iii) rapid progression through the incipient and subclinical disease to active TB, or (iv) a period of cycling through incipient and subclinical states that may precede the overt symptomatic disease or eventual disease resolution (Drain et al., 2018). Although MTB is the causative agent of pulmonary tuberculosis, evidence from real-world studies suggests environmental factors may fuel TB disease progression (Dheda et al., 2016; WHO, 2022). It is of considerable public health importance to integrate with disease spectrum, environmental factors, and epidemiological evidence to develop individualized preventive strategies to protect those with latent TB infection (LTBI)/previously treated TB infection (completed initial treatment but tendency to recur) against progressing to endogenous activated disease, due to existing estimate of the global burden of LTBI as “one-fourth” of the world population, as well as frequent relapse (Houben and Dodd, 2016; WHO, 2022).

Air pollution, undoubtedly, is an alarming concern because it has been associated with human systemic ailments diseases, notably respiratory and cardiovascular diseases (Guo et al., 2013, 2022; Liu et al., 2019). Recent evidence suggests that exposure to outdoor air pollutants might impair immune functions, resulting in a higher susceptibility to active TB (Popovic et al., 2019; Xiang et al., 2021). However, available studies also reported that ambient pollutants had insignificant effects on TB, even protective effects to some extent. Thus far, the results have been inconsistent and poorly understood (Popovic et al., 2019; Xiang et al., 2021). These results should be interpreted carefully due to a limited understanding of its clinical pathogenic spectrum of infection and disease. To date, there is a lack of accurate detectable molecular epidemiological data to evaluate its relationship based on progressing spectrum of within-host endogenous activation of MTB.

Fortunately, genetic changes usually occur steadily per generation in living organisms. For example, species with shorter generation times (i.e., the time required for bacterial amplification to double, a measurement of replication rate) tend to have faster speeds of molecular evolution (i.e., whole-genomic mutation rates) (Thomas et al., 2010). These observations have revealed that the number of mutations that occur over a defined period can be used as a “molecular clock” that estimates replication rates within a bacterial species (Colangeli et al., 2020; Ford et al., 2011; Gibson et al., 2018). The use of mutation rates described under laboratory conditions combined with measures of mutation accumulation over time in natural conditions has been applied to estimate replication rates in various bacteria (Gibson et al., 2018). Several studies have also utilized a molecular clock to study the evolution rates of MTB and confirmed that mutation rates are relatively higher when MTB is in a state of increased replication rate and reproduction activity, which might more appropriately be considered “active TB” (Colangeli et al., 2020; Ford et al., 2011). Conversely, if MTB transits into a physiological status of low mutation rates, this indicates that MTB has a low replication rate or inhibitory activity (Colangeli et al., 2020; Ford et al., 2011).

Here, we conducted a retrospective multi-center cohort to investigate the bacteriological relationship between ambient pollutants and TB by deciphering the endogenously activated progression of unique within-host MTB strain isolated from initial matched recurrent episodes before anti-tuberculosis chemotherapy.

2. Methods

2.1. Study setting and population

We collected initial matched recurrent isolates from three independent cohort studies. 1) We prospectively recruited sputum smear-positive pulmonary TB patients during a drug-resistance surveillance program from January 1, 2011 to December 31, 2020 in Lianyungang City, Jiangsu Province of Eastern China. 2) We recruited sputum smear-positive pulmonary TB patients from designated hospitals from August 2013 to December 2015 in Xuzhou, Nantong, Changzhou and Taizhou

Cities, Jiangsu Province of Eastern China (Liu et al., 2022). 3) Pulmonary TB patients that were bacteriologically confirmed sputum smear- or culture-positive were recruited from January 1, 2009 to December 31, 2020 in Wusheng County, Sichuan Province of Western China (Li et al., 2022). We collected the patient's sex, age, treatment history, culture-based drug-susceptibility test, treatment outcome, diabetes, pulmonary cavity, sputum test results at the second month of treatment, the interval between initial episodes and recurrent episodes, and residence. Based on these three cohorts, we first selected patients whose initial episodes were successfully cured and then recruited patients with initially matched recurrent culture-positive strains who succeeded in whole-genome sequencing (WGS) before anti-tuberculosis chemotherapy. The “cured” case is a bacteriologically confirmed TB patient who was smear- or culture-negative in the last month of anti-tuberculosis treatment. Recurrent TB was defined as a new episode of bacteriologically confirmed TB in a patient with a previously bacteriologically confirmed TB episode who completed treatment or was declared cured.

2.2. DNA extraction, quality control and mapping of WGS

Strains were recovered on the Lowenstein-Jensen medium at 37 °C from stored samples that were kept in -80 °C freezers, and genomic DNA was extracted with the cetyltrimethylammonium bromide (CTAB) method (Schiebelhut et al., 2017). A 300-base-pair double-ended DNA library was constructed for each isolate and sequenced on an Illumina NovaSeq 6000 with an expected sequencing depth of 200 × . Raw sequencing reads were trimmed with ‘fastp’ (version 0.20.1). We removed reads of low quality and retained good sequencing reads mapped to the reference genome (H37Rv, NC000962.3) with BWA-MEM (version 0.7.17). Freebayes (version 1.3.6) was used to detect single-nucleotide polymorphisms (SNPs), insertions and deletions (indels), and multi-nucleotide polymorphisms (MNPs) by the following criteria: Phred base quality ≥30, mapping quality ≥30, sequencing depth ≥5, no less than two reads supporting the mutated allele and frequency of the mutated allele ≥75% (Garrison and Marth, 2012).

2.3. Pairwise SNP distance analysis and phylogenetic tree reconstruction

The fixed SNPs (frequency ≥75%), excluding those in drug-resistant genes and repetitive regions of the genome (e.g., PPE/PE-PGRS family genes, phage sequence, insertion or mobile genetic elements) (Yang et al., 2022), were used to calculate the pairwise SNP distance. MTB isolates with a sequencing depth of <20 × or a genome coverage of <95% were excluded from the analysis. The identified SNPs were used to construct a phylogeny tree using MEGA (version X) with the maximum likelihood method and 1000 bootstraps. The phylogenetic trees were visualized with the Interactive Tree of Life (<https://itol.embl.de/>). Sequencing data were deposited in the Genome Sequence Archive (<https://bigd.big.ac.cn/gsa>) under accession to the numbers CRA004889 and CRA011332.

2.4. Definition of endogenous reactivation and exogenous reinfection

Recurrent TB was judged as endogenous reactivation when MTB isolate pairs extracted from the initial and relapse episodes of TB had less than 12 SNPs difference between their respective genomes. Conversely, for those with an SNP distance greater than 12 SNPs, differences between genomes were judged as indicating exogenous reinfection with a different strain (Colangeli et al., 2020; Li et al., 2022). The TB-Profiler program detected mixed infections, drug resistance, and lineage profiles (Phelan et al., 2019).

2.5. Calculation of MTB mutation rates of *Mycobacterium tuberculosis* (MTBMR)

Fixed 18-hour generation time equal to that reported for log phase growth in MTB cultures (Trojanowski et al., 2015), we estimated the MTB mutation rates (mutations/base pair [bp]/generation) as follows:

$$\mu = \frac{\sum_{i=1}^n m_i}{N \cdot \sum_{i=1}^n (t_i/g)}, \text{ where } \mu \text{ was the mutation rate, } m_i \text{ was the number of}$$

SNPs that differed in the i th paired MTB isolates out of n pairs, and N was the genome size (sequencing reads covering an average of 99.5% of the MTB genome, $N = 0.995 \times L$, where L was reference genome size with 4,411,532 bp), t_i was the time between the initial and recurrent MTB strains collected (hours), and g was the generation time (hours). Since t_i was captured in days, when estimating the mutation rate, t_i was converted to hours.

2.6. Data on air pollutant concentrations and meteorological factors

We extracted data on meteorological factors, including daily average temperature ($^{\circ}\text{C}$), daily average relative humidity (%), daily average wind speed (m/s) and daily sunshine time (hours) in six cities between January 1, 2014, and December 31, 2020, from the China Meteorological Data Sharing Center (<http://data.cma.cn/>), as well as the daily average concentrations of six ambient pollutants (<http://106.37.208.233:20035/>), including PM_{2.5} (particulate matter with aerodynamic diameter $\leq 2.5 \mu\text{m}$), PM₁₀ (particulate matter with aerodynamic diameter $\leq 10 \mu\text{m}$), sulfur dioxide (SO₂), nitrogen dioxide (NO₂), carbon monoxide (CO), and ozone (O₃) (the concentration of O₃ was the maximum 8-hour moving average) in 6 cities during the same period. The concentration unit of pollutants was measured in $\mu\text{g}/\text{m}^3$.

2.7. Statistical analysis

Isolate pairs were categorized into a higher and lower mutation rates group according to the median value of mutation rates of within-host MTB. Categorized variables are expressed as frequencies (percentages), and continuous variables are described as medians (interquartile range, IQR). We compared the differences of groups using the chi-square test, Mann-Whitney U test, or Fisher's exact test. We applied the generalized additive model (GAM) with a Poisson link function to estimate the effects of ambient pollutant exposure on the MTBMR from lag 0–1 to lag 0–12 months. Poisson models were used to obtain the mutation rates (mutations/bp/generation) by using bp \times generation as an offset. Covariates adjusted in the model included city, sex, age, diabetes, pulmonary cavity, MTB lineages, drug resistance, daily average ambient temperature, relative humidity, wind speed, and sunshine time. We also performed two sensitivity analyses to examine the robustness of the relationships between ambient pollutants exposure and MTBMR. First, we estimated the association at different lag times. Second, we included data for other ambient pollutants to construct multi-pollutant models.

Optimal maximum freedom (df) selection for the time variable (months between initial matched recurrent isolates) was made by generalized cross-validation score. First, we constructed a basic model, including all the above covariates except for meteorological factors, and added a thin plate spline function with a maximum of 6 df for the time variable to control the long-term trends of MTBMR. The generalized cross-validation score was used to identify the optimal maximum df for the time variable. We then used the thin plate spline function with an optimal maximum df for the time variable, a maximum df of 2, to build a smooth term for four meteorological factors and added them to the basic model separately to construct a single-pollutant model (Zhu et al., 2018).

We used the R package "mgcv" to construct the GAM. The strength of the relationship between factors and mutation rates of within-host MTB was described as a percentage change and 95% confidence intervals (CIs) for each 10 $\mu\text{g}/\text{m}^3$ increase in air pollutants. The significance level

was set at 0.05. All analyses were performed using R software (version 4.1.2, <https://www.r-project.org/>).

3. Results

3.1. Participant enrollment

We conducted a multi-center study based on three cohorts. In the first cohort, 2252 sputum smear-positive TB patients were recruited in Lianyungang city, Jiangsu Province, China, from January 1, 2011 to December 31, 2020 (Fig. 1A). In the second cohort, we recruited 2098 bacteriologically confirmed TB patients in designated hospitals in 4 cities in Jiangsu Province, China, from August 2013 to December 2015. (Fig. 1B). In the third cohort, there were 1728 bacteriologically confirmed cases of pulmonary TB registered in a county in Sichuan Province, China, from January 1, 2009, to December 31, 2020 (Fig. 1C). After excluding nontuberculous mycobacteria-infected patients, failed sequencing samples, failed initial treatment, and exogenous reinfections and further considering the routine monitoring of meteorological factors and ambient pollutants since January 1, 2014, we finally included 99 TB patients with available initial matched recurrent isolate pairs for analysis.

3.2. Clinical characteristics and microbiological findings in lower and higher within-host mutation rates groups

Among 99 TB patients, 49 (49.50%) had lower mutation rates ($<2.72 \times 10^{-10}$), and 50 (50.50%) had higher mutation rates ($\geq 2.72 \times 10^{-10}$). The median (IQR) times among initial matched recurrent isolate pairs in lower mutation rates and higher mutation rates groups were 18.67 (13.43–34.30) and 22.93 (15.39–39.01) months, respectively. Of these, 85 (85.86%) were men, and 14 (14.14%) were women. Their age ranged from 20 to 86 years in recurrent episodes, with a median (IQR) of 66.00 (54.00–72.00) and 62.00 (53.75–70.50) years, respectively, in lower mutation rates and higher mutation rates groups. No clinical characteristic (sex, age, pulmonary cavity, diabetes, time between isolate pairs from initial matched recurrent patients, and early/late endogenous activation), and microbiological findings (MTB lineages, sputum test at the second month of standardized combination chemotherapy, and drug resistance of isolate pairs) were statistically distinguishable between lower mutation rates and higher mutation rates groups (Table 1).

3.3. Lineages and phylogenetic relationships between MTB isolates included in the study

Lineages 2 and 4 accounted for 75.76% and 24.24% of all isolates, respectively. In addition to the main lineages, there were 7 sub-lineages in 99 isolate pairs, including 67 lineage 2.2.1 (67.68%), 7 lineage 2.2.2 (7.07%), 2 lineage 4.2 (2.02%), 2 lineage 4.2.2 (2.02%), 4 lineage 4.4 (4.04%), 10 lineage 4.4.2 (10.10%), and 7 lineage 4.5 (7.07%) (Table 1). Furthermore, we constructed phylogenetic trees for the 198 whole genomes of MTB from 99 initial matched recurrent isolate pairs. Of the 99 confirmed isolate pairs, the isolate pairs and their lineages were located next to each other on the phylogenetic tree, which confirmed that initially matched recurrent isolate pairs were extracted from unique strains (SNP <12) by excluding cases with mixed infections and reinfected with other strains (Fig. 2).

3.4. Comparisons of six air pollutants exposure levels in lower and higher within-host mutation rates groups

The air pollutants exposure concentration of NO₂ was significantly higher in groups with a higher within-host mutation rate at any lag time (from 0–6 to 0–8 months, two-sided $P < 0.05$). It indicated that air pollutants exposure might affect the host immunity and trigger

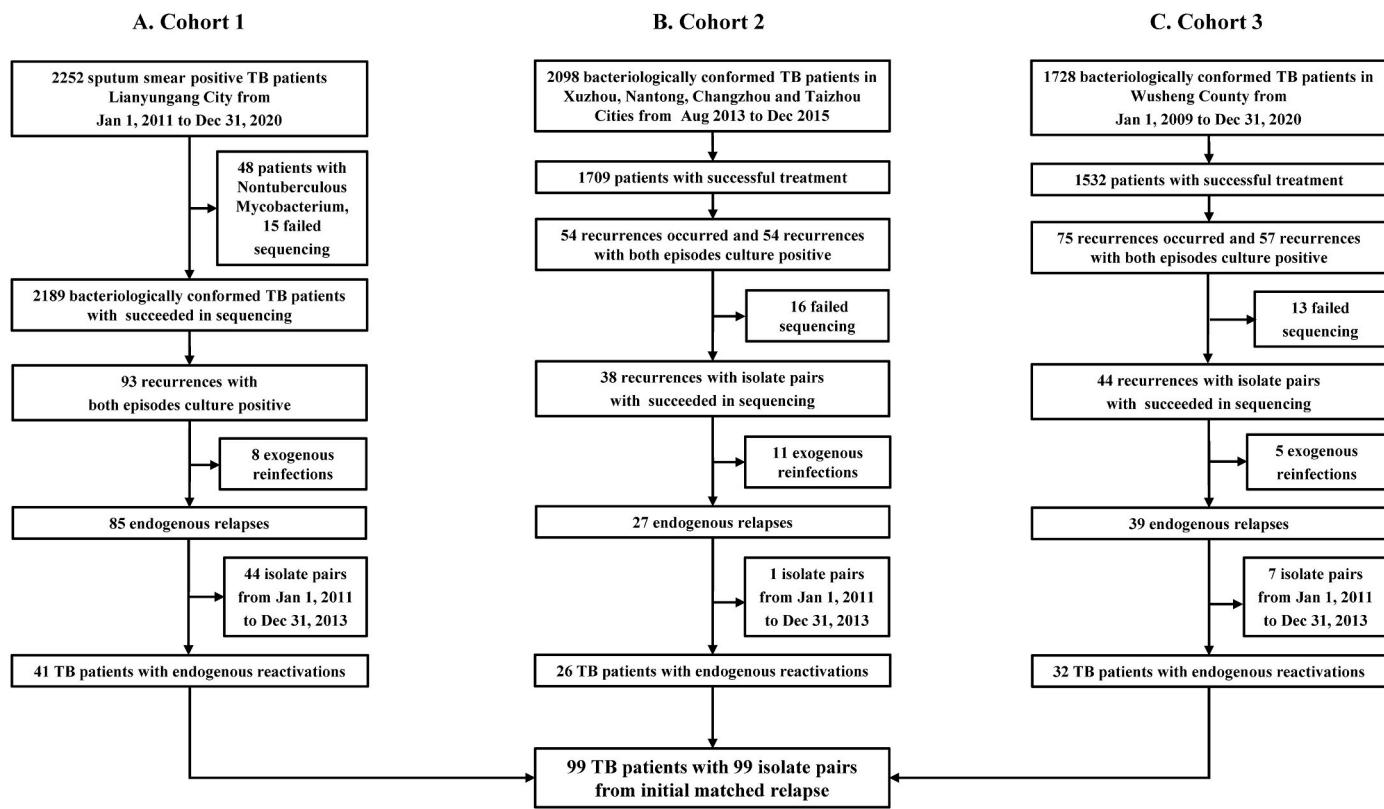


Fig. 1. Flow chart of study subject recruitment.

endogenously activated progression of within-host MTB. The concentration of PM₁₀, PM_{2.5}, SO₂, and O₃ (except for a lag of 0–6 or 0–8 months) was higher, while the concentration of CO was lower, in the group with a higher within-host mutation rate at any lag time (from 0–1 to 0–12 months), but without significant difference ($P > 0.05$) (Table 2, Supplementary Figure 1–6).

3.5. The effects of air pollution exposure on MTBMR in the single-pollutant models

In the single-pollutant models, PM₁₀ was positively associated with increased MTBMR from lag 0–1 to 0–12 months. The maximum effect was at a lag of 0–12 months, and for an increased 10 µg/m³ concentration in PM₁₀, MTBMR increased by 81.87% (95% CI: 38.38, 139.03). PM_{2.5} was positively associated with increased MTBMR from lag 0–5 months to 0–12 months. The maximum effect was at a lag of 0–12 months, and MTBMR increased by 73.91% (95% CI: 22.17, 147.55) with an increase in the 10 µg/m³ concentration in PM_{2.5} (Fig. 3, Supplementary Table 1).

SO₂ was positively associated with an increased MTBMR from lag 0–1 to 0–12 months. The maximum effect was at a lag of 0–7 months, and MTBMR increased by 132.2% (95% CI: 49.45, 260.76) with an increase of 10 µg/m³ in SO₂. NO₂ was also positively associated with an increased MTBMR from lag 0–6 to lag 0–12 months. The maximum effect was at a lag of 0–9 months, and MTBMR increased by 124.02% (95% CI: 34.5, 273.14) with an increase of 10 µg/m³ concentration in NO₂. However, O₃ was negatively correlated with an increased MTBMR from lag 0–6 to lag 0–12 months. The maximum effect was at a lag of 0–9 months, and MTBMR decreased by 6.18% (95% CI: -9.24, -3.02) with an increase of 10 µg/m³ concentration in O₃. No significant effect was observed for CO (Fig. 3, Supplementary Table 1).

3.6. Effects of air pollution on MTBMR in the multipollutant models

In the multipollutant models, PM₁₀ was positively associated with an increased MTBMR from lag 0–3 months to lag 0–12 months, and the maximum effect remained at a lag of 0–12 months (92.16%, 95% CI: 41.99, 160.06). PM_{2.5} was also positively associated with an increased MTBMR from lag 0–5 to 0–12 months. The maximum effect was at a lag of 0–9 months, and with an increase of 10 µg/m³ concentration, MTBMR increased by 67.41% (95% CI: 17.59, 138.33) (Fig. 4, Supplementary Table 2).

SO₂ was positively associated with an increased MTBMR from lag 0–1 to 0–12 months except for a lag of 0–11 months. The maximum effect was at a lag of 0–8 months, and MTBMR increased by 248.02% (95% CI: 88.10, 543.89) for each 10 µg/m³ increase in SO₂. NO₂ was positively associated with an increased MTBMR from lag 0–6 to lag 0–10 months. The maximum effect remained at a lag of 0–9 months, and MTBMR increased by 234.23% (95% CI: 80.21, 519.87) with an increase in the 10 µg/m³ concentration of NO₂ (Supplementary Table 2). O₃ was negatively correlated with an increased MTBMR from lag 0–4 to lag 0–5 months and from lag 0–7 to lag 0–11 months. The maximum effect remained at a lag of 0–9 months, and MTBMR decreased by 11.58% (95% CI: -18.73, -3.80) with an increase in the 10 µg/m³ concentration of O₃. No significant association between CO and MTBMR was observed (Fig. 4, Supplementary Table 2).

4. Discussion

In this study, we conducted a multi-center analysis on initial matched recurrent unique isolate pairs to uncover the effects of ambient pollutant exposure on the MTBMR (signs of bacterial reproduction rate), aiming to confirm its actual impacts on the progression of endogenous activation. This will be conducive to shedding light on the increasing number of population-based time-series studies, which often arrive at inconsistent conclusions on the relationship between ambient pollutants

Table 1

Clinical and Microbiological characteristics of TB patients in two mutation rates group according to median value of mutation rates (2.72×10^{-10}) of within-host mycobacterium tuberculosis.

Variables	Mutation rate1	Mutation rate2	P
	(mutations/bp/generation)	(mutations/bp/generation)	
	$<2.72 \times 10^{-10}$	$\geq 2.72 \times 10^{-10}$	
Demographics and clinical characteristics			
TB patients with isolates pairs, n (%)	49 (49.50)	50 (50.50)	
Time between isolate pairs, median (IQR) month	18.67 (13.43–34.30)	22.93 (15.39–39.01)	0.124
Early or late endogenous activation, n (%)			
Early (<2 years), n (%)	29 (59.18)	26 (52.00)	0.605
Late (≥ 2 years), n (%)	20 (40.82)	24 (48.00)	
Sex, n (%)			0.388
Female	5 (10.20)	9 (18.00)	
Male	44 (89.80)	41 (82.00)	
Age, median (IQR) years	66.00 (54.00–72.00)	62.00 (53.75–70.50)	0.506
Age group, n (%)			0.574
<60	15 (30.61)	19 (38.00)	
≥60	34 (69.39)	31 (62.00)	
Pulmonary cavity, n (%)			0.110
No	30 (61.22)	39 (78.00)	
Yes	19 (38.78)	11 (22.00)	
Diabetes			0.617
No	47 (95.92)	49 (98.00)	
Yes	2 (4.08)	1 (2.00)	
Microbiological and laboratory findings			
Main lineages, n (%)			1
Lineage 2	37 (75.51)	38 (76.00)	
Lineage 4	12 (24.49)	12 (24.00)	
Sub lineages			0.236
Lineage 2.2.1	32 (65.31)	35 (70.00)	
Lineage 2.2.2	4 (8.16)	3 (6.00)	
Lineage 4.2	2 (4.08)	0 (0)	
Lineage 4.2.2	1 (2.04)	1 (2.00)	
Lineage 4.4	4 (8.16)	0 (0)	
Lineage 4.2	4 (8.16)	6 (12.00)	
Lineage 4.5	2 (4.08)	5 (10.00)	
Sputum test at the second month of treatment, n (%)			0.059
Negative	43 (87.76)	49 (98.00)	
Positive	6 (12.24)	1 (2.00)	
Drug Resistance of initial isolates, n (%)			0.054
Sensitive	32 (65.31)	30 (60.00)	
Other	5 (10.20)	1 (2.00)	
Pre-MDR	7 (14.29)	15 (30.00)	
MDR	2 (4.08)	4 (8.00)	
Pre-XDR	3 (6.12)	0 (0)	
Drug Resistance of relapse isolates, n (%)			0.061
Sensitive	30 (61.22)	25 (50.00)	
Other	3 (6.12)	3 (6.00)	
Pre-MDR	11 (22.45)	16 (32.00)	
MDR	1 (2.04)	6 (12.00)	
Pre-XDR	4 (8.16)	0 (0)	

Notes: Data were expressed as median (interquartile range [IQR]), mean (standard deviation [SD]) or number (percentage). Comparisons between Mutation rate1 group ($<2.72 \times 10^{-10}$) and Mutation rate2 group ($\geq 2.72 \times 10^{-10}$) were made using the Kruskal-Wallis test, chi-square test, or Fisher's exact test, as appropriate. **Abbreviations:** MDR, multi-drug resistance, strains resistant to isoniazid and rifampin; Pre-MDR, pre-multidrug resistance, strains resistant to either isoniazid, rifampin, ethambutol or streptomycin, or to any combination of these drugs except isoniazid and rifampin; XDR, extensive drug resistance, on the base of MDR, the additional resistance to the fluoroquinolones and injectable medications (amikacin, kanamycin and capreomycin); Pre-XDR, pre-extensively drug-resistance, resistance to isoniazid and rifampicin and additional resistance to either any second-line injectable drug (amikacin, kanamycin and capreomycin) or any fluoroquinolone resistant; Others, the situations inconsistent with the above patterns.

and TB risk (Popovic et al., 2019; Xiang et al., 2021). In the present study, the estimated median value of MTBMR was 2.72×10^{-10} mutations/bp/generation, which was consistent with the previously reported range of 2.33×10^{-10} mutations/bp/generation (95% CI: 1.40×10^{-10} , 3.26×10^{-10}) (Walker et al., 2013). Consequently, it was rational and reliable that we applied MTBMR to dissect the relationship between ambient pollutants and TB risk. In the single-pollutant model, the short- and long-term exposure to PM₁₀ and SO₂ was positively associated with MTBMR. A positive association between PM_{2.5}, NO₂ and MTBMR was only observed for long-term exposure. In contrast, an inverse association was found for long-term exposure to O₃ and MTBMR. Similar results were also found in the multi-pollutant model.

Popovic et al. performed a systematic literature review up to July 3, 2018, by evaluating 11 epidemiological studies comprising 215,337 participants (Popovic et al., 2019) and revealed that exposure to outdoor PM₁₀, PM_{2.5}, SO₂ and NO₂ might be significantly associated with culture-positive TB status or TB-related hospital admissions. There were 6/11 studies assessing PM_{2.5}, of which 4/6 suggested a significant association with TB risk. There was some evidence of significant associations among PM₁₀, SO₂, NO₂ and TB risk, but with inconsistent results. In addition, Xiang et al. (2021) conducted another meta-analysis by searching articles up to October 1, 2020, including 17 articles and 293,678 TB patients and reported that exposure to an increased concentration of PM₁₀ (per 10 µg/m³ increase, RR = 1.058), SO₂ (per 1 ppb increase, RR = 1.016), and NO₂ (per 1 ppb increase, RR = 1.010) was associated with an increased risk of TB. However, no significant association was found between exposure to PM_{2.5} and TB risk. Since many studies have conflicting conclusions, we selected novel bioinformatics tools for whole-genomic MTBMR to explain the relationship between air pollutants and TB risk by estimating the within-host progression of endogenous activation.

An ecological study in North Carolina (Smith et al., 2014) and a spatial analysis in Peru (Carrasco-Escobar et al., 2020) suggested co-occurrences of elevated concentrations of outdoor exposure to PM and TB. Time series studies from the Cities of Lianyungang (Li et al., 2019), Lanzhou (Niu et al., 2021), Hefei (Huang et al., 2020), and Fuyang (Wang et al., 2022b) all reported that increased concentrations of PM_{2.5} and PM₁₀ increased the incidence of culture-positive TB, consistent with our results. However, other studies demonstrated that short- or long-term exposure to PM_{2.5} or PM₁₀ was not associated with active TB risk (a time series study from Ningbo (Ge et al., 2017) and two cohort studies from Taiwan (Lai et al., 2016) and Seoul (Hwang et al., 2014)). This inconsistency may be partly attributed to a disparity in air pollutant levels. The median concentration of PM₁₀ was 47.34 µg/m³ in Taiwan (Lai et al., 2016), and 62.80 µg/m³ in Seoul (Hwang et al., 2014), which were lower than the level of our study at 71.00 mg/m³, and the median concentration of PM_{2.5} was 27.79 µg/m³ in Taiwan (Lai et al., 2016), which was also lower than the level in our study. The median concentration of PM₁₀ was 71.00 (IQR: 50.00–101.00) µg/m³ in Ningbo (Ge et al., 2017), which was consistent with our study of 71.00 (IQR: 48.00–107.00) µg/m³. In addition to the disparity in air pollutant levels, other factors such as meteorological conditions, industrialization levels, and toxic substances adsorbed on PM_{2.5} or PM₁₀ may also differ in manners that impact the exposure-response effect (Zhu et al., 2018).

The inherent antibacterial properties of SO₂ make it more effective in inhibiting MTB than the clinical agent isoniazid (Malwal et al., 2012). Time-series analysis from Ningbo (Ge et al., 2017), Hefei (Huang et al., 2020), and Fuyang (Wang et al., 2022b) observed a negative association between SO₂ concentration and the initial symptom occurrence, suggesting that short-term exposure might have protective effects on TB. Nevertheless, SO₂ at elevated concentrations is known to induce oxidative damage to biomacromolecules such as proteins, lipids, and DNA (Malwal et al., 2012). Time-series analysis from Lianyungang (Li et al., 2019) and Lanzhou (Niu et al., 2021), and a cohort from Seoul (Hwang et al., 2014) observed that SO₂ was positive with the occurrence of TB, which was consistent with our findings. Malwal et al. (2012)

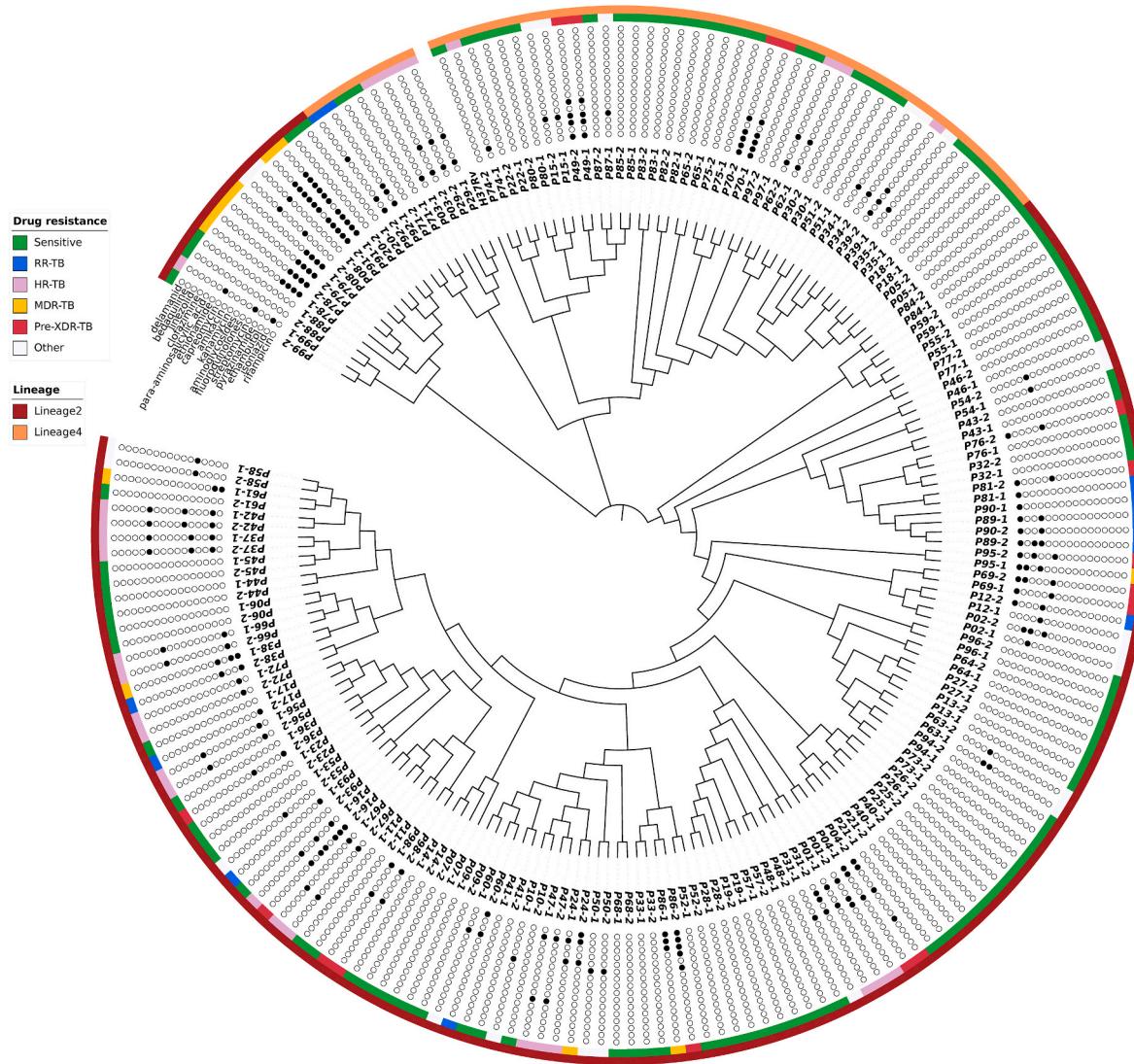


Fig. 2. Phylogeny and drug-resistance profiles of 198 *Mycobacterium tuberculosis* strain from 99 recurrent patients.

Solid circles indicated drug resistance detected by molecular drug susceptibility testing.

Solid rectangles with different colors indicated drug resistance types (sensitive, RR-TB, HR-TB, MDR-TB, Pre-XDR-TB, and others) and lineages (Lineage 2, Lineage 4).

Abbreviations: RR-TB, rifampicin-resistant tuberculosis, strains sensitive to isoniazid but resistant to rifampin; HR-TB, isoniazid-resistant tuberculosis, strains sensitive to rifampicin but resistant to isoniazid; MDR-TB, multi-drug resistant tuberculosis, strains resistant to isoniazid and rifampin; Pre-XDR-TB, pre-extensively resistant tuberculosis, resistance to isoniazid and rifampicin and additional resistance to either any second-line injectable drug (amikacin, kanamycin and capreomycin) or any fluoroquinolone resistance; Others, the situations inconsistent with the above patterns.

found that the minimum inhibitory concentration (MIC) of SO₂ on MTB H37Rv strain was at 0.05 µg/mL (0.15 µM), which was better than the MIC of isoniazid (0.05 µg/mL, 0.37 µM) determined under similar conditions (Malwal et al., 2012). After measuring unit conversion, the MIC of 0.05 µg/mL is equivalent to 50,000 µg/m³. However, inhalable concentration to pulmonary alveoli is far lower than this effective inhibitory concentration. Besides, the MTB H37Rv strain is Lineage 4 in the study by Malwal et al. (2012), but Lineage 2 accounted for more than 75% of our study. Lineage 2 strains were reported to have an increased capacity to acquire drug resistance, virulence, host response, and severity (Merker et al., 2015; Wiens et al., 2018). Those may cause the effects of oxidative damage to be more prominent than the antibacterial benefit from SO₂.

We found that the higher mutation rate group had an increased concentration of O₃ than that of the lower group, but the association should be interpreted carefully. The estimated level of O₃ from air quality monitors was a poor proxy for individual exposure since the ambient O₃ and NO were bonded by chemical coupling, and it was

authenticated that NO₂ was depleted during the formation of O₃ in the presence of sunlight (Clapp, 2001; Smith et al., 2016). To unravel the actual relationship between O₃ and the risk of TB, we then added the interaction term (O₃: NO₂: Sunshine time) into the models for adjustment. An inverse association was observed for long-term exposure to O₃ and MTBMR, with a maximum decrease of 6.18% in MTBMR at lag 0–9 months in the single-pollutant model and a maximum reduction of 11.58% in MTBMR at lag 0–9 months in the multipollutant model. Our result for O₃ was consistent with a nested case-control study in the US conducted by Smith et al. (highest quintile vs. lowest quintile, odds ratio [OR] = 0.66, 95% CI: 0.55–0.79) (Smith et al., 2016); a time-series study in Hefei, China by Huang et al. (per 10 µg/m³ increase, risk ratio [RR] = 0.96, 95% CI: 0.94–0.98) (Huang et al., 2020); and a cohort study in Taiwan, China by Lai et al. (per 10 µg/m³ increase, hazard ratio [HR] = 0.69, 95% CI: 0.49–0.98) (Lai et al., 2016). Conversely, a time-series study in Jinan, China, by Liu et al. (2018) (per 10 µg/m³ increase, RR = 1.39, 95% CI: 1.04–1.85), and time-series analysis in Wulumuqi, China, by Yang et al. (per 1 µg/m³ increase, attributable risk percentage

Table 2

Distribution of six air pollutants exposure level in two mutation rates group according to median value of mutation rates (2.72×10^{-10}) of within-host mycobacterium tuberculosis.

Pollutants	Lag time (months)	Mutation rate (mutations/bp/generation), M (Q ₂₅ , Q ₇₅)		
		Mutation rate <math><2.72 \times 10^{-10}</math>	Mutation rate >math>\geq 2.72 \times 10^{-10}</math>	P*
PM ₁₀ (μg/m ³)	0–1	77.03 (57.43–97.73)	85.42 (55.82–104.82)	0.332
	0–2	73.56 (53.25–89.56)	84.80 (59.45–105.27)	0.136
	0–3	71.73 (56.01–89.74)	78.79 (64.71–107.19)	0.077
	0–4	73.88 (60.87–91.05)	77.37 (65.23–108.58)	0.121
	0–5	71.00 (58.39–87.49)	74.09 (66.10–105.57)	0.096
	0–6	73.52 (59.55–85.98)	76.03 (67.42–101.89)	0.135
	0–7	75.15 (59.92–87.67)	79.36 (66.50–96.06)	0.153
	0–8	77.58 (61.69–84.81)	80.03 (64.03–95.56)	0.097
	0–9	76.54 (61.40–87.26)	77.90 (63.57–97.21)	0.156
	0–10	76.41 (64.28–85.07)	79.27 (65.64–95.82)	0.144
	0–11	75.81 (67.53–82.33)	80.81 (64.68–95.24)	0.170
	0–12	75.88 (67.70–81.72)	78.41 (65.70–95.17)	0.383
PM _{2.5} (μg/m ³)	0–1	41.80 (33.87–60.50)	46.53 (31.62–65.40)	0.719
	0–2	42.05 (35.30–57.77)	48.15 (34.33–62.00)	0.399
	0–3	42.29 (36.66–56.09)	49.34 (36.40–63.89)	0.320
	0–4	43.78 (37.16–60.81)	46.83 (40.36–63.17)	0.314
	0–5	43.57 (39.11–54.38)	46.60 (38.02–59.43)	0.393
	0–6	44.84 (38.01–53.18)	47.96 (37.53–57.18)	0.347
	0–7	46.53 (38.72–55.06)	47.61 (35.57–57.47)	0.470
	0–8	45.70 (37.81–57.12)	47.86 (37.52–57.12)	0.523
	0–9	46.09 (38.77–56.42)	49.76 (39.80–58.99)	0.537
	0–10	45.88 (39.37–54.08)	51.11 (39.58–58.22)	0.504
	0–11	46.33 (41.52–51.93)	50.99 (41.75–58.47)	0.401
	0–12	47.93 (42.62–50.22)	49.92 (41.09–57.33)	0.467
SO ₂ (μg/m ³)	0–1	16.47 (10.80–26.63)	20.03 (11.88–29.78)	0.122
	0–2	16.38 (10.75–28.37)	23.28 (12.23–28.83)	0.157
	0–3	15.78 (10.91–26.34)	22.79 (12.06–27.64)	0.190
	0–4	15.37 (10.88–27.25)	22.79 (11.60–26.83)	0.244
	0–5	14.93 (10.99–24.11)	21.69 (11.28–27.03)	0.243
	0–6	13.97 (11.08–24.05)	21.39 (11.33–26.65)	0.264
	0–7	13.43 (11.86–23.82)	21.64 (11.91–27.07)	0.271
	0–8	13.76 (11.81–22.93)	21.51 (12.26–26.75)	0.288
	0–9	14.39 (12.06–21.94)	21.54 (11.62–27.67)	0.383
	0–10	14.59 (12.50–21.26)	21.26 (12.11–27.42)	0.294
	0–11	15.16 (12.74–21.38)	20.51 (12.33–27.78)	0.331

Table 2 (continued)

Pollutants	Lag time (months)	Mutation rate (mutations/bp/generation), M (Q ₂₅ , Q ₇₅)		
		Mutation rate <math><2.72 \times 10^{-10}</math>	Mutation rate >math>\geq 2.72 \times 10^{-10}</math>	P*
NO ₂ (μg/m ³)	0–12	15.01 (12.68–21.19)	20.41 (12.63–27.41)	0.432
	0–1	22.77 (15.33–33.47)	25.97 (19.35–38.78)	0.166
	0–2	23.98 (15.18–33.18)	26.22 (19.88–38.99)	0.130
	0–3	23.86 (15.58–32.70)	28.24 (18.72–38.40)	0.086
	0–4	25.17 (15.85–34.25)	27.73 (18.64–37.17)	0.104
	0–5	23.69 (15.45–31.07)	26.92 (19.41–34.59)	0.076
	0–6	23.54 (15.80–31.09)	27.23 (19.41–35.16)	0.033
	0–7	23.97 (17.86–29.31)	26.62 (20.61–34.72)	0.029
	0–8	24.34 (18.83–28.45)	26.36 (21.10–35.51)	0.040
	0–9	23.91 (18.39–28.64)	27.35 (21.56–35.73)	0.052
	0–10	23.91 (20.87–29.51)	26.89 (21.66–35.54)	0.100
	0–11	23.88 (21.09–29.03)	26.44 (22.18–35.38)	0.136
O ₃ (μg/m ³)	0–12	24.44 (20.58–28.83)	26.50 (22.65–34.71)	0.219
	0–1	62.13 (43.93–112.23)	83.90 (55.07–115.72)	0.495
	0–2	69.11 (42.76–116.05)	80.60 (54.70–121.10)	0.403
	0–3	73.88 (54.47–117.55)	80.72 (60.07–118.38)	0.699
	0–4	77.07 (60.03–113.65)	85.72 (57.95–118.67)	0.806
	0–5	79.67 (63.25–107.60)	83.60 (63.37–112.36)	0.797
	0–6	84.92 (63.36–104.52)	83.21 (63.59–110.08)	0.656
	0–7	88.39 (64.76–98.95)	88.47 (68.74–112.73)	0.498
	0–8	90.66 (66.31–100.18)	89.89 (71.52–110.75)	0.390
	0–9	91.74 (70.31–102.31)	94.77 (71.78–107.71)	0.295
	0–10	90.92 (69.03–103.81)	98.57 (73.85–105.80)	0.406
	0–11	91.20 (67.98–103.20)	97.26 (72.88–105.27)	0.512
CO (μg/m ³)	0–12	91.55 (67.98–103.20)	94.22 (74.46–104.52)	0.658
	0–1	853.33 (617.37–1060.00)	805.58 (627.79–1053.33)	0.823
	0–2	846.67 (698.79–1072.08)	788.33 (691.43–1018.33)	0.634
	0–3	838.89 (688.29–1053.38)	784.94 (721.11–974.44)	0.898
	0–4	875.83 (687.81–1027.25)	807.15 (706.82–950.00)	0.860
	0–5	849.33 (700.00–1050.67)	829.26 (703.00–984.32)	0.706
	0–6	836.67 (723.61–1083.06)	823.89 (712.22–962.22)	0.525
	0–7	848.15 (740.83–1074.64)	833.81 (723.17–948.10)	0.313
	0–8	880.53 (759.35–1150.10)	830.83 (741.26–980.83)	0.188
	0–9	880.64 (768.52–1147.04)	821.30 (741.70–944.72)	0.123
	0–10	864.13 (773.00–1143.17)	832.42 (752.37–925.32)	0.203
	0–11	876.36 (791.94–1120.15)	845.51 (763.33–921.53)	0.242
7	0–12	889.76 (795.66–1096.81)	841.10 (761.39–914.17)	0.197

Notes: *Wilcoxon's rank-sum test; M (Q₂₅, Q₇₅), medians (lower quartile, upper quartile).

[ARP]: 0.57%) reported that an increased concentration of O₃ was associated with an altered TB risk (Yang et al., 2020). However, no significant association between O₃ and TB risk was found in the Seoul metropolitan area, South Korea (Hwang et al., 2014), and Los Angeles County, US (Jassal et al., 2013).

Our results may help to resolve the contradictory findings for O₃ by suggesting that the above studies are all correct. There are two contrary biological effects of O₃ on TB. On the one hand, O₃ exposure in human alveolar macrophages was linked to decreased phagocytosis and impaired antimicrobial host defense (Al-Hegelan et al., 2011; Li et al., 2013). On the other hand, O₃ exposure can kill bacteria, including multidrug-resistant bacteria (Wang et al., 2022a; Wentworth et al., 2003). The effects of O₃ on TB might be determined by its delicate balance of adverse and protective effects. In our study, O₃ exposure had an inverse impact on MTBMR, which is responsible for the antimycobacterial activity of O₃ on the MTB inhibitory activity or bacterial growth.

Following the inhalation of higher concentrations of NO₂ can stimulate the oxidative stress of the respiratory tract and is directly linked to an elevated risk of respiratory infections. An increased concentration of NO₂ contributed to the increased incidence of active TB in Lianyungang (Li et al., 2019), Lanzhou (Niu et al., 2021), Hefei (Huang et al., 2020), and Fuyang (Wang et al., 2022b), and Taiwan (Lai et al., 2016), China. A meta-analysis by Xiang et al. (2021) also confirmed that NO₂ (per 1 ppb increase, RR = 1.010) was associated with an increased risk of TB. Our results revealed a positive link between NO₂ exposure and within-host MTBMR, suggesting that increased concentration of NO₂ exposure can contribute to increased TB risk.

Concerning CO, we did not find a significant association with

MTBMR, regardless of short-term or long-term exposure. Similar insignificant effects of CO on TB have been reported in northern California (Smith et al., 2016), the Seoul metropolitan area of South Korea (Hwang et al., 2014), and a meta-analysis by Xiang et al. (2021). On the contrary, Studies in Fuyang (Wang et al., 2022b) and Wuhan (Xu et al., 2019) in China showed positive effects of CO on TB. Heme oxygenase is produced by carbon monoxide, inhibiting the host's immune response and inflammation (Chin and Otterbein, 2009). Microbiological studies have shown that atmospheric CO oxidation supports bacterial survival during nutrient limitation (Kumar et al., 2008). Mycobacterium increases the transcription and synthesis of molybdenum-copper carbon monoxide dehydrogenase by 50-fold in response to organic carbon limitation (Cordero et al., 2019). However, most organisms, including mycobacterium, use this enzyme to support survival rather than growth (Cordero et al., 2019). Hence, we did not observe the positive effects of CO on MTBMR due to its low physiological growth or replication status.

When hosts are in a normal immune defense state, the immune response limits infection to the point that there are no clinical or radiographic signs of overt TB disease (Barry et al., 2009; Gill et al., 2009). This is associated with dramatically slowing or ceasing bacterial growth (Barry et al., 2009; Ford et al., 2011). In contrast, MTB replication throughout the suppressed immune system is substantially higher than that restrained by the normal immune system (Gibson et al., 2018; Gill et al., 2009; Weller and Wu, 2015). Due to genetic changes often occurring at a steady rate per generation in living organisms (Weller and Wu, 2015), when MTB undergoes active replication, this indicates that MTB has more generations and tends to have a higher rate of within-host molecular evolution and mutation rates (Colangeli et al., 2020; Gibson et al., 2018; Weller and Wu, 2015). Consequently, it is reasonable that exposure to increased concentrations of air pollutants, such as (PM_{2.5}, PM₁₀, SO₂, and NO₂), may suppress the immune defense, increasing an individual's susceptibility to developing active TB, as well as the

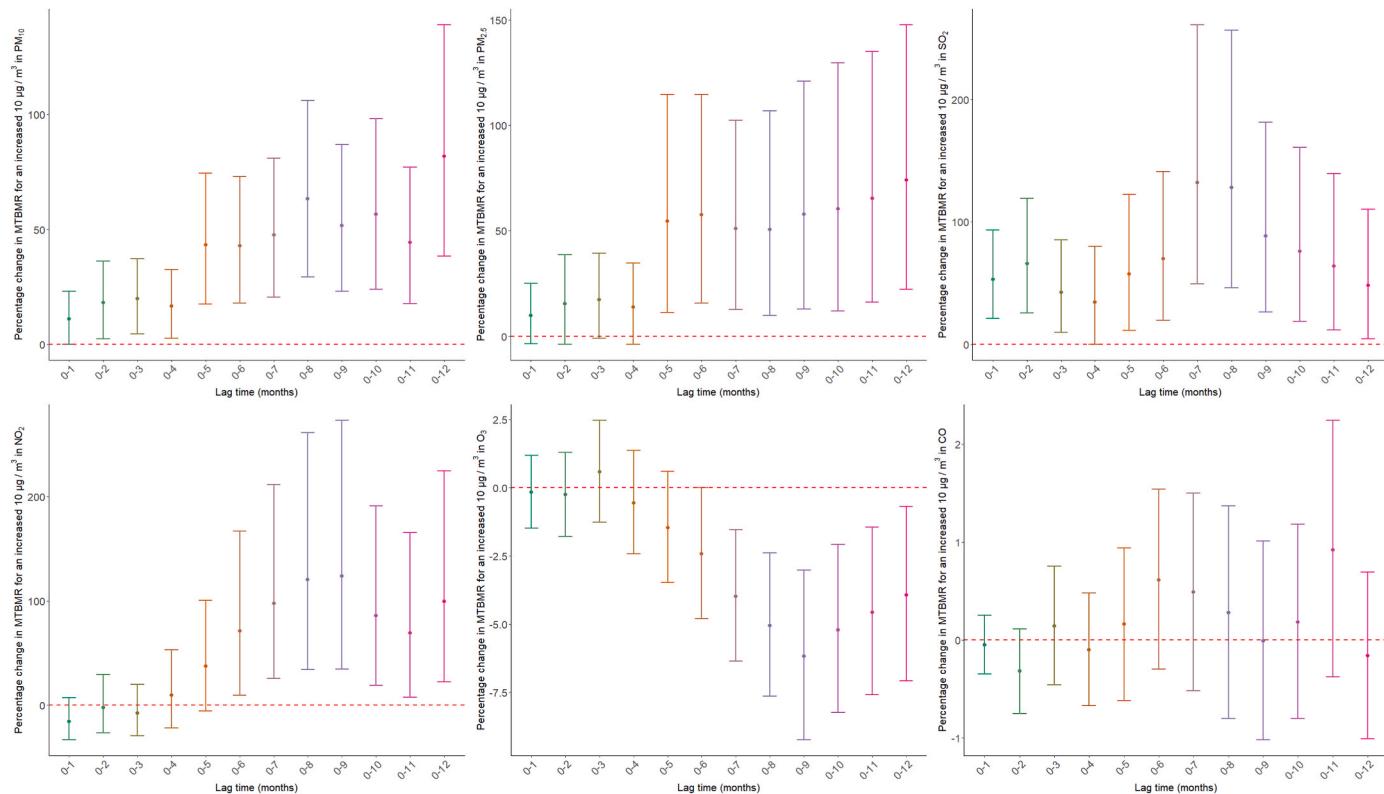


Fig. 3. The effects of air pollution exposure on MTBMR in the single-pollutant models.

The strength of the relationship between factors and mutation rates of within-host MTB was described as a percentage change and 95% confidence intervals (CIs) for each 10 $\mu\text{g}/\text{m}^3$ increase in air pollutants.

Abbreviations: MTBMR, mutation rates of MTB.

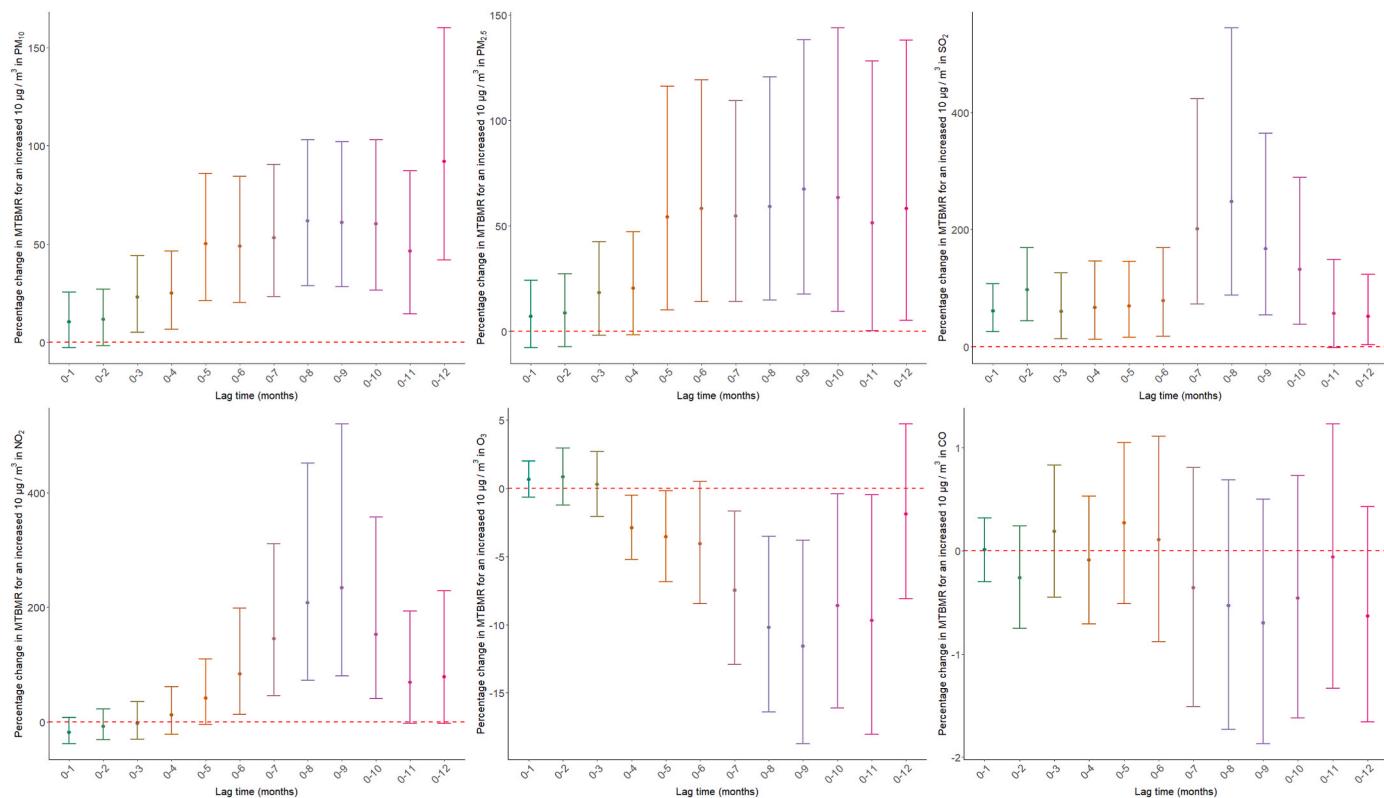


Fig. 4. The effects of air pollution exposure on MTBMR in the multi-pollutant models.

The strength of the relationship between factors and mutation rates of within-host MTB was described as a percentage change and 95% confidence intervals (CIs) for each $10 \mu\text{g}/\text{m}^3$ increase in air pollutants.

Abbreviations: MTBMR, mutation rates of MTB.

increased whole-genomic mutation rates. The mechanisms of air pollutants involved in the pathogenesis progression of endogenous reactivation of within-host MTB remain to be further studied and verified in a larger population.

Our study has several major strengths. First, to our knowledge, this is the first molecular epidemiology research with innovativeness centered on evaluating the relationship between ambient pollutant exposure and TB risk based on deciphering the progression of within-host endogenous activation. Second, we excluded TB patients with mixed and exogenous reinfections and included only TB pairs infected with unique strains from initially matched recurrent episodes. This avoids misclassification induced by infection from circulating MTB around community transmission. However, several limitations to our study are worth emphasizing. First, we could not obtain the HIV status of the TB patients in our study because TB patients in China are not routinely tested for HIV, which may affect the MTB microevolution (Ford et al., 2011). Fortunately, previous evidence suggested that 95% of TB recurrences in HIV-negative TB patients were due to endogenous activation, the majority of TB recurrences (75%) in HIV-positive TB patients were due to reinfection (Shanmugam et al., 2021), and the prevalence of HIV in China is relatively low. Due to all subjects enrolled in our study being isolated from a unique MTB strain with reinfection excluded, the unknown HIV status of our TB patients may not affect the reliability of our findings. Second, since not all notified TB patients had the culture-positive disease, we could not consider those without MTB isolates available for sequencing in our analyses. Thus, we are unsure whether our findings apply to TB patients with the culture-negative disease.

5. Conclusions

We considered integrating the whole-genomic mutation rates and air

pollutants data to help us better understand the effects of air pollutants on the risk of endogenous activation of within-host MTB. Our results suggested that exposure to air pollutants, particularly PM₁₀, PM_{2.5}, SO₂, and NO₂, was positively correlated with within-host MTBMR, which accounted for the increased TB risk observed in clinical and epidemiological practices due to microbiologic evidence of a faster bacterial replication rate. The inverse effects of O₃ on the MTBMR may result from the inherent physicochemical antibacterial properties of O₃, which helped to slow or cease bacterial growth and proliferation and thus induced no clinical or radiographic abnormalities of overt TB disease. In view of the large number of people worldwide who have LTBI and are exposed to ambient pollutants, our novel findings may have potential public health implications.

Credit author statement

Bilin Tao: Conceptualization, Investigation, Formal analysis, Methodology, Data curation, Writing-original draft, Writing. Zhongqi Li: Formal analysis, Investigation. Yuting Wang: Data curation, Writing-review & editing. Jizhou Wu, Xinling Shi: Data curation. Jinyan Shi: Investigation, Methodology, Data curation. Qiao Liu: Conceptualization, Investigation, Resources, Data curation, Project administration. Jianming Wang: Conceptualization, Investigation, Supervision, Funding acquisition, Project administration, Writing-review & editing.

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Jiangsu Health Commission (M2020020/ZD2021052).

Ethical approval

This study was approved by the ethics committee of Nanjing Medical University. After informed consent was obtained from all participants, questionnaires were used to collect demographic data.

Patient consent for publication

Not required.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.envres.2023.115695>.

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