

# Impact of prophylactic and 'rescue pack' antibiotics on the airway microbiome in chronic lung disease

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## ABSTRACT

The management of many chronic lung diseases involves multiple antibiotic prescriptions either to treat acute exacerbations or as prophylactic therapy to reduce the frequency of exacerbations and improve patients' quality of life.

**Aim** To investigate the effects of antibiotics on the homeostasis of bacterial communities in the airways, and how this may contribute to antimicrobial resistance (AMR) among respiratory pathogens and microbiota.

**Methods** Within an observational cohort study, sputum was collected from 84 patients with chronic obstructive pulmonary disease and/or bronchiectasis at stable state: 47 were receiving antibiotic prophylaxis therapy. V3-V4 16S-rRNA sequencing on Illumina MiSeq, quantitative PCR for typical respiratory pathogens, bacteriology cultures and antimicrobial susceptibility testing of sputum isolates, resistome analysis on a subset of 17 sputum samples using MinION metagenomics sequencing were performed.

**Finding** The phylogenetic  $\alpha$ -diversity and the total bacterial density in sputum were significantly lower in patients receiving prophylactic antibiotics ( $p=0.014$  and  $0.029$ , respectively). Antibiotic prophylaxis was associated with significantly lower relative abundance of respiratory pathogens such as *Pseudomonas aeruginosa*, *Moraxella catarrhalis* and members of family Enterobacteriaceae in the airway microbiome, but not *Haemophilus influenzae* and *Streptococcus pneumoniae*. No major definite directional shifts in the microbiota composition were identified with prophylactic antibiotic use at the cohort level. Surveillance of AMR and resistome analysis revealed a high frequency of resistance to macrolide and tetracycline in the cohort. AMR expressed by pathogenic bacterial isolates was associated with antibiotics prescribed as 'rescue packs' for prompt initiation of self-treatment of exacerbations (Spearman's  $\rho=0.408$ ,  $p=0.02$ ).

**Conclusions** Antibiotic prophylactic therapy suppresses recognised pathogenic bacteria in the sputum of patients with chronic lung disease. The use of antibiotic rescue packs may be driving AMR in this cohort rather than prophylactic antibiotics.

## INTRODUCTION

Chronic lung diseases such as bronchiectasis and chronic obstructive pulmonary disease (COPD) are associated with both a

## WHAT IS ALREADY KNOWN ON THIS TOPIC

- ⇒ In the management of chronic lung diseases, substantial amounts of antibiotics are prescribed either as prophylaxis or treatment of acute exacerbations.
- ⇒ Substantial scientific evidence supports the clinical usefulness of prophylactic antibiotic use, especially macrolides which can reduce the rate of exacerbations.
- ⇒ A key question is which of these strategies leads to a greater risk of altering the microbiome and/or the development of antimicrobial resistance.

## WHAT THIS STUDY ADDS

- ⇒ We found that antibiotic prophylaxis suppressed common pathogenic bacteria such as *Pseudomonas aeruginosa* in sputum from patients with chronic lung diseases; however, use of antibiotic rescue packs to self-treat exacerbations may be associated with antimicrobial resistance in our cohort.

## HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- ⇒ The potential impact of prescribed antibiotic rescue packs is concerning and requires review, especially in patients with frequent exacerbations who might benefit more from antibiotic prophylaxis.

considerable socioeconomic burden and impacts on patients' lives.<sup>1</sup> Many people with chronic lung conditions also periodically experience intermittent acute deteriorations in respiratory health (exacerbations) which cause significant morbidity, impact significantly on quality of life and necessitate a change in regular medication.<sup>2 3</sup> Frequent exacerbations have been associated with progressive lung damage, faster decline in lung function and worse quality of life.<sup>4 5</sup>

Guidelines for bronchiectasis and COPD recommend that patients have a self-management plan (SMP) in place for acute exacerbations. Within these SMPs, antibiotics are often prescribed and kept at home by patients as a 'rescue pack' to allow for prompt start of antibiotic treatment.<sup>2 3</sup>



Reducing the rate of exacerbations has been a major aim of patient management.<sup>6</sup> Evidence from systematic reviews and meta-analyses of randomised placebo-controlled clinical trials has shown the clinical usefulness of prophylactic antibiotics in reducing the frequency of exacerbations in COPD<sup>7,8</sup> and bronchiectasis.<sup>9,10</sup> Azithromycin specifically has very good penetration into respiratory tissues, and a long serum half-life (up to 60 hours), permitting once-daily and even thrice-weekly dosing, with a characteristic postantibiotic effect<sup>11</sup>—making it the macrolide of choice.

Nevertheless, the adverse effects of long-term use of antimicrobials in chronic respiratory disease has not been fully investigated. Concern exists that the selection pressure on microbiota from a prolonged antibiotic regimen may alter host microbial homeostasis. In addition, there is the risk of AMR emergence in human microbiota, which may act as a reservoir for antimicrobial resistance dissemination into the wider population.<sup>12</sup> Given that one aim of prophylactic antibiotics is to reduce the need and use of acute ‘rescue’ antibiotics in people with chronic lung diseases, a key question is which of these strategies leads to a greater risk of altering the microbiome and/or the development of antimicrobial resistance?

Here, we investigated the impact of prolonged antibiotic prophylactic therapy on the airway microbiome in patients with chronic lung disease, and the influence of prophylactic antibiotics and acute ‘rescue packs’ on the resistome and AMR in sputum bacterial isolates.

## METHODS

### Study design and population

Participants with diagnosis of bronchiectasis (on CT) or COPD (on spirometry) were recruited between February 2017 and February 2018, participants were followed-up for 12 months. This was a convenience sample. The inclusion criteria were: age  $\geq 20$  years, confirmed diagnosis of COPD and/or bronchiectasis, history of frequent exacerbations ( $\geq 2$ /year), ability to spontaneously expectorate sputum and consent to participate. The exclusion criteria were diagnosis of cystic fibrosis or lung cancer, history of lung transplantation, known tuberculosis or HIV infections at the time of recruitment and clinical instability (sputum collected within 1 week before and 2 weeks after antibiotic treatment of an exacerbation or any other acute infection was excluded in the presented work). Clinical data collection included interviewing participants, and reviewing medical records and daily diary cards filled by participants reporting changes in the symptoms and treatment.

### Patient and public involvement

Our research proposal was discussed with academics, physicians and students at University College London and with a sample of patients and their families/carers in pulmonary rehabilitation programme held in Peckwater Centre, London prior to submitting the ethics

application. We adjusted the design in the light of their comments. Feedback on the study’s procedure was obtained from participants during clinic visits. Any concerning findings in sputum microbiology results were communicated with the treating physician.

### Sputum processing and bacteriology cultures

Details on the methods are provided in online supplemental data. Sputum samples were processed with Sputasol (Oxoid, UK), comprehensive bacteriology cultures were obtained by plating sputum on Columbia agar with chocolate horse blood, Columbia colistin-nalidixic acid agar and cystine-lactose-electrolyte-deficient agar. All morphologically distinct colonies on the three cultures were isolated, purified and identified using matrix-assisted laser desorption/ionisation-time-of-flight (MALDI-TOF) mass spectrometry. Antimicrobial susceptibility of bacterial isolates was determined using the Kirby-Bauer agar diffusion technique according to the standards of the European Committee on Antimicrobial Susceptibility Testing (EUCAST).<sup>13</sup>

### DNA extraction

DNA was then extracted from 1 mL sputum samples that had been centrifuged, pellet heated at 95°C for 30 min, then mechanically disrupted by bead-beating step, on the automated LIAISON Ixt extraction platform using DiaSorin Arrow-DNA extraction kit.

The total bacterial density in sputum indicated by the number of copies of the 16S rRNA gene per  $\mu\text{L}$ ; in addition, the bacterial loads of *Pseudomonas aeruginosa*,<sup>14</sup> and *Haemophilus influenzae*, *Moraxella catarrhalis* and *Streptococcus pneumoniae*<sup>15</sup> were quantified using two multiplex quantitative PCR (qPCR) Taq-Man assays on a Qiagen Rotor-gene 6000 machine (online supplemental table S1).<sup>16</sup> A mean of technical triplicates was taken for each sample. An internal amplification control, SPUD A, was used to test for PCR inhibition.

### 16S rRNA profiling

A sequence library was created by amplification of V3-V4 regions of the bacterial 16S rRNA gene using 341 forward-primer and 805 reverse-primer. The PCR products (584 bp amplicons) were cleaned using Agencourt AMPure XP beads (Beckman Coulter, UK). The samples were pooled in an equimolar ratio at 8 nM into one library which was checked by TapeStation (Agilent, USA). Sequencing was performed using Illumina MiSeq Platform using costume sequencing primers, and MiSeq Reagent Kit v2 (500 cycles).<sup>17</sup>

An extraction negative control and a no-template PCR control (water) were run alongside each batch of samples throughout the process as negative controls. A laboratory-prepared mock community was run as a positive control.

We adopted the workflow established by Microbiome helper<sup>18</sup> using QIIME pipeline V.1.9.1.<sup>19</sup> The appropriate

statistical significance tests were calculated using QIIME wrapper scripts. STAMP (V.2.1.3)<sup>20</sup> was used to visualise the results and testing the differential abundances of taxa between the two study groups using White's non-parametric t-test.<sup>21</sup> All p values were corrected using Benjamini-Hochberg false discovery rate method for multiple comparisons. The statistical analysis was performed using IBM SPSS V.25.0.<sup>22</sup>

### Resistome analysis

Metagenomic sequencing was carried out on a subset of 17 sputum samples: 8 were from patients with bronchiectasis on prophylactic antibiotic therapy and 9 from the comparator group, using Oxford Nanopore MinION system. Human DNA was depleted as per the published method by Charalampous *et al.*<sup>23</sup> Metagenomics libraries of six multiplexed samples were prepared using the Rapid Barcoding Kit (Ref: SQK-RLB004, ONT). Sequencing was performed on MinION flow cell (R.9.4.1) for 48 hours. The genomes were assembled using the miniasm/minimap pipeline.<sup>24</sup> A Basic Local Alignment Search Tool (BLAST) search against the ResFinder database performed<sup>25</sup> to detect AMR genes. The alignments with accuracy <90% have been excluded. The prevalence of AMR genes within the samples was measured in parts per million reads (ppm), that is, the number of sequence reads identified as AMR genes relative to the total number of reads representing the sample.

### RESULTS

The clinical and demographic characteristics of patients who were and were not on prescribed antibiotic prophylaxis therapy are shown in table 1. Forty-seven of 84 participants were prescribed antibiotic prophylaxis therapy for over a year, in the context of their routine medical care; in 66% these were macrolides, specifically, 29 patients were on azithromycin 250 mg thrice weekly and 8 on 500 mg thrice weekly; 2 were on clarithromycin (250 mg twice daily). Other prescribed antibiotics were co-trimoxazole (960 mg once daily or 1920 mg, divided into two doses, thrice weekly), ciprofloxacin (250 mg twice daily), doxycycline (100 mg once daily), amoxicillin (250 mg twice daily), cephalexin (500 mg twice daily) and phenoxymethyl penicillin (250 mg twice daily).

There were no significant differences in the proportions of patients with COPD and bronchiectasis in the two groups ( $p=0.157$ ). All participants were regularly receiving the influenza vaccine and had pneumococcal vaccine. Lung function as indicated by the spirometry results was comparable between the two study groups (table 1).

A significantly higher proportion of patients with common variable immunodeficiency (CVID) were on antibiotic prophylaxis therapy (66%), compared with seven in the comparator group (19%) ( $p<0.0001$ ); 90% of the patients with CVID were on immunoglobulin replacement therapy.

Seventy-nine per cent of the participants were prescribed a rescue pack of antibiotics to keep at home as an SMP; 47% of the rescue packs contained amoxicillin/clavulanic acid, 20% doxycycline, 14% quinolones (either ciprofloxacin, levofloxacin or moxifloxacin), 15% amoxicillin and 7.5% had macrolides either azithromycin or clarithromycin.

For comparing the microbiome of patients who were and were not receiving antibiotic prophylaxis therapy, one sputum sample per participant was selected to represent the microbiome profile at stable state. To avoid biases due to exacerbations and antibiotic treatment, sputum samples collected within 1 week before and 2 weeks after exacerbation and/or breakthrough antibiotic treatment were excluded.

The total bacteria density and the phylogenetic  $\alpha$ -diversity were significantly lower in the sputum collected from patients on prophylactic antibiotics in comparison with those who were not (figure 1). The median 16S rRNA gene copies (IQR) was 6.20 (5.54–6.73)  $\log_{10}$  copies/ $\mu$ L in the antibiotic prophylaxis group vs 6.60 (6.17–6.92)  $\log_{10}$  copies/ $\mu$ L in the comparator group ( $p=0.029$ ) (figure 1A). The difference of means of the phylogenetic  $\alpha$ -diversity index PD whole tree in the two populations (SD) was 1.4 (0.56),  $p=0.014$ . This was despite the greater proportion of patients with bronchiectasis in the prophylactic antibiotic group (table 1) and the fact that the total bacterial density was significantly higher in the patients with bronchiectasis compared with those with COPD, where the median 16S rRNA gene copies (IQR) were 6.63 (6.18–6.96) vs 6.18 (5.41–6.88)  $\log_{10}$  copies/ $\mu$ L ( $p=0.042$ ), respectively (figure 1B).

Antibiotic prophylaxis was the only significant covariate in weighted-UniFrac  $\beta$ -diversity index ( $p=0.038$  by permutational multivariate analysis of variance (PERMANOVA)) and unweighted-UniFrac  $\beta$ -diversity index ( $p=0.012$  by PERMANOVA) (online supplemental data, online supplemental figure S1). Chronic lung disease (whether bronchiectasis or COPD) was a significant covariate in unweighted-UniFrac  $\beta$ -diversity index ( $p=0.01$  by PERMANOVA) but not in weighted-UniFrac  $\beta$ -diversity index ( $p=0.152$  by PERMANOVA). Primary immunodeficiency status (CVID) was not a significant covariate in both weighted and unweighted-UniFrac  $\beta$ -diversity indices ( $p>0.05$  by PERMANOVA) (online supplemental data, online supplemental figure S2).

The bacterial community structure was shifted towards more *Proteobacteria* ( $p=0.041$ ) in patients with bronchiectasis while in patients with COPD it was shifted towards more *Firmicutes* ( $p=0.048$ ). *Bacteroidetes* was slightly lower in patients with COPD and was significantly associated with airflow obstruction ( $p=0.049$ ) (online supplemental figures S3 and S4).

The microbiome profiles demonstrated that the microbiota composition in sputum was similar in patients who did or did not use prophylactic antibiotics at the phylum level. Only the phylum *Synergistetes*, which is represented by a minor taxon present at relative abundance (RA) of

**Table 1** Demographic and clinical characteristics of participants

		On prophylactic antibiotic treatment (n=47)	Comparator group (n=37)	P value
Prophylactic antibiotics*				
Macrolides	Azithromycin	29 (62%)		
	Clarithromycin	2 (4%)		
Quinolones	Ciprofloxacin	2 (4%)		
Tetracyclines	Doxycycline	4 (9%)		
β-Lactams		3 (6%)		
Sulfonamides	Co-trimoxazole	7 (15%)		
Age† (years)		67 (12)	62 (19)	0.39‡
Sex*	Males	22 (47%)	18 (49%)	0.87§
BMI (kg/m <sup>2</sup> )†		27.79 (4.40)	27.48 (9.14)	0.30‡
Chronic lung disease*				
	Bronchiectasis	37 (79%)	24 (65%)	0.16§
	COPD	10 (21%)	13 (35%)	
CVID*		31 (66%)	7 (19%)	<0.001§
Smoking*	Smokers	1 (2%)	5 (14%)	0.14¶
	Ex-smokers	11 (23%)	9 (24%)	
	Never-smokers	35 (74%)	23 (62%)	
Passive smoking*		10 (63%)	12 (60%)	0.88§
Number of exacerbation events/year**		2 (2–3)	3 (2–5)	0.18‡
Frequency of rescue pack consumption/year**		2 (2–3)	2.5 (2–4)	0.20‡
Rescue pack*		42 (89%)	24 (65%)	0.007§
Rescue pack antibiotics*				
β-Lactams	Co-amoxiclav	23 (49%)	8 (22%)	
	Amoxicillin	3 (6%)	7 (19%)	
FQ	Ciprofloxacin, levofloxacin, moxifloxacin	7 (15%)	2 (5%)	
Tetracyclines	Doxycycline	6 (13%)	7 (19%)	
Macrolides	Azithromycin, clarithromycin	3 (6%)	2 (5%)	
Rescue pack corticosteroids*		9 (19%)	5 (14%)	0.025§
Prescribed CIP course for <i>Pseudomonas aeruginosa</i> infection*		11 (23%)	7 (19%)	0.62§
Oral corticosteroids (<10 mg/day)*		10 (21%)	5 (14%)	0.36§
Carbocisteine*		10 (21%)	7 (19%)	0.79§
Oxygen therapy*		4 (9%)	3 (8%)	1.00¶
Inhaled respiratory medication*				
SABA		16 (34%)	16 (43%)	0.39§
LABA		19 (40%)	16 (43%)	0.80§
LAMA		9 (19%)	13 (35%)	0.10§
ICS*		21 (45%)	17 (46%)	0.91§
FEV1 (L)**		1.96 (1.06–2.91)	1.52 (0.94–2.72)	0.37‡
FEV1 % predicted**		80.5% (43.8%–109%)	75% (46%–92%)	0.56‡
FVC (L)**		2.99 (2.31–3.59)	2.29 (1.72–3.72)	0.22‡
FVC % predicted**		105.9% (70.8%–122%)	91% (73%–122%)	0.45‡

Continued

**Table 1** Continued

	On prophylactic antibiotic treatment (n=47)	Comparator group (n=37)	P value
FEV1/FVC**	0.73 (0.50–0.80)	0.67 (0.51–0.74)	0.58‡

\*N (%).

‡Mean (SD).

‡P value by Mann-Whitney U test.

§P value by  $\chi^2$  test.

¶P value by Fisher's exact test.

\*\*Median (IQR).

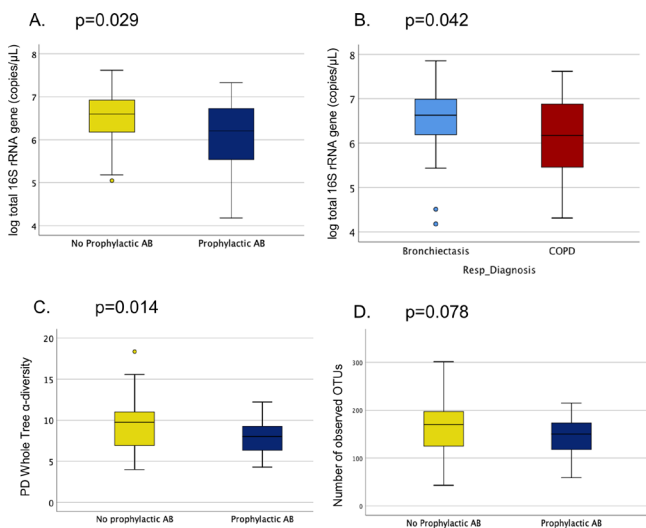
BMI, body mass index; CIP, Ciprofloxacin; COPD, chronic obstructive pulmonary disease; CVID, common variable immunodeficiency; FEV1, forced expiratory volume in 1 s; FQ, fluoroquinolones; FVC, forced vital capacity; ICS, Inhaled Corticosteroids; LABA, Long-Acting Beta-2 Agonists; LAMA, Long-Acting Muscarinic receptor Antagonists; SABA, Short-Acting Beta-2 Agonists.

<0.5% in all samples, was significantly less abundant in the patients on prophylactic antibiotic therapy ( $p=0.002$ ) (figure 2). At the genus level, the major taxa constituting the microbiome profiles and representing the core respiratory microbiota were similar in the patients who did or did not use prophylactic antibiotics (figure 3). Nevertheless, potentially pathogenic taxa were significantly less abundant in patients on prophylactic antibiotic therapy including: *Pseudomonas* ( $p=0.027$ ), Enterobacteriaceae ( $p=0.021$ ), *Klebsiella* ( $p=0.046$ ), *Pasteurella* ( $p=0.012$ ) and *Morganella* ( $p<0.0001$ ). The genus *Moraxella* tended to be less abundant in patients on prophylactic antibiotic therapy; however, the observed difference did not reach

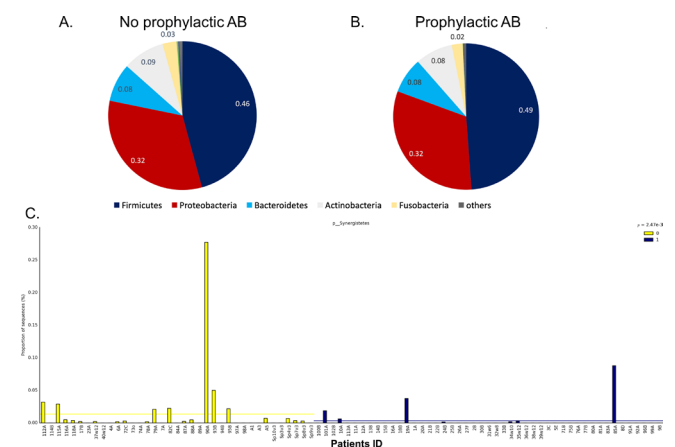
statistical significance ( $p=0.087$ ) (figure 4). Some minor taxa with RA <1% such as *Bacteroidetes* ( $p=0.02$ ), *Schwartzia* ( $p=0.001$ ) and *Sphaerochaeta* ( $p=0.01$ ) were also significantly lower in the antibiotic prophylaxis group.

qPCR confirmed that *P. aeruginosa* load was significantly suppressed in the sputum of patients on antibiotic prophylaxis; the median load (IQR) was 2.89 (2.62–3.64)  $\log_{10}$  CFU/mL vs 7.23 (3.44–7.95)  $\log_{10}$  CFU/mL in those who were not ( $p=0.001$ ), even though *P. aeruginosa* was more frequently detected in the antibiotic prophylaxis group; 61% vs 44% in the comparator group (figure 5D). *M. catarrhalis* was significantly less prevalent in the sputum of patients on antibiotic prophylaxis (4%) compared with 20% in those who were not ( $p=0.039$ ). However, when detected, the load of *M. catarrhalis* was no different between the two groups (figure 5A,C). The prevalence and loads of both *H. influenzae* and *S. pneumoniae* were similar in both groups.

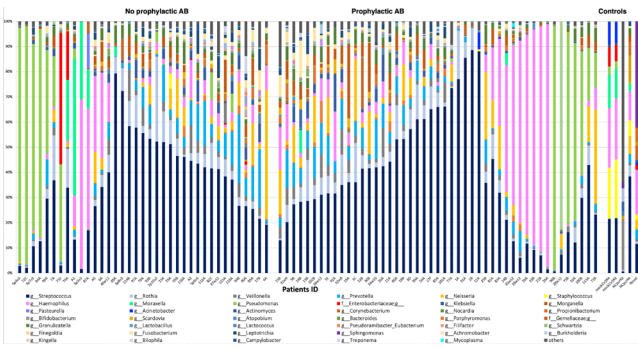
Co-existence of two respiratory pathogens was detected in 39% of the examined sputum samples (figure 5B).



**Figure 1** The total bacterial density,  $\alpha$ -diversity and richness of airway bacterial community in patients with prophylactic antibiotics (AB) (n=47, navy blue) and those who were not (n=37, yellow). (A) Total number of 16S rRNA copies (a proxy to total bacterial density in sputum) was significantly lower in the prophylactic antibiotic group ( $p=0.029$ ); however, (B) it was significantly higher in the patients with bronchiectasis (sky blue, n=50) compared with patients with chronic obstructive pulmonary disease (COPD) (red, n=23) ( $p=0.042$ ). (C)  $\alpha$ -Diversity expressed by phylogenetic diversity index: PD whole tree ( $p=0.014$ ) and (D) richness expressed by total number of observed Operational Taxonomic Units (OTUs) ( $p=0.078$ ).



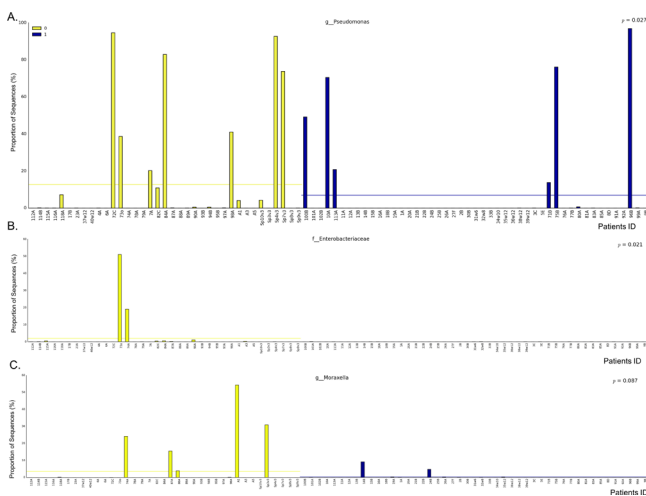
**Figure 2** Comparison between the average airway microbiome profile of patients not receiving antibiotic (AB) prophylaxis (comparator group) (A) and those on prophylactic AB therapy (B) at the phylum level ( $p>0.05$ ); (C) the relative abundance of phylum *Synergistetes* was significantly lower in patients receiving antibiotic prophylaxis therapy ( $p=0.00247$ ); yellow: comparator group, navy blue: AB prophylactic group.



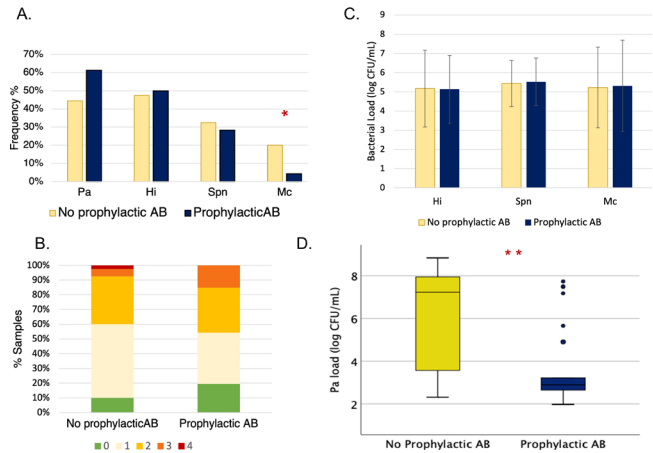
**Figure 3** Airway microbiome profiles of patients with chronic lung disease who were receiving antibiotic (AB) prophylaxis therapy in comparison with patients in the comparator group; not receiving antibiotic prophylaxis; profiles of the Mock community resulted from two sequencing runs R3 and R4. f, family; g, genus; NC, no template negative control; NCext, extraction negative control.

The most common combinations were *P. aeruginosa*+*H. influenzae* (12%) and *H. influenzae*+*S. pneumoniae* (9%). The co-existence of these three pathogens was detected in 7% of samples. Four pathogens were detected in one patient with bronchiectasis who was not receiving antibiotic prophylaxis. However, there were no significant differences in the co-existence of these respiratory pathogens in the sputum of patients who were or were not on antibiotic prophylaxis ( $p=0.203$ ).

In bacterial cultures, a total of 187 distinct bacterial isolates were isolated from sputum cultures and identified by MALDI-TOF. The prevalence of the 68 sputum isolates; 36 from patients on prophylactic antibiotics and 32 from the comparator group are shown in figure 6. The variety and frequency of bacterial pathogens isolated from the

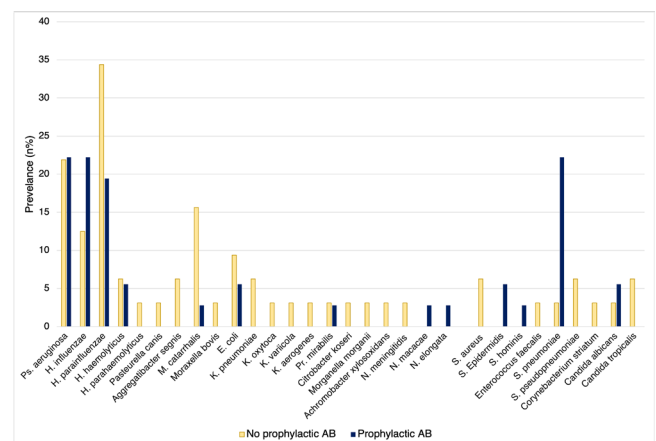


**Figure 4** Suppression of potential pathogenic taxa in the sputum of patients on prophylactic antibiotic therapy (navy blue) compared with others in the comparator group (yellow): (A) genus *Pseudomonas* ( $p=0.027$ ), (B) family Enterobacteriaceae ( $p=0.021$ ), (C) genus *Moraxella* ( $p=0.087$ ).

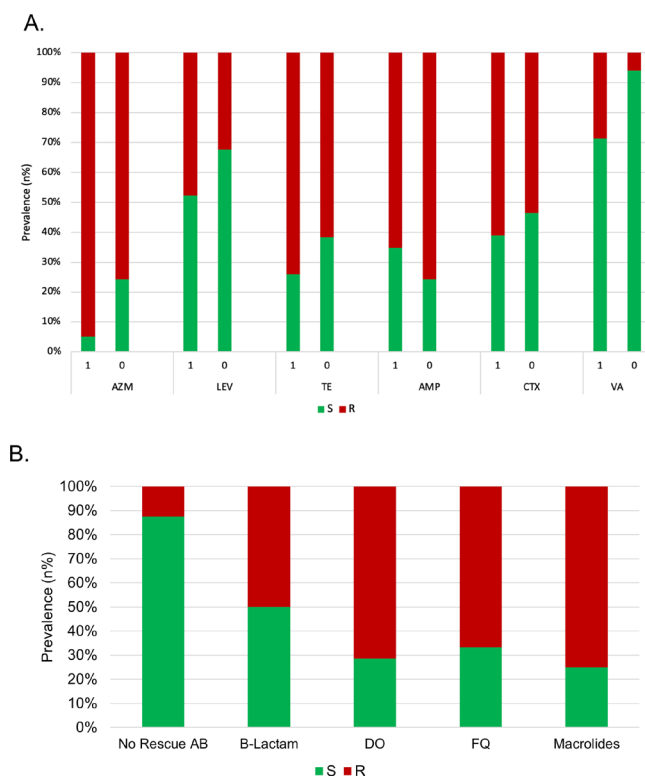


**Figure 5** Prevalence, co-existence and bacterial loads of pathogenic airway bacteria in patients with chronic lung disease who were receiving antibiotic (AB) prophylaxis therapy (navy blue) and those not (comparator group, yellow) as determined by the multiplex quantitative PCRs. (A) Percentage of samples tested positive for the tested pathogens, (B) number of organisms tested positive within the same sputum samples ( $p=0.203$ ), (C) mean bacterial loads of *Haemophilus influenzae* (Hi), *Moraxella catarrhalis* (Mc), *Streptococcus pneumoniae* (Spn) were similar in both groups ( $p=0.189$ , multivariate analysis of variance). (D) *Pseudomonas aeruginosa* (Pa) load was significantly lower in the prophylactic AB group ( $p=0.001$ ). Bacterial load was measured in CFU/mL of original sputum sample for each bacteria. Samples which gave negative results for a given bacteria were excluded from the analysis. Error bars show  $\pm 1$  SD. \* $P<0.05$ , \*\* $p\leq 0.01$ .

sputum of patients using prophylactic antibiotics were significantly less than those not receiving prophylactic antibiotics in the comparator group. Twenty-six per cent of the Gram-negative isolates were isolated from sputum cultures of patients on prophylactic antibiotics compared with 48% from the rest of the cohort ( $p=0.011$ ).



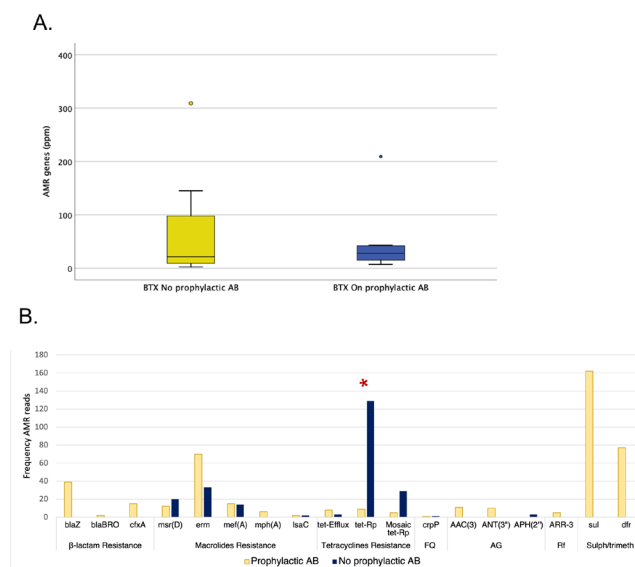
**Figure 6** The taxa isolated from the sputum of patients with chronic lung disease and their prevalence among the patients who were receiving antibiotic (AB) prophylaxis therapy ( $n=36$ , navy blue) and those not (comparator group, yellow,  $n=32$ ).



**Figure 7** (A) Comparison between the antimicrobial resistance (AMR) in the commensal viridans streptococci group isolated from the sputum of patients receiving antibiotic (AB) prophylaxis therapy (group 1) and those not (comparator (group 0) ( $p < 0.0001$ ), (B) association between the AMR in pathogenic sputum isolates and the prescribed ABs in the rescue packs for the self-treatment of acute exacerbations ( $p = 0.04$ ) prevalence: percentage of the resistant isolates. AZM, azithromycin; AMP, ampicillin; CTX, cefotaxime; DO, doxycycline, FQ, fluoroquinolone; LEV, levofloxacin; R, resistant (red); S, sensitive (green); TE, tetracycline; VA, vancomycin.

Antibiotic susceptibility testing revealed high proportions of resistance to macrolide (64% and 29%), tetracycline (45% and 34%) and ampicillin (10% and 67%) of the tested Gram-positive and Gram-negative isolates, respectively in the whole cohort. Ciprofloxacin and cefotaxime resistance was detected in 21% and 17% of the Gram-negative isolates, respectively (online supplemental figures S5 and S6). The observed AMR in the bacterial isolates was associated with the corresponding prescribed antibiotics in the rescue packs for self-treatment of acute exacerbations (Spearman's correlation  $\rho = 0.408$ ,  $p = 0.02$ ) but not with the prophylactic antibiotics used (Spearman's correlation  $\rho = -0.419$ ,  $p = 0.136$ ) (figure 7B).

Among 119 tested viridans streptococci isolates, resistance to multiple antibiotics was common in both groups such as: azithromycin (95% in the antibiotic prophylaxis group vs 76% in the comparator group), tetracycline (74% vs 62%, respectively), ampicillin (65% vs 76%, respectively), cefotaxime (61% vs 54%, respectively), levofloxacin (48% vs 32%, respectively) and vancomycin (29% vs 6%, respectively). Nevertheless, resistance was



**Figure 8** (A) Comparison between the relative abundance of the total antimicrobial resistance (AMR) genes (measured in ppm sequence reads) in sputum samples from patients with bronchiectasis (BTX) on antibiotic (AB) prophylaxis (blue,  $n = 8$ ) and other patients with BTX having frequent exacerbations treated with AB rescue packs but not receiving AB prophylaxis therapy (yellow,  $n = 9$ ) ( $p = 0.48$  by Mann–Whitney). (B) The prevalence of AMR genes in the patients on antibiotic prophylaxis and those not ( $*p < 0.05$  by MW). AG, aminoglycosides resistance; FQ, fluoroquinolones resistance; Rf, rifamycin resistance; Rp, ribosomal protection proteins; Sulph/trimeth, sulfonamides/trimethoprim.

significantly more frequently detected in the antibiotic prophylaxis group compared with the comparator group ( $p < 0.0001$ ) (figure 7A).

In the resistome analysis, no significant differences in the prevalence of AMR genes were found in the sputum samples of patients with bronchiectasis who were on prophylactic antibiotics (seven were on 250 mg azithromycin thrice weekly and one on ciprofloxacin 250 mg twice daily) compared with those who were not ( $n = 9$ ) ( $p = 0.48$ ). Although not statistically significant due to the small sample sizes, a trend was observed in which the patients who were not receiving antibiotic prophylaxis therapy but had frequent exacerbations, median frequency of exacerbations/year 4 (IQR 2–5), exhibited a broader range of AMR gene prevalence: the median (IQR) was 21 ppm (6–121 ppm,  $n = 9$ ) compared with those on prophylactic antibiotics which was 28 ppm (IQR 14–43 ppm,  $n = 8$ ) (figure 8A). Also, a greater diversity of AMR genes conferring resistance to multiple antibiotic classes was observed in the frequent exacerbator patients using rescue packs and not on prophylactic antibiotics (figure 8B).

## DISCUSSION

Antibiotic prophylaxis is a common approach in the management of chronic lung diseases including COPD



and bronchiectasis, especially in advanced cases with frequent and/or severe exacerbations. Here, we report the impact of prolonged antibiotic prophylaxis therapy on the airway microbiome within a cohort of 84 patients with chronic respiratory conditions: COPD and/or bronchiectasis, 47 received prophylactic antibiotics prescribed for at least 1 year prior to joining the study as part of their routine clinical care. We also relate AMR in sputum-resistome and sputum-derived bacterial isolates to antibiotics: prophylactic and acute 'rescue' prescription.

Antibiotic prophylaxis was associated with lower  $\alpha$ -diversity of the airway bacterial community, as indicated by the PD whole tree (a quantitative measure of the phylogenetic diversity within an ecosystem)<sup>26</sup> and decline in the total bacterial burden in sputum. Previous studies have also reported a decline in richness and  $\alpha$ -diversity in bronchoalveolar lavage (BAL) samples from patients with COPD and moderate-to-severe asthma who were receiving azithromycin prophylaxis therapy.<sup>27 28</sup>

The microbiome profiles at the phylum level were similar between those prescribed and not prescribed prophylactic antibiotics. *Synergistetes* (a minor phylum) was the only phylum significantly less abundant in the antibiotic prophylaxis group. *Synergistetes* is a recently recognised bacterial phylum which has been detected in various body microbiomes such as the oral cavity, gut, umbilicus and vagina. Since its presence has been associated with disease in sites such as periodontitis, abscesses and cysts, *Synergistetes* are best considered opportunistic pathogens.<sup>29</sup>

There were no significant differences in the relative abundances of any of the genera that would normally be defined as components of the healthy microbiota, between the patients receiving antibiotic prophylaxis and those who did not. Similarly, Rogers *et al* reported that there were no significant differences in microbiota composition between erythromycin treatment and placebo arms in the bronchiectasis and low-dose erythromycin study (BLESS).<sup>30</sup>

Some pathogenic genera such as *Pseudomonas*, Enterobacteriaceae, *Klebsiella*, *Pasteurella* and *Morganella* were significantly less abundant in the patients receiving antibiotic prophylaxis compared with those who did not. Sequencing data suggested that *Moraxella* was also less abundant within the antibiotic prophylaxis group, although statistical significance was not achieved. Nevertheless, this observation was confirmed by specific qPCR ( $p=0.039$ ).

qPCR is more sensitive and specific compared with 16S rRNA sequencing, data on the sensitivity and specificity of the methods used is presented in the online supplemental data, online supplemental figure S7, online supplemental tables S2 and S3. Apart from *S. pneumoniae* which could not be distinguished by V3-V4 16S rRNA sequencing from the viridans streptococci that are abundant in sputum, a strong significant correlation between the qPCR results of *H. influenzae* (Spearman's  $\rho=0.798$   $p=8.4E-39$ ) and *M. catarrhalis* ( $\rho=0.621$ ,  $p=3.2E-15$ ) and

the corresponding taxa in the sequencing results of V3-V4 variable regions of 16S rRNA gene was found (online supplemental data, online supplemental figure S8). This correlation was less for *P. aeruginosa* ( $\rho=0.238$   $p=2.9E-4$ ). Although *P. aeruginosa* was more frequently detected by qPCR in the antibiotic prophylaxis group, as a significantly higher proportion of CVID patients were in this study group and *P. aeruginosa* was significantly more prevalent in the patients with CVID (68%,  $p=0.01$ ) compared with the rest of the cohort, the load of *P. aeruginosa* was in fact significantly lower in patients receiving prophylactic antibiotics ( $p=0.001$ ). The same trend was observed after excluding the patients with CVID, although the sample size in this case was too small to assess true statistical significance (online supplemental figure S9).

The data from molecular methods were reflected in the bacterial culture results where pathogenic bacteria were isolated significantly less frequently from patients' sputum using prophylactic antibiotic therapy compared with the other participants. This is in line with previous clinical trials which evaluated the efficacy of antibiotic prophylaxis therapy in various chronic lung diseases, and reported that respiratory colonisation with typical respiratory bacterial pathogens was either eliminated or inhibited following introduction of the prophylactic antibiotic.<sup>31-33</sup> It is noteworthy that microbiology was a secondary outcome in these trials and the results in most cases were based on culture-dependent techniques only, which are less holistic compared with the microbiome approach and can be insensitive when detecting bacteria present at low loads.

This finding may explain how prophylactic antibiotics reduce the risk of exacerbations in chronic lung disease, where the mechanism would be long-term suppression of pathogenic bacteria within the airway microbiome rather than 'snapshot' elimination of pathogens at acute exacerbation events. This would support the use of antibiotic prophylaxis in the management of chronic lung disease.

Most participants (62%) in the antibiotic prophylaxis group in our cohort were on an intermittent azithromycin regimen (thrice weekly). Macrolides are reported to have additional anti-inflammatory and immunomodulatory properties<sup>34</sup>; therefore, the mechanism of action may not be mediated solely through antimicrobial activity. For example, macrolides have poor antibacterial activity against *P. aeruginosa*, but azithromycin was found to inhibit its biofilm formation by interfering with essential quorum sensing pathways.<sup>35 36</sup> Segal *et al* have demonstrated that azithromycin could modulate the metabolome of the microbiome which in turn mediated the anti-inflammatory and immunomodulatory activity in the BAL samples of patients with COPD receiving azithromycin prophylaxis therapy.<sup>27</sup>

Surveillance of AMR revealed a high frequency of resistance across the whole cohort; however, it was significantly greater in the antibiotic prophylaxis group. For example, azithromycin resistance was detected in 95% of the viridans streptococci isolates in the antibiotic prophylaxis



group vs 75% in the comparator group. Previous studies confirmed that the oropharyngeal carriage of macrolide resistant viridans streptococci is common in general populations; it was estimated as 71% in a Belgian cohort of healthy adults and 94% in another Spanish cohort. In the Belgian study, co-resistance to tetracycline was identified in 73% of the isolates.<sup>37 38</sup>

The resistome analysis revealed similar trends in which AMR genes were slightly richer (in terms of frequency of detection) in the samples from patients with bronchiectasis on prophylactic antibiotics, however, the broadest diversity of AMR genes was observed in those patients who had frequent exacerbations events treated with antibiotic rescue packs but were not receiving antibiotic prophylaxis. Nevertheless, due to the small sample sizes, the study lacked the power to demonstrate that any of the observed trends were statistically significant. The acquired and/or inherent resistance of the microbiota might explain the resilience of the airway microbial community in response to antimicrobial treatment.

The relationship between the development of AMR and use of antibiotic prophylaxis is unclear within the current literature. Many studies report a significant rise in the acquisition of AMR, or a significant elevation in the minimum inhibitory concentrations of clinical isolates, in response to antibiotic prophylaxis.<sup>31 39 40</sup> The viridans streptococci develop resistance rapidly, especially to macrolides and tetracyclines.<sup>41 42</sup> Whole genome sequencing of commensal streptococci in one study revealed that macrolide transmissible resistance genes were carried with tetracycline-resistance determinants on transposable elements.<sup>42</sup> Commensal streptococci were also reported to carry the same macrolide resistance genes as pathogenic streptococci.<sup>43</sup> Therefore, microbiota may be regarded as a reservoir for AMR in the microbial ecosystem.

On the other hand, some studies reported no significant differences in the detection rates of the antibiotic resistance between patients receiving antibiotic prophylaxis therapy and those who did not.<sup>32 44</sup> Other studies have found that the resistance acquired during the antibiotic prophylaxis therapy was temporary, this suggested that acquired resistance is at the cost of fitness and does not persist.<sup>45 46</sup>

In current clinical practice, courses of antibiotics are often prescribed in 'rescue packs' kept by patients to initiate treatment of acute exacerbations promptly. The decision on antibiotic choice is based on clinician preference and may consider the individual's clinical condition, drug tolerance and allergies, previous sputum bacteriology culture results and the antimicrobial susceptibility profile (antibiogram) of previous sputum pathogenic bacterial isolates. Nevertheless, the benefit of antibiotics in the treatment of acute exacerbation, especially in COPD, remains controversial, and the evidence supporting the universal treatment of exacerbation with antibiotics is limited.<sup>2</sup> In our results, AMR detected in sputum isolates was more closely related to antibiotic rescue packs rather

than to prophylactic antibiotics. Therefore, the practice of prescribing antibiotic rescue packs might be more concerning in this sector rather than antibiotic prophylaxis therapy, especially in patients who suffer frequent exacerbations. This may justify the benefit of antibiotic prophylaxis in these patients. Patients' education on the rational use of antibiotics during exacerbations is crucial (such as having clear criteria when to start and stop them). Also, rotating prescriptions between different classes of antibiotics may theoretically help to mitigate AMR in this sector, although the available options may be limited and our data cannot inform on this.

There are several limitations to our results. As this was an observational study, participants were not randomly assigned to receive prophylactic antibiotics, therefore, our results demonstrate association but cannot prove causation. The decision to place a patient on antibiotic prophylaxis therapy was a clinical decision taken by the treating clinicians prior to joining the study. Antimicrobial prescription behaviour can be subjective and clinicians have different thresholds for prescribing antibiotic prophylaxis, although in most cases this decision is reserved for severe cases. This may explain why the frequency of exacerbations was comparable in both study groups despite the evidence that antibiotic prophylaxis therapy reduces the rate of exacerbations. Our cohort included patients with COPD, bronchiectasis and 45% of participants had an underlying diagnosis of CVID (the majority of whom were receiving immunoglobulin replacement). A relatively higher proportion of participants had bronchiectasis compared with COPD in the whole cohort; nevertheless, similar proportions of patients with bronchiectasis and COPD were present in both study groups. Apart from azithromycin, the small number of participants in the other six antibiotic prophylaxis regimes did not allow for comparisons between different antibiotics. The residual antimicrobial activity in the samples could have biased the bacteriology cultures in the antibiotic prophylaxis group but the molecular methods confirmed the findings of the culture-based approach. All these sources of variability might have masked some significant trends in the data.

In conclusion, antibiotic prophylaxis therapy was associated with reduced phylogenetic  $\alpha$ -diversity of the bacterial communities and lower bacterial density in sputum. It selectively suppressed specific taxa that represent bacterial respiratory pathogens without disrupting the homeostasis of the respiratory microbiota. It did not induce a definitive compositional shift in the airway microbiota composition at the cohort level. In general, macrolide resistance was high in the whole cohort, nevertheless, it was significantly greater in patients receiving antibiotic prophylaxis. The practice of antibiotic rescue packs may be driving AMR in this cohort since the detected AMR expressed by sputum bacterial isolates was associated with prescribed antibiotics in the rescue packs kept by patients for self-treatment of exacerbations. Therefore, the clinical decision of antibiotic prophylaxis in the

management of chronic lung disease should be carefully considered on an individual basis by weighing the benefits of suppressing pathogenic bacteria and reducing the rate of exacerbations, and hence need for acute antibiotic treatment courses, against the risk of enriching resistance to the prescribed antibiotic among bacterial populations and the considerable adverse effects that can result from the long-term use of these antimicrobial chemotherapeutic agents.

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