Total sputum nitrate/nitrite is associated with exacerbations and *Pseudomonas aeruginosa* colonisation in bronchiectasis

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Abstract

Background Sputum nitrate/nitrite, which is the main component of reactive nitrogen species, is a potential biomarker of disease severity and progression in bronchiectasis. This study aimed to determine the association between nitrate/nitrite and exacerbations and airway microbiota in bronchiectasis.

Methods We measured total nitrate/nitrite concentration in sputum samples collected from 85 patients with stable bronchiectasis, performed 16S ribosomal RNA sequencing of sputum samples and predicted the denitrification ability of airway microbiota using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt). Relationships between sputum total nitrate/nitrite and disease severity, exacerbations and airway microbiota were examined.

Results Higher total sputum nitrate/nitrite was associated with more severe bronchiectasis defined by E-FACED (exacerbation, forced expiratory volume in 1 s, age, chronic colonisation by *Pseudomonas aeruginosa*, radiological extension and dyspnoea) (p=0.003) or Bronchiectasis Severity Index (p=0.006) and more exacerbations in the prior 12 months (p=0.005). Moreover, total sputum nitrate/nitrite was significantly higher in patients with worse cough score (p=0.03), worse sputum purulence score (p=0.01) and worse Medical Research Council dyspnoea score (p=0.02). In addition, the total sputum nitrate/nitrite of the *P. aeruginosa* colonised (PA) group was higher than that of the non-*P. aeruginosa* colonised (NPA) group (p=0.04), and the relative abundance of *P. aeruginosa* was positively correlated with total nitrate/nitrite (r=0.337, p=0.002). Denitrification module (M00529) was also significantly enriched in the PA group compared to the NPA group through PICRUSt analyses. Using receiver-operating characteristic analysis, total nitrate/nitrite was associated with exacerbations during 1-year follow-up (area under the curve 0.741, p=0.014).

Conclusions Sputum nitrate/nitrite is a biomarker of disease severity and associated with *P. aeruginosa* colonisation in bronchiectasis.

Introduction

Bronchiectasis is pathologically defined as permanent abnormal dilatation of one or more bronchi, which manifests clinically as a condition with variable symptoms of cough, sputum production and pulmonary exacerbations. Bronchiectasis is the result of a complex interaction between impaired mucociliary clearance, pathogen infection or colonisation, chronic airway inflammation, airway damage and abnormal remodelling [1, 2]. In addition, substantial evidence showed that oxidative and nitrosative stress have also been implicated in the pathogenesis of many pulmonary diseases including chronic inflammatory lung disorders such as asthma, COPD and bronchiectasis [3, 4]. Nitrosative stress is manifested as increased production of reactive nitrogen species (RNS), such as nitrate, nitrite and peroxynitrite, which can result in increased or amplified inflammation, DNA damage, inhibition of mitochondrial respiration, protein dysfunction and cell damage, as well as structural changes in the airways [5, 6].

Nitrate and nitrite are the main components of RNS, and the main source is nitric oxide (NO), which is synthesised by inducible nitric oxide synthase (iNOS) using L-arginine as a substrate. iNOS is induced in the presence of inflammation by a variety of pro-inflammatory cytokines. This enzyme, once expressed, continues to produce large quantities of NO, which is short-lived *in vivo* and can be easily transformed into nitrate/nitrite by its fast reaction with superoxide (O_2^-) [7, 8]. Previous studies suggested that bronchiectasis is mainly characterised by chronic neutrophilic airway inflammation, manifested as elevated release of interleukin (IL)-8, IL-1β, interferon (IFN)-γ and tumour necrosis factor (TNF)-α [9]. These cytokines have been proven to promote the expression of iNOS [10], which results in nitrate/nitrite production. Consistently, increased levels of nitrate/nitrite were found in the airways of patients with various chronic airway diseases [11–14]. Additionally, a series of studies showed that nitrate/nitrite as terminal electron acceptor can be utilised by Gammaproteobacteria for their anaerobic respiration and growth by denitrification [15, 16]. Gammaproteobacteria, represented by *Pseudomonas aeruginosa*, is significantly enriched in the airway microbiota of bronchiectasis, which is a marker of airway microbiota dysbiosis, and closely associated with the progression of bronchiectasis [17, 18].

We hypothesised that nitrate/nitrite, the main product of nitrosative stress, may be involved in altering respiratory homoeostasis of bronchiectasis, which could contribute to the progressive deterioration of bronchiectasis. Therefore, in this study, we prospectively explored the association between sputum nitrate/nitrite and progression of bronchiectasis and investigated sputum nitrate/nitrite as a biomarker of disease severity.

Methods

Study participants

A cohort of 85 patients with stable bronchiectasis was recruited from Union Hospital (Wuhan, China) between August 2020 and August 2021. Inclusion criteria were age >18 years, high-resolution computed tomography-confirmed bronchiectasis, and clinical symptoms consistent with bronchiectasis (cough, sputum production or respiratory infection). Exclusion criteria were inability to give informed consent, traction bronchiectasis, lack of important clinical information, malignancy and inability to complete this study because of serious dysfunction of heart, brain, kidney and other important organs. Ethical approval for the study was given by Wuhan Union Hospital (approval number 2020-0288). Severity of disease was evaluated using the E-FACED (exacerbation; forced expiratory volume in 1 s, age, chronic colonisation by *Pseudomonas aeruginosa*, radiological extension and dyspnoea) score [19] and the Bronchiectasis Severity Index (BSI) [20]. Exacerbation is defined as the requirement for antibiotics in the presence of one or more symptoms of increasing cough, increasing sputum volume, worsening sputum purulence, worsening dyspnoea, increased fatigue/malaise, fever and haemoptysis, and severe exacerbation is defined according to British Thoracic Society guidelines as unscheduled hospitalisations or emergency department visits for exacerbations or complications as recorded from patient histories [21]. Chronic *P. aeruginosa* colonisation is defined by isolation of the same pathogen in two or more cultures ⩾3 months apart in 12 months [22]. The severity of symptoms was assessed using Medical Research Council (MRC) grade [23], cough score [24], sputum volume score [25], sputum purulence score [25, 26] and haemoptysis score [27], and lung radiological severity was assessed using the modified Reiff score [28] (supplementary table e1).

Sputum collection

Spontaneous sputum samples from 85 patients with stable bronchiectasis were collected (one sputum sample per patient). Each sputum sample was divided into two parts; one part was immediately stored at −80°C for microbiota sequencing, and the other part was diluted in PBS (TBD, China) and centrifuged at 12 000×*g* for 5 min, and the supernatant stored at −80°C for measurement of nitrate and nitrite and other inflammatory markers.

Measurement of total nitrate/nitrite, IL-1β and IL-8

The concentration of nitrate/nitrite in sputum was measured in 85 sputum samples using the Griess reaction [29] (Beyotime Biotech, China) according to the manufacturer's recommendations. From each sputum sample, 100 μL was immediately diluted ×10 in PBS, and supernatant transferred to a 96-well microtitre plate. Nitrite concentration was estimated by addition of the Griess reagent for 10 min, whereby nitrite was converted into a purple azo-compound, which was quantitated by the optical density at 540 nm measured using an ELISA plate reader (EnSpire Multimode Plate Reader, USA). The total nitrate and nitrite was estimated by a two-step analysis process: the first step converted nitrate to nitrite utilising nitrate reductase. After incubation for 2 h, the next step involved the addition of the Griess reagent, whereby nitrite was converted into a purple azo-compound. After incubation with Griess reagent for 10 min the optical density at 540 nm was measured using an ELISA plate reader (EnSpire Multimode Plate Reader). Commercially available ELISA kits were used to measure sputum IL-1β and IL-8 (4A Biotech, China).

DNA extraction and 16S rRNA gene sequencing

Microbial community genomic DNA was extracted using the E.Z.N.A. soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's instructions. The DNA extract was checked on 1% agarose gel, and DNA concentration and purity were determined with NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The hypervariable region V3–V4 of the bacterial 16S ribosomal (r)RNA gene was amplified with primer pairs 338F (5′-ACTCCTACGGGAGGC AGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) using an ABI GeneAmp 9700 PCR thermocycler (ABI, CA, USA). The PCR amplification of the 16S rRNA gene was performed as follows: initial denaturation at 95°C for 3 min, followed by 27 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 45 s, single extension at 72°C for 10 min, ending at 4°C. The PCR product was extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions and quantified using Quantus Fluorometer (Promega, USA).

Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq PE300 platform/NovaSeq PE250 platform (Illumina, San Diego, CA, USA) according to the standard protocols by Majorbio Bio-Pharm Technology (Shanghai, China). The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered by fastp version 0.20.0 and merged by FLASH version 1.2.7 with the following criteria: 1) the 300 bp reads were truncated at any site receiving an average quality score of <20 over a 50 bp sliding window, and the truncated reads <50 bp were discarded, reads containing ambiguous characters were also discarded; 2) only overlapping sequences >10 bp were assembled according to their overlapped sequence. The maximum mismatch ratio of overlap region is 0.2. Reads that could not be assembled were discarded; 3) samples were distinguished according to the barcode and primers, and the sequence direction was adjusted, exact barcode matching, two nucleotide mismatch in primer matching. Operational taxonomic units (OTUs) with 97% similarity cut-off were clustered using UPARSE version 7.1, and chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analysed using RDP Classifier version 2.2 against the 16S rRNA database using confidence threshold of 0.7.

Functional profiling using PICRUSt

The functional prediction of the bacterial communities was performed using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt 1.1.4). The PICRUSt tool was designed to infer metagenomics information from 16S rRNA sequencing data. Module and enzyme (Enzyme Commission (EC)) information were obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database, and the abundance of each functional category was calculated based on OTU abundance.

Statistical analysis

Categorical data were analysed by determining the frequency and percentage in each category, whereas continuous data were displayed using median (interquartile range (IQR)) or mean±SD. We used the Mann–Whitney test or Kruskal–Wallis test for analysis of continuous variables, and used the Chi-squared test for analysis of categorical variables. Correlations were analysed using Spearman's rank-order correlation. β-diversity was calculated using the Bray–Curtis distance. Discrimination for disease severity and exacerbations at 1 year was analysed using the area under a receiver operating characteristic curve (AUC). Statistical analysis was performed using SPSS version 25.0 and GraphPad Prism version 9. A two-sided p-value <0.05 was considered statistically significant.

Results

Demographics and characteristics of the participants

The demographic data and clinical characteristics of the participants included in this study are shown in table 1. The median (IQR) age of 85 patients with stable bronchiectasis was 57.0 (45.5–66.5) years, with 31 (36.5%) men and 54 (63.5%) women. The most common aetiology was idiopathic bronchiectasis (n=59, 69.4%), followed by post-infective bronchiectasis (n=23, 27.0%). Hypertension (n=8, 9.4%) and diabetes (n=8, 9.4%) were the two most common comorbidities of bronchiectasis. The median (IQR) BSI score was 12.0 (7.5–16.0) and the E-FACED score was 4.0 (3.0–6.0). The median duration of bronchiectasis was 10 years, the longest was 50 years. The median (IQR) number of exacerbations in the prior 12 months was 2.5 (1.0–5.0), and the number of hospitalisations in the prior 12 months was 1.0 (1.0–2.0). Through traditional sputum culture, the most common pathogen was *P. aeruginosa* (n=42, 49.4%), followed by *Staphylococcus aureus* (n=2, 2.4%) and *Haemophilus influenzae* (n=2, 2.4%). Lung function, laboratory tests and imaging scores are summarised in supplementary table e1.

TABLE 1 Demographic and clinical characteristics of 85 patients with bronchiectasis	
Age years	57.0 (45.5–66.5)
Gender	
Male	31 (36.5)
Female	54 (63.5)
Aetiology of bronchiectasis	
Idiopathic	59 (69.4)
Post-infective	23 (27.0)
Previous ABPA	1 (1.2)
Previous NTM infection	1 (1.2)
Primary immunodeficiency	1 (1.2)
Comorbidities	
Hypertension	8 (9.4)
Diabetes	8 (9.4)
Cardiovascular disease	5 (5.9)
Neurological disease	2 (2.4)
Smoking history	
Never	71 (83.5)
Past	9 (10.6)
Current	5 (5.9)
BMI kg·m^{-2}	21.1 (18.4–23.6)
BSI	12.0 (7.5–16.0)
Mild	16 (18.8)
Moderate	10 (11.8)
Severe	59 (69.4)
E-FACED	4.0 (3.0–6.0)
Mild	32 (37.6)
Moderate	35 (41.2)
Severe	18 (21.2)
Duration of bronchiectasis years	10.0 (3.0–30.0)
Exacerbations in prior 12 months	2.5 (1.0–5.0)
Hospitalisations in prior 12 months	1.0 (1.0–2.0)
MRC	2.0 (1.0–4.0)
Cough score	1.0 (1.0–2.0)
Sputum volume score	1.0 (1.0–2.0)
Sputum purulence score	6.0 (4.0–6.0)
Haemoptysis score	0.0 (0.0–1.0)
Medication	
Inhaled corticosteroids	10 (11.8)
Low-dose macrolides	6 (7.1)
Inhaled LAMA or/and LABA	18 (21.2)
Traditional cultured pathogens	
Pseudomonas aeruginosa	42 (49.4)
Staphylococcus aureus	2 (2.4)
Haemophilus influenzae	2 (2.4)
Escherichia coli	1 (1.2)
Streptococcus pneumoniae	1 (1.2)
Klebsiella pneumoniae	1 (1.2)
Aspergillus flavus	2 (2.4)

Data are presented as median (interquartile range) or n (%). ABPA: allergic bronchopulmonary aspergillosis; NTM: nontuberculosis mycobacteria; BMI: body mass index; BSI: Bronchiectasis Severity Index; E-FACED: exacerbation, forced expiratory volume in 1 s, age, chronic colonisation by *Pseudomonas aeruginosa*, radiological extension and dyspnoea; MRC: Medical Research Council; LAMA: long-acting muscarinic antagonist; LABA: long-acting β$_2$-agonist.

Total sputum nitrate/nitrite was associated with severity of bronchiectasis

Sputum nitrate/nitrite was measured in 85 patients with stable bronchiectasis and the median value were 352.7 µM. The sputum total nitrate/nitrite in severe group was higher compared with mild group (559.8 µM *versus* 291.9 µM, p=0.003) or moderate group (559.8 µM *versus* 343.2 µM, p=0.035) using the E-FACED score (figure 1a). The sputum total nitrate/nitrite in severe group was higher compared with the mild group (425.7 µM *versus* 192.9 µM, p=0.006) using the BSI score (figure 1b). Meanwhile, the association between increased total nitrate/nitrite and increased exacerbations frequency in the prior
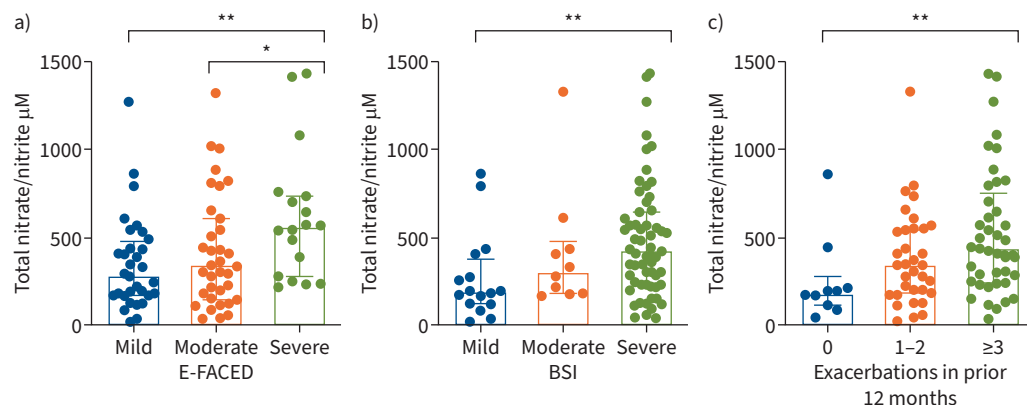
FIGURE 1 Association between total nitrate/nitrite in sputum and severity of disease. a) E-FACED (exacerbation, forced expiratory volume in 1 s, age, chronic colonisation by *Pseudomonas aeruginosa*, radiological extension and dyspnoea) score; b) Bronchiectasis Severity Index (BSI) score; c) exacerbations in prior 12 months. Data are presented as median (interquartile range). Mann–Whitney. *: p<0.05, **: p<0.01.

12 months was also significant (181.4 µM *versus* 433.1 µM, p=0.005) (figure 1c). In addition, we found a certain correlation between total nitrate/nitrite and symptom scores in patients with bronchiectasis. For instance, total nitrate/nitrite was significantly higher in patients with worse cough scores (median 417.9 µM, IQR 285.3–611.0 µM) than those with better cough scores (281.8 µM, 146.3–548.6 µM) (p=0.030). Total nitrate/nitrite was also significantly higher in patients with worse sputum purulence scores (410.8 µM, IQR 237.2–620.9 µM) and worse MRC scores (457.8 µM, IQR 280.6–774.5 µM) than those with better sputum purulence scores (156.1 µM, IQR 48.6–220.9 µM) (p=0.011) and better MRC scores (306.1 µM, IQR 173.6–549.3 µM) (p=0.017). Although there was no significant difference in total nitrate/ nitrite between different subgroups of sputum volume score and haemoptysis score, total nitrate/nitrite was higher in patients with a worse sputum volume score or a worse haemoptysis score (figure 2). However, total nitrate/nitrite was not significantly related to lung function, laboratory tests and the modified Reiff score (supplementary figure e1), and also not related to smoking history or different etiologies (supplementary figure e2). We found that sputum nitrate/nitrite was higher in patients with bronchiectasis complicated with hypertension (778.7 µM *versus* 343.2 µM, p=0.039) or diabetes (705.4 µM *versus* 343.2 µM, p=0.016) (supplementary figure e2). To further explore the relationships between nitrate/nitrite and other inflammatory markers, we also found the sputum nitrate/nitrite is significantly correlated with IL-1β (r=0.608, p<0.001) and IL-8 (r=0.782, p<0.001) in sputum from patients with bronchiectasis (supplementary figure e3).

The relationship between total sputum nitrate/nitrite and sputum microbiota in bronchiectasis

We further performed 16S rRNA sequencing for sputum samples from 85 patients with bronchiectasis. We found that total nitrate/nitrite is not significantly associated with α-diversity measured by five different methods (Chao, ACE, Shannon, Simpson and coverage) (figure 3a). At the phylum level, the total nitrate/ nitrite concentration was negatively correlated with Bacteroidota (r=−0.279, p=0.010) (figure 3b). At the class level, the total nitrate/nitrite concentration was negatively correlated with Bacteroidia (r=−0.279, p=0.010) (figure 3c). At the genus level, the total nitrate/nitrite concentration was positively correlated with *Pseudomonas* (r=0.335, p=0.002), and negatively correlated with *Haemophilus* (r=−0.250, p=0.021) and *Prevotella* (r=−0.267, p=0.013) (figure 3d). In addition, total sputum nitrate/nitrite concentration of the *P. aeruginosa* colonised (PA) group (median 439.9 µM, IQR 241.4–651.5) was higher than that of the non-*P. aeruginosa* colonised (NPA) group (283.8 µM, 181.1–489.9 µM) (p<0.05) (figure 4). We further divided the data into a low nitrate/nitrite group (<352.7 µM) and a high nitrate/nitrite group (⩾352.7 µM) based on the median sputum nitrate/nitrite in our study. Higher nitrate/nitrite group was also not significant association with reduced α-diversity. Principal coordinates analysis of Bray–Curtis distances indicated difference in the sputum microbiota between the high nitrate/nitrite group and the low nitrate/nitrite group (p=0.016). The relative abundance of *Pseudomonas* was significantly increased in the high nitrate/nitrite group compared with the low nitrate/nitrite group (42.7% *versus* 25.3%, p=0.019) (figure 5).

Denitrification functional predictions from sputum microbiota in bronchiectasis: PICRUSt analyses

To identify microbial denitrification functions in the sputum microbiota from stable bronchiectasis, PICRUSt was used to predict denitrification function in KEGG based on OTU representative sequences.
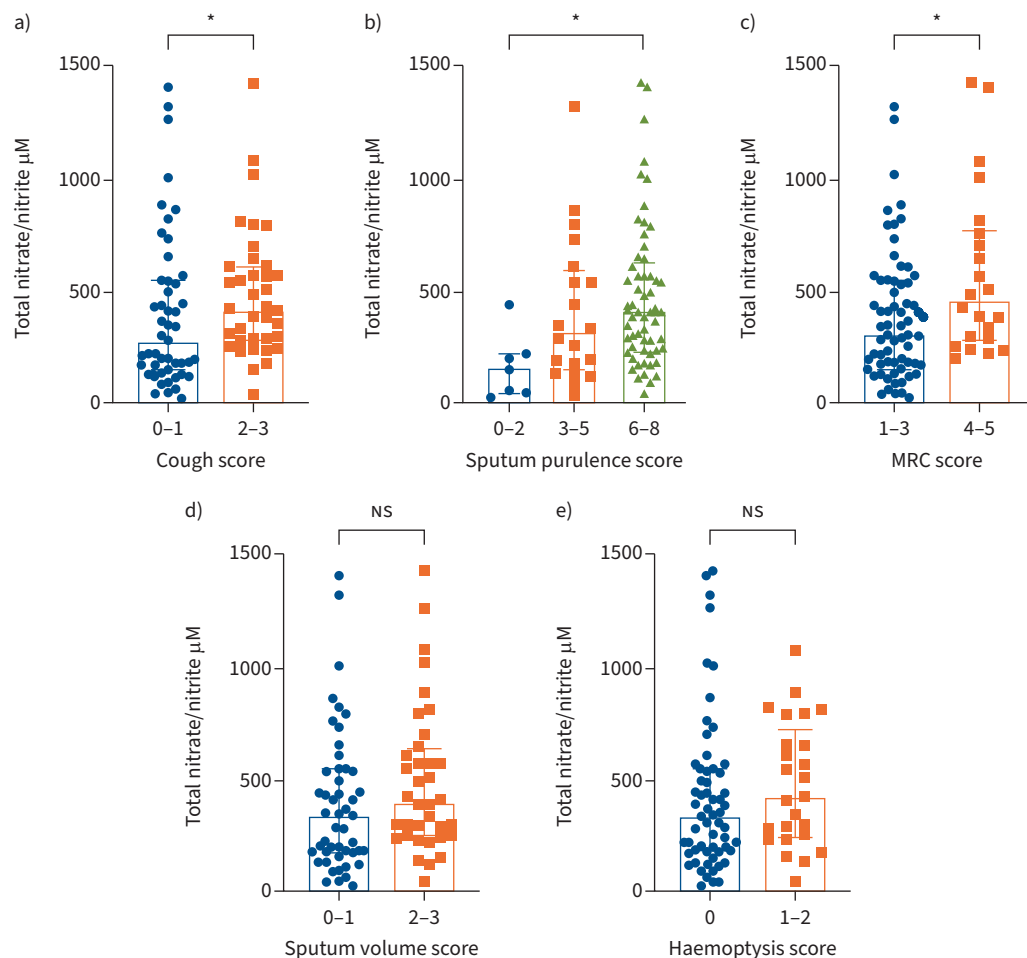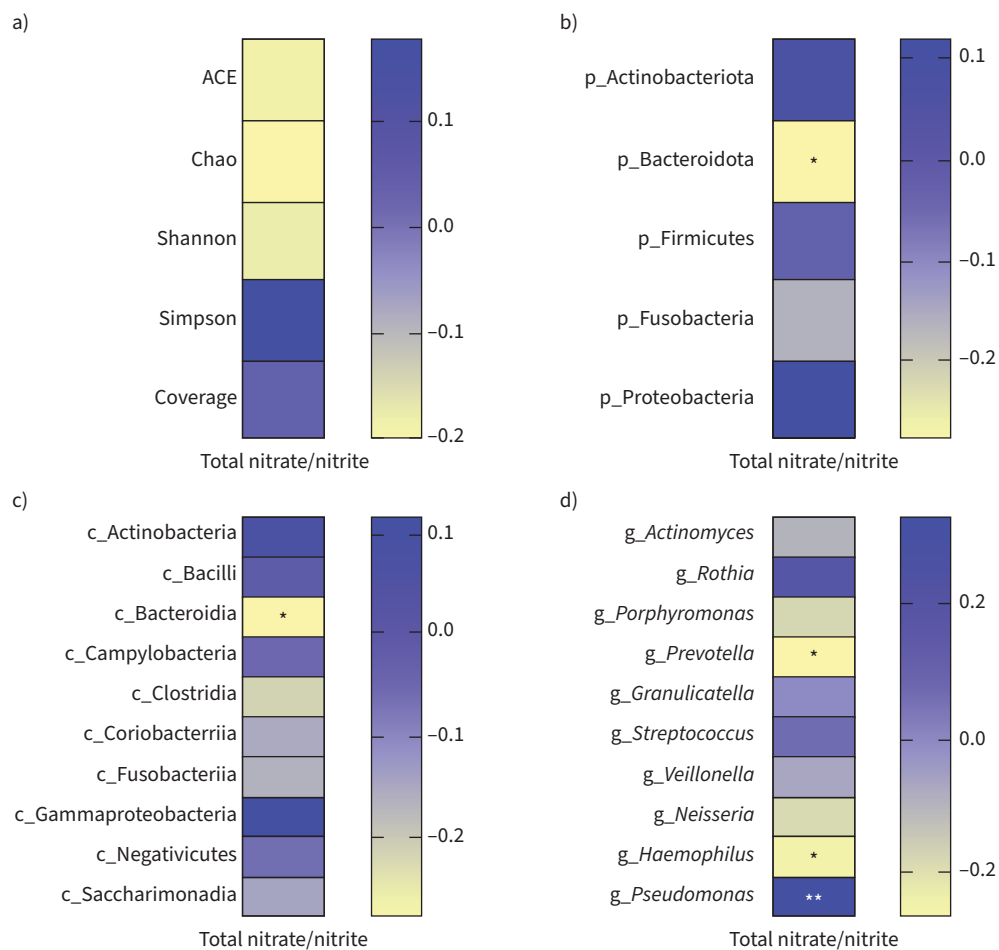
FIGURE 2 Total nitrate/nitrite in sputum of patients with bronchiectasis from different symptom groups.
a) Cough score; b) sputum purulence score; c) Medical Research Council (MRC) score; d) sputum volume score;
e) haemoptysis score. NS: nonsignificant. Data are presented as median (interquartile range). Mann–Whitney.
*: $p<0.05$, NS: $p\geqslant0.05$.

Denitrification pathway in KEGG include KEGG module (M00529) and KEGG enzyme (EC 1.7.2.5, EC 1.7.2.4, EC 1.7.99.4 and EC 1.7.2.1). We further compared the expression differences of module and enzymes during denitrification between the PA group and the NPA group. Our study found that denitrification module (M00529) is significantly enriched in PA group compared with NPA group at the level of the KEGG module. In addition, the expression of enzymes involved in the denitrification process was also significantly abundant in PA group, including nitric-oxide reductase (cytochrome c) (EC 1.7.2.5) and nitrous-oxide reductase (EC 1.7.2.4). Nitrate reductase (EC 1.7.99.4) and nitrite reductase (NO-forming) (EC 1.7.2.1) had an increasing trend in the PA group compared with the NPA group, although there was no significant difference (figure 6).

*Sputum nitrate/nitrite and longitudinal clinical outcomes*
In the cohort, the mortality rate was 2.4%; 8.2% of patients experienced mild or moderate exacerbations; and 80.0% of patients had hospital admissions for severe exacerbations during 1 year of follow-up. To further assess the ability of the standardised nitrate/nitrite in distinguishing severe bronchiectasis from mild and moderate patients according to E-FACED, the accuracy was 0.714 (AUC=0.714, p=0.006) as demonstrated using receiver operating characteristic analysis (figure 7a). To further distinguish severe bronchiectasis from mild and moderate patients according to BSI, the accuracy was 0.672 (AUC=0.672, p=0.012) as demonstrated using receiver operating characteristic analysis (figure 7b). All subjects in this study were followed-up for 1 year; nitrate/nitrite was associated with exacerbations during follow-up (AUC=0.741, p=0.014) (figure 7c).

FIGURE 3 Relationship between total nitrate/nitrite in sputum and airway microbiota in patients with bronchiectasis. Spearman's rank correlation coefficient is indicated using a colour gradient: blue indicates positive correlation, yellow indicates negative correlation. a) α-diversity; b) phylum level; c) class level; d) genus level. *: p<0.05, **: p<0.01.
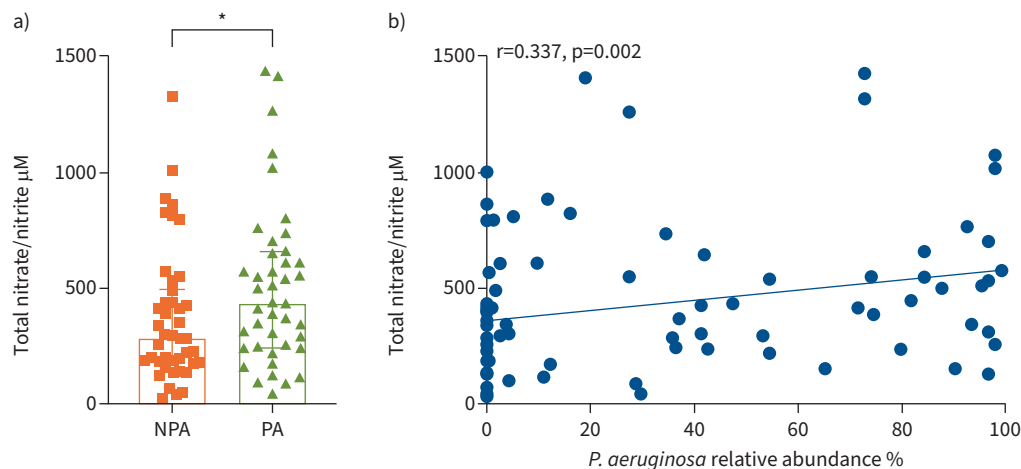
FIGURE 4 Relationship between total nitrate/nitrite in sputum and *Pseudomonas aeruginosa* in patients with bronchiectasis. PA: *P. aeruginosa* colonised; NPA: non-*P. aeruginosa* colonised. Data are presented as median (interquartile range). Mann–Whitney. *: p<0.05.
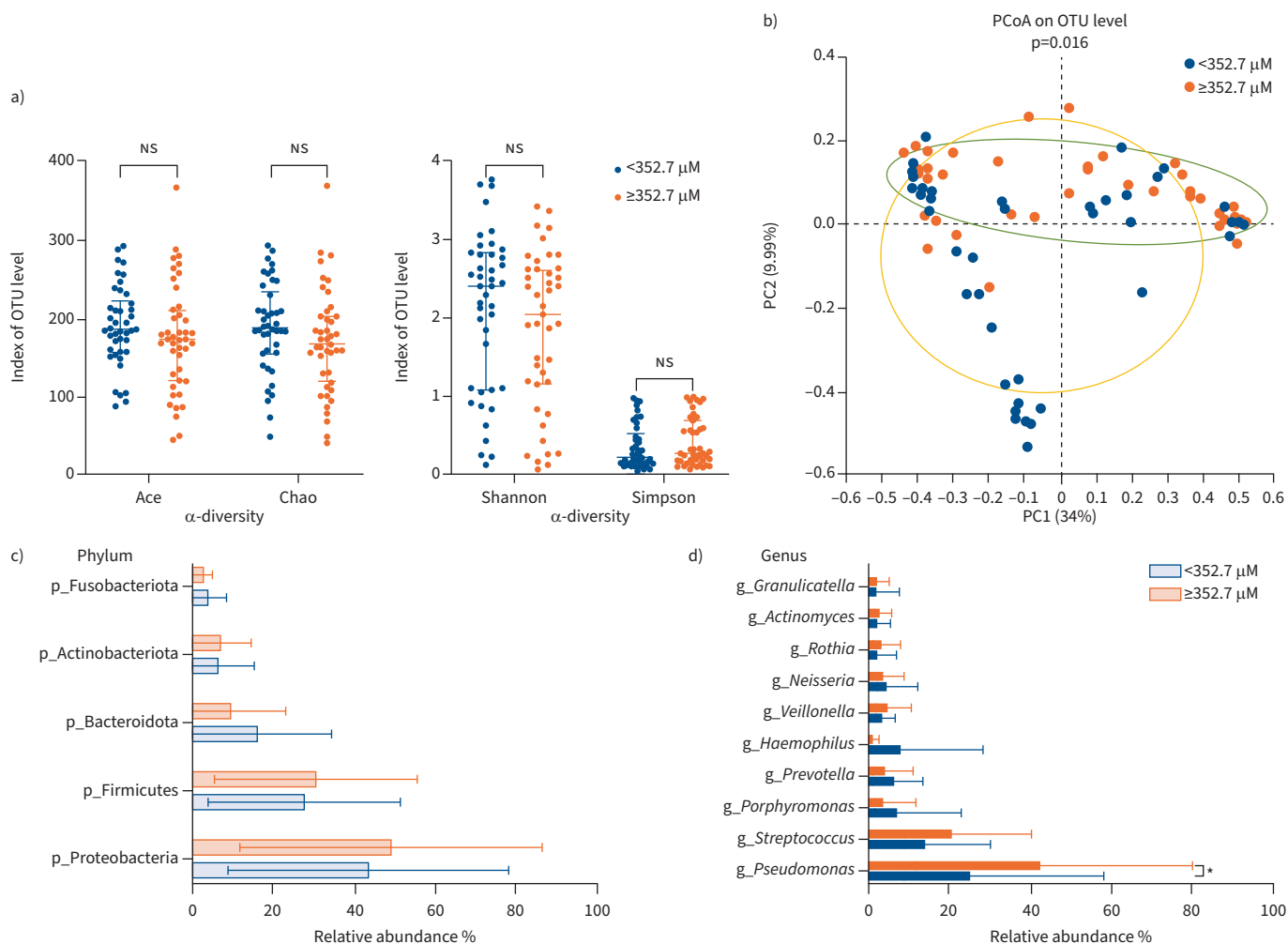
FIGURE 5 Differences of the sputum microbiome between high nitrate/nitrite group and low nitrate/nitrite group. a) α-diversity; b) β-diversity assessed by principal coordinates analysis (PCoA) of Bray–Curtis distances; c) taxonomic differences at phylum level; d) taxonomic differences at genus level. OTU: operational taxonomic units; NS: nonsignificant. Wilcoxon rank-sum test. *: p<0.05.

Discussion

As far as we know, this is the first study to investigate the level of nitrosative stress in sputum samples from patients with stable bronchiectasis and identify relationships between RNS, disease severity and airway microbiota of bronchiectasis. Our study indicated that sputum nitrate/nitrite is closely associated with symptoms, severity of disease and airway microbiota, and sputum nitrate/nitrite could be as a biomarker of disease severity and disease progression of bronchiectasis.

As we all know, nitrate and nitrite are the important products of nitrosative stress. Our research found that the total sputum nitrate/nitrite in patients with bronchiectasis is significantly elevated (supplementary figure e4), and along with worsening disease, the nitrate/nitrite gradually increases, which suggests that enlarged nitrosative stress occurring in the airways of patients with bronchiectasis. The increased nitrate/nitrite level has been found previously in other airway diseases including COPD, asthma and pneumonia [11, 30]. However, existing nitrate/nitrite studies on bronchiectasis mainly focus on exhaled breath condensate [11]. Studies indicate that oral cavity facultative anaerobic bacteria in the deep crypts of the base of the tongue can reduce nitrate to nitrite in the course of respiration, and some of this nitrite is reduced to NO in the oral cavity, influencing total nitrate/nitrite level [31]. The specimens of the lower respiratory tract are closer to the most realistic state of the lung environment. Alveolar lavage fluid is affected by variable degrees of dilution and more difficult to collect than sputum; therefore, we focused our analysis on sputum nitrate/nitrite. In addition to airway disease, some studies showed that nitrate/nitrite also plays an important role in patients with hypertension [32], diabetes [33] and neoplasm [34]. In an inflammatory microenvironment,
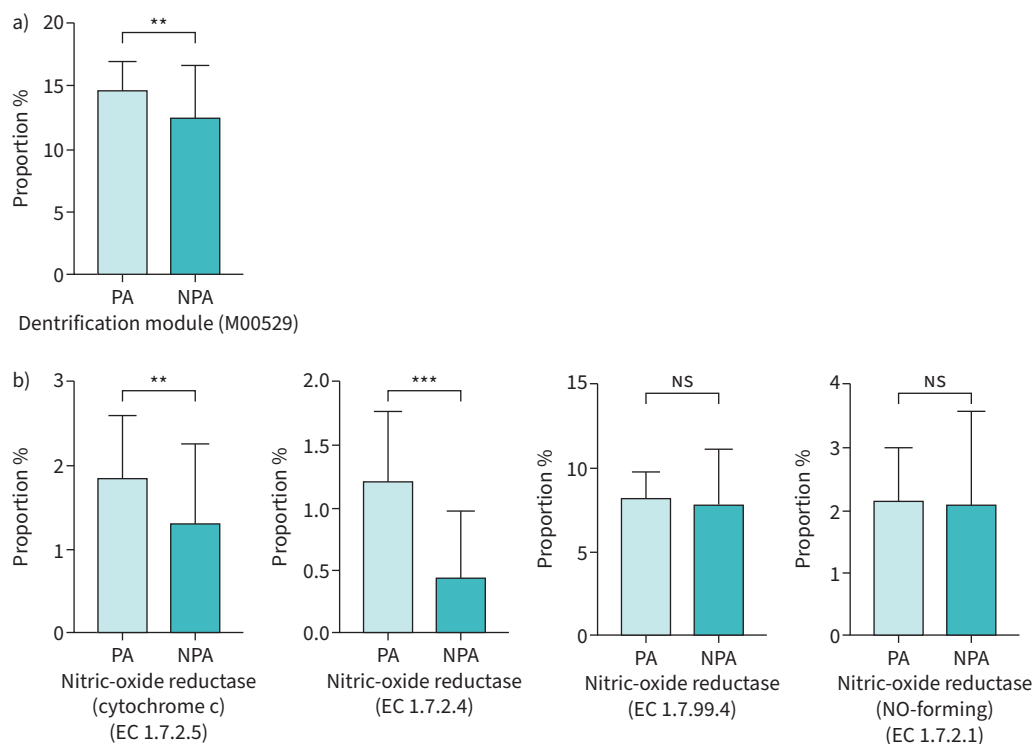
FIGURE 6 Denitrification functional predictions through Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analyses from sputum microbiota between the *Pseudomonas aeruginosa* colonised (PA) group and the non-*P. aeruginosa* colonised (NPA) group in bronchiectasis. a) Kyoto Encyclopedia of Genes and Genomes (KEGG) module level; b) KEGG enzyme level. EC: Enzyme Commission; ns: nonsignificant; NO: nitric oxide. **: p<0.01, ***: p<0.001, ns: p⩾0.05.

inflammatory cells may become activated and generate oxidants in response to various stimuli ("oxidative stress"). Simultaneously, exaggerated production of NO in the presence of oxidative stress may lead to the formation of RNS, such as nitrate, nitrite and peroxynitrite [5]. These RNS can react with a variety of biological molecules, including proteins, lipids and nucleic acids, which can lead to cell dysfunction or
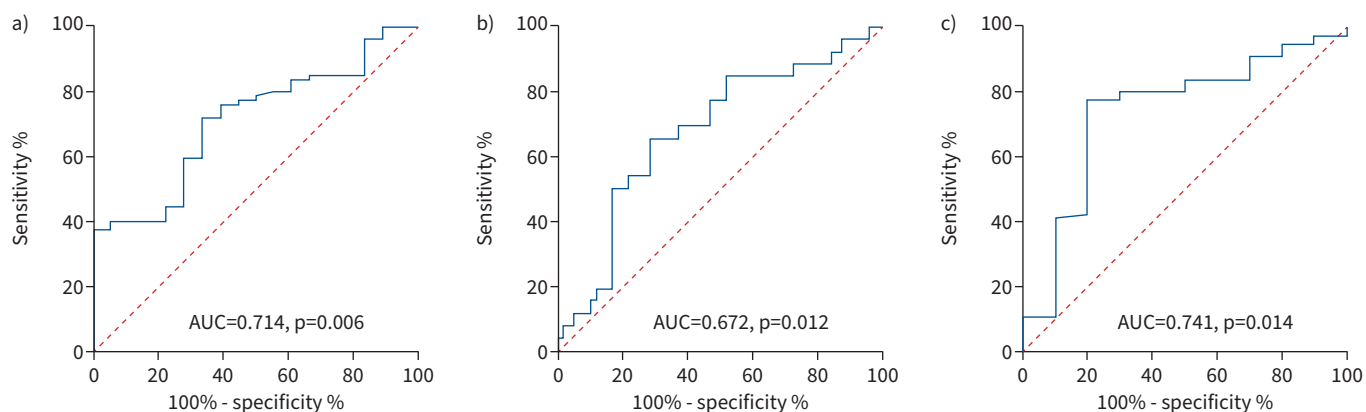
FIGURE 7 Receiver operating characteristic curve for the validity of the nitrate/nitrite to discriminate different groups of patients with bronchiectasis. a) Severe bronchiectasis *versus* mild-to-moderate bronchiectasis according to E-FACED (exacerbation, forced expiratory volume in 1 s, age, chronic colonisation by *Pseudomonas aeruginosa*, radiological extension and dyspnoea); b) severe bronchiectasis *versus* mild-to-moderate bronchiectasis according to Bronchiectasis Severity Index (BSI); c) exacerbations *versus* non-exacerbations during 1 year of follow-up. AUC: area under the curve.

death, as well as damage to the lung extracellular matrix [35]. In addition, RNS also decrease numbers and function of epithelial cilia, increase mucus production, and promote inflammation by activating NF-κB, which orchestrates the expression of multiple inflammatory genes [36, 37]. Therefore, nitrosative stress plays an important role in the etiology and progression of numerous human diseases including bronchiectasis.

Bronchiectasis is predominantly a neutrophilic inflammatory disease [38], and multiple cytokines are elevated in the airway of bronchiectasis, such as IL-6, IL-8, TNF-α and IL-1β, which may participate in promoting the expression of iNOS [10]. The host cell localisation of iNOS promoting the production of nitrate/nitrite has been mainly in macrophages and neutrophils and O_2^- has been mainly produced by neutrophil respiratory burst [39]. These effects together promote the production of nitrate/nitrite. Therefore, neutrophils and airway inflammation play important roles in the production of nitrate/nitrite in patients with bronchiectasis. In the study, we confirmed the positive correlation between nitrate/nitrite and IL-8, IL-1β; however, there was not significant correlation between circulating neutrophils and sputum nitrate/nitrite. We will further explore the relationship between airway neutrophils, other inflammatory factors and nitrate/nitrite.

In addition, our study found that nitrate/nitrite is significantly correlated with P. aeruginosa. We all know that the most common pathogen of bronchiectasis was P. aeruginosa (n=42, 49.4%), which is more easily detected in the sputum of severe patients as well as being associated with poor prognosis of bronchiectasis [9]. Combining previous research findings, P. aeruginosa is capable of both aerobic and anaerobic respiration as well as fermentation of arginine and pyruvate [40]. Under oxygen-limiting conditions, P. aeruginosa encodes a fully functional electron transport system that utilises nitrate or nitrite as a terminal electron receptor for anaerobic respiration to promote growth by denitrification [41]. In bronchiectasis, impaired mucociliary clearance and increased mucus plugs cause airway obstruction and dilatation, leading to a low oxygen concentration of airway environment [42]. Therefore, nitrate/nitrite may promote the growth and colonisation of P. aeruginosa by providing terminal electron acceptors in airway of bronchiectasis, leading to gradual progression of disease. Consistently, our study indicated that denitrification ability was relatively higher in the PA group compared with the NPA group through PICRUSt functional prediction. Simultaneously, the interactions of P. aeruginosa lipopolysaccharides, alginates, flagellin or pilin with inflammasome-forming NOD-like receptors on macrophages and dendritic cells activate host inflammatory and innate immune responses [9], which will promote the expression of iNOS to produce nitrate/nitrite. In summary, the nitrate/nitrite and P. aeruginosa can interact and self-perpetuate, participating in the progression of bronchiectasis. In addition to the positive correlation between the relative abundance of Pseudomonas and total nitrate/nitrite, we also found the relative abundance of Haemophilus and Prevotella are negatively correlated with total nitrate/nitrite. Previous research showed that Haemophilus and Prevotella belong to nitrate-reducing bacteria [43], but cannot utilise nitrate/nitrite as terminal electron acceptors for respiration and growth. H. influenzae possesses a versatile respiratory chain and uses a specialised type of metabolism termed respiration-assisted fermentation instead of denitrification [44]. ALTEMANI et al. [45] found that nitrate supplementation is able to decrease the relative abundance of Prevotella with nitrate-reducing capability. This may be a possible mechanism for the weak negative correlation between nitrate/nitrite and Haemophilus or Prevotella.

In summary, nitrosative stress and the associated oxidative stress play important roles in the progression of bronchiectasis. Therefore, antioxidant therapy may have a beneficial effect on the prognosis of bronchiectasis. Antioxidants are exogenous or endogenous molecules that mitigate any form of oxidative/ nitrosative stress or its consequences. Antioxidant supplements might reduce oxidative damage and build up levels of antioxidants [46]. For oral supplements, current studies showed that oral supplementation with N-acetylcysteine and glutathione showed beneficial effects by preventing the lung function deterioration [47]. For inhaled supplements, inhalation of N-acetylcysteine and glutathione into the lungs of people with cystic fibrosis reduced P. aeruginosa abundance and resulted in a significantly positive effect on lung function in the adult population [47].

During 1 year of follow-up, the mortality rate was 2.4%, and 80.0% of patients who had hospital admissions for severe exacerbations, which was higher than observational data from international registries. The consequence may be affected by the relatively small sample size and high proportion of moderate and severe bronchiectasis according to BSI or E-FACED. In our study, total nitrate/nitrite was associated with exacerbations during 1 year of follow-up, which indicates that nitrate/nitrite could be as a biomarker of progression and prognosis of bronchiectasis. Subsequently, we will further expand the sample size and develop multicentre studies to explore the specific mechanism of nitrate/nitrite in bronchiectasis.

Our study has several limitations. Firstly, the sample size was relatively small and the proportion of moderate and severe cases was relatively high. Secondly, only nitrate/nitrite was measured, but other

metabolites of oxidative/nitrosative stress in the airway and circulation contributing to the pathogenesis of bronchiectasis and bacterial growth will be further investigated. Thirdly, our study was a prospective observation study and only established the relationships between nitrate/nitrite, severity of bronchiectasis and *P. aeruginosa*. The in-depth mechanism is worth exploring further. Nitrate/nitrite may be a useful biomarker of progression in bronchiectasis and a potential therapeutic target for refractory *P. aeruginosa* colonisation. Finally, our research period was after the coronavirus disease 2019 (COVID-19) pandemic in China, which has certainly influenced exacerbation frequency during follow-up due to the introduction of social distancing and mitigation measures that reduced person-to-person interaction and reduced the circulation of respiratory viruses such as influenza and rhinovirus. However, China implemented unified prevention measures during the research period, and all recruited populations in our study came from Wuhan Union Hospital in Hubei province, which may minimise the impact of COVID-19 on this study.

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