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**Airway ‘Resistotypes’ and Clinical Outcomes in Bronchiectasis**

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AT A GLANCE COMMENTARY

Scientific knowledge on the subject: The microbiome in bronchiectasis demonstrates geographic variability, correlates clinically with disease progression and outcomes, and incorporates a distinct repertoire of antimicrobial resistance genes known as the resistome. The clinical and therapeutic implications of the resistome in bronchiectasis remain unclear.

What this study adds to the field: We present the largest study of the bronchiectasis resistome to date, including patients from five countries in clinically ‘matched’ cohorts of Asian and European origin. We demonstrate the relevance of the resistome to clinical outcomes in bronchiectasis and outline novel ‘resistotypes’ with therapeutic implications. Targeted eradication of specific airway microbes that exhibit broad resistance genes profiles mediate reversion to more clinically favourable resistotypes suggesting that bronchiectasis resistomes can be modified for therapeutic benefit.

This article has an online data supplement, which is accessible from this issue’s table of content online at www.atsjournals.org.
ABSTRACT

Introduction: Application of whole-genome shotgun metagenomics to the airway microbiome in bronchiectasis highlights a diverse pool of antimicrobial resistance genes: the 'resistome', the clinical significance of which remains unclear.

Methods: Individuals with bronchiectasis were prospectively recruited into cross-sectional and longitudinal cohorts (n=280) including the international multicentre cross-sectional Cohort of Asian and Matched European Bronchiectasis 2 study (CAMEB 2; n=251) and two independent cohorts, one describing patients experiencing acute exacerbation and a further cohort of patients undergoing P. aeruginosa eradication treatment. Sputum was subjected to metagenomic sequencing and the bronchiectasis resistome evaluated in association with clinical outcomes and underlying host microbiomes.

Results: The bronchiectasis resistome features a unique resistance gene profile and elevated counts of aminoglycoside, bicyclomycin, phenicol, triclosan and multi-drug resistance genes. Longitudinally, it exhibits within-patient stability over time and during exacerbations despite between-patient heterogeneity. Proportional differences in baseline resistome profiles including increased macrolide and multi-drug resistance genes associate with shorter intervals to next exacerbation, while distinct resistome archetypes associate with frequent exacerbations, poorer lung function, geographic origin, and the host microbiome. Unsupervised analysis of resistome profiles identified two clinically relevant ‘resistotypes’ RT1 and RT2, the latter characterized by poor clinical outcomes, increased multi-drug resistance and P. aeruginosa. Successful targeted eradication in P. aeruginosa-colonized individuals mediated reversion from RT2 to RT1, a more clinically favourable resistome profile demonstrating reduced resistance gene diversity.
**Conclusion:** The bronchiectasis resistome associates with clinical outcomes, geographic origin, and the underlying host microbiome. Bronchiectasis ‘resistotypes’ link to clinical disease and are modifiable through targeted antimicrobial therapy.

**Key words:** Bronchiectasis; microbiome; metagenomics; resistome; resistotype.
INTRODUCTION

Emerging work on the lung microbiome underscores its centrality to infection, inflammation, and disease progression in bronchiectasis (1, 2). Principle to this are the functional capabilities of its constituent microbial taxa, including the expression of antimicrobial resistance (AMR) (3, 4).

Antibiotic use in bronchiectasis, while important, has lacked the anticipated impact for this ‘infective’ condition suggesting a more complex, dynamic and evolving understanding of pathogenesis, advanced by the ‘vicious vortex’ hypothesis (1). Mechanisms through which antibiotics function in bronchiectasis, going beyond microbial killing, remain unclear, reflected by the repeated failure of several multi-centre antibiotic-focused clinical trials (5-9). Considering other influences such as geographic heterogeneity, airway inflammation and underlying AMR will allow an improved understanding of antibiotics in bronchiectasis leading to better clinical success (10-12). Current clinical practice relies heavily on the detection of dominant culturable pathogens, which presents a limited perspective (2, 13).

The airway microbiome, characterised by diverse antimicrobial resistance genes (ARGs), exhibits variability across chronic respiratory disease states including bronchiectasis (10, 14-16). Innovations in sequencing technologies and bioinformatics have accelerated the functional appraisal of the microbiome, including its resistome (4, 10). The chronicity of bronchiectasis coupled to its repeated cycles of recurrent infection and antimicrobial therapy, likely have important implications on the airway resistome: one that defines the survival and colonization capabilities of individual microorganisms within a host microbiome. Higher levels of AMR favour overgrowth of drug-resistant microorganisms, potentially shaping microbiome composition, function, and disease outcome (14-16). Following antibiotics, microbial alteration ensues leading to changes in microbial burden, gene expression and/or metabolite production (17, 18).
Despite established links between the bronchiectasis microbiome and clinical outcomes, the role and influence of the resistome remains unclear. Here, we report the largest study of the bronchiectasis resistome to date, encompassing both cross-sectional and longitudinally sampled cohorts. We present assessment of stable patients from five countries in clinically ‘matched’ cohorts of Asian and European origin allowing assessment of clinical association with respect to geographic variability while carefully assessing resistome stability in response to alternative antimicrobial treatment regimes in serially sampled subjects. We describe the clinical correlates of the resistome and outline novel ‘resistotypes’ with therapeutic implications. Some of the results of these studies have been previously reported in the form of abstracts (19-21).

**METHODS**

**Study populations.** The study was designed to investigate the variability of the resistome and its clinical implications in bronchiectasis. To this end, four clinical cohorts were recruited to this study, each receiving appropriate ethical approval (see supplementary methods). All recruited individuals provided written informed consent for participation. The four cohorts are summarized as follows:

(1) Non-diseased (comparator) cohort: to provide a baseline comparator of the ‘healthy’ resistome in non-diseased individuals for reference, n=25 never smoking individuals with no history of respiratory or other known medical diseases and with normal spirometry were recruited in Singapore with clinical data and (induced) sputum sampling performed.

(2) Cross-sectional Cohort of Asian and Matched European Bronchiectasis 2 (CAMEB2): to comprehensively assess the variability and clinical correlates of the airway resistome in bronchiectasis, two-hundred and fifty-one patients with bronchiectasis (confirmed in
accordance with British Thoracic Society guidelines (22)) were prospectively recruited across four countries (Singapore, Malaysia, Scotland and Italy) forming part of the cross-sectional CAMEB2 study; a follow-up cohort from the original CAMEB study (23). Here, n=130 individuals with stable bronchiectasis were recruited, with complete clinical data collation and sputum sampling, across four Asian sites: three in Singapore (i.e. Singapore General Hospital, Changi General Hospital and Tan Tock Seng Hospital) and one in Malaysia (University of Malaya Medical Centre), a subset of which (n=97) were matched based on age, sex, exacerbation frequency, and lung function (as FEV$_1$ % predicted) to a European cohort (n=112) recruited at two sites: one in Scotland (i.e. Ninewells Hospital, Dundee, UK) and one in Italy (Foundazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico, Milan) (Table 1). Patients were recruited during periods of clinical stability, defined as the absence of new symptoms, and/or change in bronchiectasis therapy with no exacerbations and/or acute antibiotic use in the preceding four-week period. Exclusion criteria included other concurrent major respiratory diagnosis including asthma or COPD as established by international criteria including spirometry (24, 25). Individuals receiving chemotherapy and those with acute and/or recent infection requiring short-term antibiotic therapy (oral or intravenous) in the four weeks preceding outpatient attendance were excluded.

(3) Bronchiectasis exacerbation cohorts: to assess the stability of the airway resistome over time and in response to acute antimicrobial treatment, two independent bronchiectasis cohorts were recruited: one in Singapore that was followed during stability for between 3-7 months with repeated sputum sampling and a second from two sites in the east of Scotland (n=18) sampled before, during and post-exacerbation. For the latter cohort, patients were enrolled during clinical stability and asked to provide a spontaneous sputum sample at baseline (pre-exacerbation sample) and then followed closely, with repeat sputum sampling
performed ≤24 hours after commencing antibiotic therapy (exacerbation sample), and then following 14 days of antibiotic treatment (post-exacerbation sample). The final post-exacerbation sample was obtained following cessation of antibiotic therapy on day 14 once clinical recovery was achieved and confirmed clinically. The presence of an exacerbation was defined in accordance with established consensus (26).

4) *Pseudomonas aeruginosa* eradication cohort: to quantify the effect of targeted antimicrobial eradication therapy on the resistome, individuals with bronchiectasis were recruited at the Bronchiectasis Outpatient Clinic of the 5th respiratory department of Sotiria Chest Hospital, Greece, with new *P. aeruginosa* infection defined by culture-based isolation from sputum for the first documented occasion. Patients were treated with 14 days of ciprofloxacin followed by sequential 3-month treatment with inhaled colistin in accordance with ERS eradication recommendations (27). Spontaneous sputum sampling was performed at baseline, before antimicrobial treatment, and after eradication. Baseline and follow up samples were assessed by both *P. aeruginosa*-specific qPCR and metagenomics as described in the online supplement.

A schematic figure summarising all cohorts and the overall study design can be found in the online supplement (Supplementary Figure E1) together with details on ethics approvals and clinical, radiological, and functional evaluation of all participants.

**Whole-genome shotgun analysis of the airway resistome.** Sputum DNA was extracted, and its integrity confirmed before undergoing shotgun sequencing on a HiSeq 2500 platform. Adapter and quality trimming was performed using Trimmomatic, with non-human reads identified via Bowtie2 alignment against the human genome (28, 29). For resistome profiling, non-host reads were analyzed for antimicrobial resistance genes (ARGs) using ShortBRED
against the CARD database to quantify ARG abundance (30, 31). We define 'resistome' as the ARG profile observed in each patient while 'resistotype' denotes specific clusters or groupings within the resistome, characterized by distinct resistance gene profiles, delineated by spectral clustering of Bray-Curtis distances, defined by maximal silhouette score. Further details on sputum collection, DNA extraction, metagenomics sequencing and additional downstream bioinformatic analysis are detailed in the online supplement. Negative controls for sequencing are illustrated in Supplementary Figure E2 and raw data have been uploaded to the National Center for Biotechnology Information (NCBI) sequence read archives under the project accession numbers PRJNA595703, PRJNA590225 and PRJNA982297.

Reviewer link: https://dataview-ncbi-nlm-nih.gov.elib.tcd.ie/object/PRJNA982297?reviewer=ftn4p37ipaoonf8nmp869tibs

**Statistical analysis.** Distributional differences in patient demographics were assessed using the Wilcoxon test (non-parametric) for continuous variables and the chi-squared test for categorical data (Table 1). A p-value of <0.05 was considered statistically significant for these tests. For group comparisons of differential resistance gene categories (normalised by relative abundance), the Kruskal-Wallis test was employed to assess non-diseased and bronchiectasis subjects as well as across clinical subgroups of the CAMEB2 cohort, with statistical significance set at an FDR-adjusted p-value of <0.05. Antimicrobial resistome, microbiome and virome relative abundance profiles were visualised by stacked bar plots or Principal Coordinates Analysis (PCoA) plots of Bray-Curtis dissimilarity. Permutational Multivariate Analysis of Variance (PERMANOVA) on derived patient-dissimilarity matrices was employed across relative groups in cross-sectional analysis. Geographic variability in the CAMEB2 cohort was assessed by PERMANOVA adjusting for major geographic covariates (identified...
in Table 1) with a significance threshold of p<0.05 used to assess the overall effect of geography on the resistome. In longitudinal analyses, paired-PERMANOVA was employed to account for within patient variability across repeated measures to assess for temporal variation in response to antimicrobial treatment, also using a p-value of < 0.05. Spectral clustering was performed on Bray-Curtis distance between patient ARG profiles with the stability and robustness of the clusters evaluated by maximizing average silhouette scores (32). Differential abundance of resistance genes was assessed using Linear discriminant analysis Effect Size (LefSe) with default parameters, where a threshold for significance was set at an LDA score (log10) of 2.0 or higher (33). Cramer's V and the contingency coefficient were calculated as measures of the strength of association between nominal clinical variables, with a significance level of p < 0.05. All statistical analysis was performed using custom scripts written in R. A repository describing all processed datasets and analytical codes to reproduce results described in this manuscript, (including R package version numbers) is accessible at the following link: https://github.com/RespiratoryMicrobiome/BE-Resistome.

RESULTS

In our initial comparison of patients from CAMEB2 (n=251) with our non-diseased comparator group (un-matched, n=25), the bronchiectasis resistome was characterized by distinct ARG profiles. These encompassed a range of drug classes, notably including therapeutic antibiotics such as aminoglycosides, beta-lactams, fluoroquinolones, and tetracyclines, alongside natural products not primarily developed for therapeutic use, such as acridine dyes, antibacterial free fatty acids, bicyclomycin and triclosan. Resistome signatures in bronchiectasis exhibit marked increases in the relative abundance of multidrug and aminoglycoside resistance genes as well
as genes encoding for resistance to phenicol, triclosan, and peptide antibiotics of lesser direct therapeutic relevance (Figure 1A-B; PERMANOVA p<0.001). When assessed longitudinally in repeated measures analyses either during stable disease (Figure 1C) or across exacerbations (Figure 1D-E), temporal within-patient resistome profiles demonstrate temporal stability in the bronchiectasis exacerbation cohort (n=21). In contrast, between-patient profiles differ significantly, and exhibit significant association with time to next exacerbation (Figure 1F and 1G; PERMANOVA p=0.026). Here, individuals experiencing shorter intervals between exacerbations demonstrate a distinct resistome profile (paired PERMANOVA, P < 0.05) and an apparent increasing trend related to the ratio of multidrug to macrolide resistance genes (Figure 1F-1G). Taken together, ‘within-patient’ resistome dynamics appears temporally stable while ‘between-patient’ variation exists and associates with shorter intervals between exacerbations in bronchiectasis in the longitudinal exacerbation cohort.

Having established resistome stability over time and during exacerbations, we next sought to explore the relationship between resistome profiles and key clinical features of bronchiectasis extant in the CAMEB2 cohort (Figure 2). By assessing the abundance of resistance genes (grouped by drug class), we identified significant associations between several resistance gene classes and exacerbation frequency, lung function and underlying bronchiectasis aetiology in the cross-sectional CAMEB2 cohort (Kruskal-Wallis, p < 0.05, FDR-corrected). However, importantly, no association with bronchiectasis severity, using the bronchiectasis severity index, was observed (Figure 2). Variability in resistome profiles was most evident at phenotypic extremities where frequent exacerbators (i.e. those experiencing >=3 exacerbations in the preceding year) exhibit significant differences in aminoglycoside, bicyclomycin, phenicol and triclosan resistance (Figure 2A). Individuals with poorest lung function (i.e. FEV1 % predicted <50%) exhibit increased bicyclomycin, triclosan, peptide, sulphonamide and
multi-drug resistance (Figure 2B) while interestingly, underlying aetiology associated with an altered resistome, largely conferred by β-lactam and fosfomycin resistance in those with post-tuberculosis bronchiectasis (Figure 2D).

Having determined that resistomes show association with exacerbation frequency, poorer lung function and underlying aetiology in bronchiectasis, we next sought to interrogate the relationship with aetiology as this is an established contributor to clinical and geographic heterogeneity observed in large bronchiectasis registry studies (34, 35). To achieve this, we leveraged the matched design of the prospectively recruited CAMEB2 cohort which includes patients from Asia (Singapore and Kuala Lumpur, Malaysia) with matching by age, sex, exacerbation frequency and lung function to patients recruited from two European sites (Dundee, Scotland and Milan, Italy) (n=209, Figure 3A, Table 1). Importantly, the CAMEB2 cohort differs from the original CAMEB cohort that matched patients on age, sex and disease severity, the latter factor was shown not to be related to differences in resistome profiles (Figure 2C) (23, 32, 36, 37). Therefore, by design, CAMEB2 allowed matching of patients based on exacerbation frequency and lung function, both independently related to resistome profiles (c.f. Figure 2A-B) across geographic regions allowing for a deeper exploration of true associations between aetiology and resistomes (Figure 3A). Within the CAMEB2 cohort significant geographic covariates were observed including median BMI, aetiology, radiological severity, hospital admissions and colonization with organisms other than *Pseudomonas*. Additionally, inhaled corticosteroid use, and long-term antibiotic use showed significant differences, being higher in the DD-MI group (Table 1). Bronchiectasis aetiology and geographic patient origin were tightly linked (38, 39), and notably aetiology strongly correlated to geographic patient origin, representing an important covariate ($\chi^2 = 42.6$, df = 9, $p < 0.001$) with moderate association indicated by Cramer's V ($V = 0.29$) and contingency coefficients ($C = 0.449$) in
matched subjects of CAMEB2. Assessment of bronchiectasis resistomes, at the level of individual genes, demonstrated a highly significant effect for geographic patient origin (p=0.006), one that remained even after accounting for aetiology (Figure 3B). PERMANOVA also confirmed 'BMI' (p=0.040), and aetiology (p=0.020) as significant predictors. These results indicate that geographic differences, alongside individual health characteristics play a significant role in the variability of antimicrobial resistance genes. Despite geographic variability to therapy, the resistome was not significantly different in users of inhaled corticosteroid and/or long-term macrolides (Supplementary Figure E3).

Interestingly, while Singapore and Kuala Lumpur appear to have overlapping resistome profiles, Dundee, and Milan, both in Europe, appeared different, in line with the reported variation in bronchiectasis across Europe through the EMBARC registry (Figure 3B) (35). In contrast, aetiological association with resistance gene profiles failed to reach statistical significance in PERMANOVA analysis of matched patients suggesting that resistomes relate most strongly to a patient’s country of origin rather than underlying aetiology (Figure 3C). Reported ancestries are related to country of origin and varied within Singapore (76% Chinese) and Kuala Lumpur (68% Malay) relative to Scotland and Italy, both with 100% European ancestry. We failed to detect any significant differences in resistome composition associated with within-region ethnic diversity (p= 0.636). Resolution to individual ARGs reveals that individuals recruited to CAMEB2 from Europe could be distinguished by the presence of specific multidrug and fluoroquinolone resistance genes *hmrM* and *patA* and the tetracycline resistance gene *tetO* (Supplementary Figure E4). Conversely, Asians in CAMEB2 exhibit an increased prevalence of broad-spectrum multidrug efflux systems as well as the macrolide resistance gene *ErmF*, aminoglycoside resistance genes (*APH(3”)-Ib* and *APH(3”)-Ib*) and both β-lactam (*CfxA2*) and fluoroquinolone (*oqxAB*) resistance mechanisms (Supplementary Figure
E4), reflecting a diversity of resistance potential related to geographic origin. Lung microbiomes are intricately linked to resistomes as resident microbes express ARGs that contribute to the resistome (10). Consequently, lung microbiomes from CAMEB2 exhibit significant geographic variability, based on country of origin (Supplementary Figure E5A-B) in line with comparable findings from resistome analysis (Figure 3B), while no such relationship was evident comparing aetiologies (Supplementary Figure E5C-D). Most noticeably, individuals from Asia exhibit a higher relative abundance of *Klebsiella pneumoniae* while Europeans exhibit marked increases in *Haemophilus influenzae* (Supplementary Figure E5A-B). Finally, we applied a metagenomic pipeline for the analysis of viral content (i.e. DNA viruses) (see online supplement and Supplementary Figure E6) to evaluate for bacteriophages that may be contributing to our findings. Bacteriophages belonging to the families *Iridoviridae*, *Siphoviridae*, *Myoviridae*, *Phycondnaviridae* and *Polydnaviridae* are detectable in bronchiectasis, and include several *Streptococcal*, *Pseudomonal* and *Staphylococcal* phages however, when considered independently, bacteriophage profiles did not demonstrate any significant or direct relationships with clinical outcomes of interest, however, they did vary based on geographic patient origin in line with our prior findings in Supplementary Figure E7.

Since the bronchiectasis resistome likely contains signatures amenable to improve patient risk stratification, we next performed an unsupervised analysis of resistome profiles observed in the CAMEB2 cohort leading to the identification of two distinct ‘resistotype’ clusters, RT1 and RT2 (Figure 4A-B), delineated by spectral clustering of Bray-Curtis distances, with a mean misclassification ratio of 3.4%, indicating a cluster robustness of 96.6% (based on 100 bootstrap iterations). Comparable microbial read depths were observed across both clusters suggesting minimal influence of sequencing depth, despite a significant association with ARG diversity across patient samples. (Supplementary Figure E8). The RT1 cluster was broadly defined by an increased abundance of genes conferring resistance to tetracyclines, β-lactams
and macrolide-lincosamide-streptogramin B class (MLS) antibiotics. RT2 exhibits an increased relative abundance of aminoglycoside, phenicol and multi-drug resistance genes as well as genes conferring resistance to peptide, fosfomycin and bicyclomycin antibiotics (Figure 4A). Correlative gene-specific linear discriminant analysis (LEfSe) highlights fluoroquinolone, tetracycline, and MLS-specific resistance genes as characteristic of RT1 while RT2 is defined by aminoglycoside, phenicol, bicyclomycin and most strikingly multi-drug resistance genes (Figure 4C). Importantly, the identified resistotypes significantly associate with clinical outcomes in bronchiectasis, where RT2 associates with increased exacerbations, poorer lung function and greater disease severity (Figure 4D-F). Interestingly, resistotypes were not evenly distributed across recruitment sites with Dundee exhibiting the lowest proportion of RT2 patients compared to Singapore, Kuala-Lumpur, and Milan respectively, corroborating our previously described geographic differences (Figure 4G). Genes encoding resistance to antimicrobials such as triclosan, phenicol, and certain peptide classes, which are not widely used in the clinical settings, suggests that environmental factors and potentially indirect selection pressures may play a role in shaping resistomes.

We next evaluated microbiomes associated with the observed resistotypes where RT1 is characterized by an increased abundance of *H. influenzae*, *Rothia mucilaginosa*, and several *Streptococcal* species while RT2 is distinguished by a predominance of *P. aeruginosa* and *K. pneumoniae* (Figure 5A-C). The observed relationship between *P. aeruginosa* and *H. influenzae* is further supported by quantitative data confirming increased ratios of *P. aeruginosa* to *H. influenzae* microbial burden within RT2, determined by confirmatory qPCR analysis, further underscoring resistome-bacteriome associations (Supplementary Figure E9). Apparent exceptions were noted such as *H. influenzae* dominance in some RT2 and *P. aeruginosa* dominance in some RT1 patients by qPCR, illustrating a further level of complexity to interpreting microbe-resistome dynamics (Supplementary Figure E9). In addition, n=10
patients in the RT1 cluster exhibit *P. aeruginosa* positivity by microbial culture. To disentangle the potential effects of *P. aeruginosa* and resistomes on the observed disease outcomes associated with RT2, we performed sub-analyses excluding all *P. aeruginosa*-positive patients (14%). Results were consistent with our initial analysis (Figure 4), except for exacerbations that trended toward significance (Supplementary Figure E10).

In addition to short-read taxonomic classification (through ShortBRED and Kaiju classifiers (31, 40)) we additionally performed metagenomic contig assembly to define the genetic context of the identified resistance genes and further elucidate taxonomic associations. This allowed us to describe the number of resistance genes associated with each major bacterial taxa in bronchiectasis as well as the distribution of the most prevalent genes across these bacteria (Figure 5D-E). Several major bacterial taxa exhibit a high abundance of resistance genes including *P. aeruginosa*, *K. pneumoniae*, *Serratia marcescens* and *H. influenzae*. Highly notable were the >50 mapped resistance genes in *P. aeruginosa* and *K. pneumoniae* while other less abundant species include *Escherichia coli*, *Stenotrophomonas maltophilia* and *Staphylococcus aureus* that all exhibit extensive resistance gene repertoires (Figure 5D). Commensals including *Streptococcus*, *Neisseria*, *Veilonella*, *Rothia* and *Prevotella* were all shown to be important carriers of antimicrobial resistance in bronchiectasis (Figure 5D). Among the most widely distributed resistance genes were the fluoroquinolone resistance genes *patA* and *patB*, observed in association with up to 15 and 29 distinct bacterial species respectively, and both associated with RT1. Other resistance genes associated with RT1 (i.e. *patA*, *patB*, *tetQ*, *tet60(A)*, *tetW*, *rlmA(II)*, *tet46(B)* and *pmrA*, c.f. Figure 4C) were among the most widely distributed across bacterial taxa (present in >=10 species) while those associated with RT2 were overall less widely distributed with the exception of *bcr-1*, *pmpM* and *mexB* respectively (Figure 5E). Altered microbial interaction metrics were observed in the RT2
cluster that exhibited overall increased interactions (142 vs 70). Notably, the observed interaction network implicates *P. aeruginosa* and *K. pneumoniae* (among others, including *R. mucilaginosa*) as keystone microbes of this resistotype. Corresponding decreases in several commensal taxa including *Streptococci*, *Fusobacteria* and *Veillonellaceae* were observed, all of which represent important nodes for the RT1 interaction network (Supplementary Figure E11).

Having determined the clinical relevance of bronchiectasis resistotypes, we next assessed if targeted therapeutic intervention with antibiotics can influence the resistome and its underlying resistotype. To investigate this, we prospectively studied *n=8* culture-positive *P. aeruginosa* infected individuals undergoing protocol-driven *P. aeruginosa* eradication involving an initial two-week treatment with oral ciprofloxacin followed by nebulized colistin for three consecutive months (longitudinal bronchiectasis eradication cohort). Sampling for analysis was obtained at baseline (pre-eradication) and then at 6-months post-eradication therapy (*n=8* patients with 16 samples). Successful eradication of *P. aeruginosa* was confirmed by qPCR verifying eradication in 5 of 8 patients, and a reduction in *P. aeruginosa* burden in all but a single patient (Supplementary Figure E12). This was accompanied by a reduction in the relative abundance of *P. aeruginosa* by metagenomic analysis from 32% (IQR: 15-67%) pre-eradication to 1.7% (IQR:0-5%) post-eradication (Figure 6A). Concomitant increases in the relative abundance of *Haemophilus* spp., several *Streptococci* and *K. pneumoniae* (in a single patient) were apparent post-eradication (Figure 6A). These microbial changes were accompanied by appreciable shifts in resistome profiles where the ratio of multidrug to MLS resistance gene abundance decreased upon completion of eradication therapy (Figure 6B). Interestingly, resistome profiles observed in the pre- versus post-eradication therapy states bore remarkable resemblance to the RT2 and RT1 clusters respectively, as previously characterised.
(c.f. Figure 4A) with overrepresentation of multidrug resistance genes in the pre-eradication state (RT2-like) compared to a greater abundance of MLS and fluoroquinolone resistance genes in the post-eradication state (RT1-like). The average number of identified resistance genes was lower post-eradication therapy (9 versus 13) as was resistance gene diversity (average Shannon diversity index 1.6 versus 2.3). In contrast to acute antibiotic bursts to treat acute exacerbations (c.f. Figure 1D), these findings suggest that prolonged and targeted eradication therapy causes appreciable shifts in resistome profiles and can potentially mediate reversion from an unfavourable RT2- to RT1 resistotype (Figure 6C). This is substantiated by the significant shift in β-diversity observed following eradication therapy in contrast to patients treated for acute exacerbation (c.f. Figure 1D-E), where no significant difference is observed (Figure 6D).

**DISCUSSION**

The bronchiectasis microbiome exhibits resistance gene signatures, linked to underlying taxonomic composition, with important clinical and therapeutic implications (2). Discordance between in vitro antimicrobial sensitivity testing of individual pathogens and clinical efficacy and outcome in practice is well recognized in bronchiectasis, evidenced by the repeated failure of antimicrobial clinical trials (5-9). While many factors are advanced to explain such inconsistency, the resistome, a collection of all ARGs within the host microbiome offers new insight (4, 10, 32). Here, we perform the largest and most comprehensive analysis of the bronchiectasis resistome to date, including patients from five countries in clinically ‘matched’ cohorts of Asian and European origin to evaluate its clinical significance.

The bronchiectasis resistome is distinct to that within a healthy lung, a likely consequence of microbial dysbiosis related to recurrent cycles of exacerbation and antibiotic therapy (2).
Interestingly, within patient profiles remain stable over time and during exacerbations, suggesting that microbial dysbiosis, once established, remains, and therefore can be leveraged for prognosis, patient stratification and precision therapy (32, 41-43). Importantly, between patient resistome profiles markedly differ, allowing resistome signatures (at baseline) to associate with time to next exacerbation. Individuals demonstrating a shorter time to next exacerbation associate with variable relative abundance of macrolide and multi-drug resistance gene determinants, while an increased MLS to multidrug resistance ratio linked to more clinically favourable outcomes bearing similarity to resistomes of non-diseased (healthy) controls. These findings are consistent with the prior identification of a core macrolide resistome, even in healthy individuals (10, 44).

Exacerbation frequency associates with poor clinical outcomes and mortality in bronchiectasis and remains a key endpoint in clinical trials (45, 46). Links between the bronchiectasis microbiome and exacerbation frequency are established and supported by prior clinical studies (32, 42, 47-49). Here, we reveal that resistome profiles of frequent exacerbators have distinct profiles characterized by increased abundance of ARGs related to aminoglycoside, bicyclomycin, phenicol and triclosan resistance, a likely consequence of repeated and varied antimicrobial exposure over time. Whether such profiles can be leveraged for therapeutic decision-making in practice, especially in frequent exacerbators, is an important clinical and research question necessitating future prospective study. Notably, resistome profiles further associate with other demographic and clinical parameters in bronchiectasis including lung function and disease aetiology, however, for the latter, this relationship is driven by geographic patient origin, rather than aetiology, a finding uncovered by leveraging the CAMEB 2 cohort that ‘matched’ individuals across Asian and European sites by age, sex, exacerbation frequency and lung function. Using CAMEB 2 allowed detailed interrogation of the specific contributions
of disease aetiology and geographic patient origin to resistome dynamics by controlling for, through meticulous patient matching, for exacerbation frequency and lung function, two other determinants of resistome composition. Our design thus addresses the comparison of diverse cohorts, overcoming challenges noted in previous studies.(5, 7, 8)

Geographic variation remains a key determinant observed disease heterogeneity in bronchiectasis, which includes aetiology, but additionally other features such as microbiology and the microbiome (2, 11, 13, 38, 52-54). The recent European Multicentre Bronchiectasis Audit and Research Collaboration (EMBARC) report reveals that even across a single continent, Europe, that significant disease heterogeneity exists in microbiology, exacerbations, and treatment response (35). Reports from other regions, including the USA, Australia, India, and Korea, further affirm such inherent differences, which significantly contribute to the clinical variability and negative outcomes seen across clinical trials in bronchiectasis (5-9, 11, 34, 53, 55-57). Our microbiome results are consistent with the reports from EMBARC based on culture, specifically with a higher frequency of *H. influenzae* in Northern Europe, increased *P. aeruginosa* in Southern Europe, and higher *K. pneumoniae* in Asia all replicated here using next generation sequencing (58). The interplay between these factors is complex and varies globally, suggesting that a deeper understanding of these relationships is crucial in clinical practice and future trial design, particularly in disentangling the independent effects of the resistome.

We next sought to establish unbiased signals within the bronchiectasis resistome conferring clinical and biological relevance for translational application. Unsupervised analysis of AMR profiles revealed two bronchiectasis ‘resistotypes’ RT1 and RT2, each demonstrating a distinct resistance pattern and clinical correlates. RT1 is characterized by favourable clinical outcomes
and fluoroquinolone, tetracycline, and MLS resistance with microbiomes dominated by *H. influenzae*, *R. mucilaginosa* and several *Streptococcal* species. RT1 exhibits resistome profiles characteristic of those observed in our non-diseased comparator cohort despite significant demographic differences, including age. Indeed, existing literature suggests a relative stability of the airway microbiome across age, suggesting that this may not be the primary factor driving pronounced bronchiectasis resistome signatures such as those seen in RT2. The RT2 resistotype associates with more exacerbations, poorer lung function and greater disease severity. RT2 resistance profiles are typified by aminoglycoside, phenicol, bicyclomycin and most significantly multi-drug resistance with their corresponding microbiomes governed by a high abundance of *P. aeruginosa* and *K. pneumoniae*, both recognised organisms conferring high antimicrobial resistance and adverse clinical outcome in bronchiectasis (34, 59, 60).

Metagenomic assembly further confirmed *P. aeruginosa* as the top microbial taxa harbouring the greatest amount of resistance genes, consistent with its high abundance within the RT2 cluster. Conversely, resistance gene distribution across other bacterial taxa highlights that macrolide, fluoroquinolone, β-lactam and tetracycline resistance gene determinants, largely characteristic of RT1, are comparable to the previously described core airway resistome even seen in a non-disease (healthy) state (10). Importantly, *R. mucilaginosa* has protective anti-inflammatory properties, while *H. influenzae* and *Streptococci* link with improved bronchiectasis outcomes compared to *P. aeruginosa* reflecting the microbial influences on the resistome of the RT1 and RT2 clusters respectively (48, 61). It is assumed that antibiotic use would result in unfavourable changes to the resistome but our data suggests this is not universally true. Remarkably, targeted *P. aeruginosa* eradication in chronically colonized individuals promoted a measurable shift from an unfavourable RT2 to RT1 profile suggesting the therapeutic potential of manipulating microbiomes in a targeted and specific manner to alter underlying resistomes (13). In contrast to pathogen-directed antimicrobial targeting, the
BLESS trial underscores the importance of indirect, non-bactericidal effects related to macrolide therapy, given the success of these agents in patients colonised by *P. aeruginosa* – an intrinsically macrolide-resistant pathogen. This further highlights the complex interplay between antibiotic therapy, the microbiome and resistome whereby alternate mechanisms, such as anti-inflammatory effects and/or interference with bacterial quorum sensing, further contribute to therapeutic response (13, 47). In line with our reported findings, successful *P. aeruginosa* eradication promoted reversion from RT2 to an RT1 phenotype, highlighting distinct changes in microbiome and associated resistome profiles. Therefore, considering resistomes and subsequent targeted microbiome-directed therapy may prevent treatment failure, while acknowledging the broader impact of targeted therapies on the microbiome including non-antibiotic effects. Notably eradication therapy directed toward key pathogens like *P. aeruginosa* or *K. pneumoniae* may simultaneously reshape resistotypes, suggesting that bacterial load and resistance gene patterns are linked. This highlights that therapeutic strategies targeting specific pathogens can have broader implications on resistome dynamics, beyond simply altering taxonomic abundance. Finally, we noted that several patients harbouring *P. aeruginosa* in sputum culture exhibit RT1 resistotypes. This indicates that resistome profiles are not always defined by the presence of a single pathogen such as *P. aeruginosa* but may reflect a broader spectrum of complex microbial interactions and resistome dynamics.

While novel with several strengths, our work does have limitations. Firstly, while we report profiles of identified resistance genes in the airway, we do not make any inference about phenotypic resistance of the microbiome as this requires detailed functional *in vitro* experimentation. Although automated microbial genomic resistance prediction has been validated for specific pathogens (62), comprehensive standardization and validation of such predictions, pertaining to metagenomic analysis of the resistome, is yet to be achieved on comparable scales and this remains a challenge. While metagenomics has many advantages, it
does have limitations including the lack of detection for low abundance resistance genes, due to low coverage, which may in practice contribute to clinical resistance and disease outcomes. Related to this, our metagenomic assembly approach, while providing added novelty, is susceptible to ‘drop out’ of lower coverage genes and taxa that may be underrepresented. While our experimental approach clearly provides important insight into microbial origins of the resistome, the mobile nature of chromosomal resistance genes in association with mobile genetic elements must be considered and therefore detected microbe-gene associations cautiously interpreted (63). Our analysis did not include absolute quantification of resistance genes. Therefore, shifts in gene relative abundance may mask true levels of certain resistance genes (e.g., lower proportional representation of fluoroquinolone resistance genes in RT2), highlighting the interpretive limitations of metagenomic data when relying on relative abundance. Our short-read analytical pipeline for resistome characterisation is dependent on the CARD ARG database which while comprehensive and internationally recognized is not exhaustive and, capable of identifying only known resistance genes.(30) This approach may be further biased by taxa that are well described and annotated in terms of their ARGs, undoubtedly the case for *P. aeruginosa* in the RT2 resistotype. Furthermore, the CARD 'multi-drug' category is broad and includes genes that provide broad resistance to antibiotics including fluoroquinolones, even amid a lower detected abundance of typical fluoroquinolone resistance, as observed in the RT2 resistotype. Despite an appreciable trend for macrolide resistance (Figure E2C), we failed to detect a significant effect of inhaled corticosteroids or long-term macrolide use on the resistome. However, we caution that our study was observational and therefore not specifically designed to test for systematic effects of therapy, which requires further analysis. It is also challenging to discern causation in terms of whether resistotype clusters precede the onset of disease activity, such as exacerbation, or if patients with different disease endotypes undergo treatments that potentially lead to altered resistotypes. Geographic
variations in bronchiectasis treatment across study sites, including preferred antibiotics, thresholds for antibiotic use, and criteria for treatment based on culture results or symptoms alone, may contribute to observed resistome and microbiome heterogeneity. While a detailed analysis of these factors is beyond the scope of this study, we recognize them as potential limitations. In addition, our current knowledge of the airway ‘bacteriophagome’ remains limited and, although we did not identify clinical associations in this analysis, further work in this area may be warranted as greater airway metagenomic data becomes available. Finally, despite emerging evidence for the importance of a gut-lung axis in bronchiectasis, we did not investigate gut microbiomes as a potential correlate of disease or reservoir for resistance (64). Given the profound impact of antibiotics on the gut microbiome, the inclusion of gut microbiome samples in future studies is warranted.

In conclusion, we describe the largest and most comprehensive assessment of the resistome in bronchiectasis to date, including its clinical relevance and translational potential. Our identification of novel ‘resistotypes’ provides a fresh approach to targeting the microbiome for therapeutic benefit with potential application in patient care, antimicrobial stewardship, and clinical trial design.

ACKNOWLEDGEMENTS

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REFERENCES


Table 1. Demographic table summarizing the CAMEB2 bronchiectasis cohort.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Non-diseased (n=25)</th>
<th>Bronchiectasis (n=251)</th>
<th>Matched (n=209) SG-KL (n=97)</th>
<th>Matched (n=209) DD-MI (n=112)</th>
<th>p-value (SG-KL vs DD-MI)</th>
</tr>
</thead>
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<tr>
<td>Geographic origin: n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td>Singapore (SG)</td>
<td>25 (100%)</td>
<td>95 (38%)</td>
<td>62 (64%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Kuala Lumpur (KL)</td>
<td>0</td>
<td>35 (14%)</td>
<td>35 (36%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Dundee (DD)</td>
<td>0</td>
<td>96 (38%)</td>
<td>0 (0%)</td>
<td>88 (79%)</td>
<td></td>
</tr>
<tr>
<td>Milan (MI)</td>
<td>0</td>
<td>25 (10%)</td>
<td>0 (0%)</td>
<td>24 (21%)</td>
<td></td>
</tr>
<tr>
<td>Matching criteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age: median (IQR)</td>
<td>34 (30-39)</td>
<td>65 (59-73)</td>
<td>67 (59-74)</td>
<td>64 (59-72)</td>
<td>ns</td>
</tr>
<tr>
<td>Sex: n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ns</td>
</tr>
<tr>
<td>Male</td>
<td>14 (56%)</td>
<td>101 (40%)</td>
<td>38 (39%)</td>
<td>46 (41%)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>11 (44%)</td>
<td>150 (60%)</td>
<td>59 (61%)</td>
<td>66 (59%)</td>
<td></td>
</tr>
<tr>
<td>Exacerbator status: n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>0</td>
<td>-</td>
<td>85 (34%)</td>
<td>33 (34%)</td>
<td>34 (30%)</td>
<td></td>
</tr>
<tr>
<td>1 to 2</td>
<td>-</td>
<td>108 (43%)</td>
<td>41 (42%)</td>
<td>49 (44%)</td>
<td></td>
</tr>
<tr>
<td>3 or more</td>
<td>-</td>
<td>58 (23%)</td>
<td>23 (24%)</td>
<td>29 (26%)</td>
<td></td>
</tr>
<tr>
<td>FEV1 % predicted: median (IQR)</td>
<td>100 (96-109)</td>
<td>72 (56-87)</td>
<td>71 (56-84)</td>
<td>76 (61-94)</td>
<td>ns</td>
</tr>
<tr>
<td>Other disease characteristics (not matched)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disease severity: n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ns</td>
</tr>
<tr>
<td>Severe (BSI: 9+)</td>
<td>-</td>
<td>143 (57%)</td>
<td>62 (64%)</td>
<td>61 (54%)</td>
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</tr>
<tr>
<td>Moderate (BSI: 5-8)</td>
<td>-</td>
<td>74 (29.5%)</td>
<td>24 (25%)</td>
<td>33 (30%)</td>
<td></td>
</tr>
<tr>
<td>Mild (BSI 0-4)</td>
<td>-</td>
<td>34 (13.5%)</td>
<td>11 (11%)</td>
<td>18 (16%)</td>
<td></td>
</tr>
<tr>
<td>Aetiology n (%)</td>
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<td></td>
<td></td>
<td></td>
<td>0.001 **</td>
</tr>
<tr>
<td>Idiopathic</td>
<td>-</td>
<td>157 (62.5%)</td>
<td>70 (72.2%)</td>
<td>75 (67%)</td>
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</tr>
<tr>
<td>Post-infection</td>
<td>-</td>
<td>34 (13.5%)</td>
<td>10 (10.3%)</td>
<td>18 (16%)</td>
<td></td>
</tr>
<tr>
<td>Post-TB</td>
<td>-</td>
<td>13 (5%)</td>
<td>9 (9.3%)</td>
<td>0 (0%)</td>
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</tr>
<tr>
<td>Other</td>
<td>-</td>
<td>47 (19%)</td>
<td>8 (8.2%)</td>
<td>19 (17%)</td>
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</tr>
<tr>
<td>BMI (kg/m²): median (IQR)</td>
<td>22 (18-27)</td>
<td>19 (18-20)</td>
<td>27 (22-29)</td>
<td>&lt;0.001 ***</td>
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</tr>
<tr>
<td>MRC dyspnoea score: n (%)</td>
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<td></td>
<td></td>
<td></td>
<td>0.042 *</td>
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<td>1 to 3</td>
<td>-</td>
<td>228 (91.6%)</td>
<td>91 (96%)</td>
<td>96 (86%)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>19 (7.6%)</td>
<td>4 (4%)</td>
<td>14 (13%)</td>
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</tr>
<tr>
<td>5</td>
<td>-</td>
<td>2 (0.8%)</td>
<td>0 (0%)</td>
<td>2 (1%)</td>
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<tr>
<td>Radiological severity: n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.022 *</td>
</tr>
<tr>
<td>1-2 lobes involved</td>
<td>-</td>
<td>93 (38%)</td>
<td>28 (30%)</td>
<td>55 (49%)</td>
<td></td>
</tr>
<tr>
<td>&gt; 3 lobes involved</td>
<td>-</td>
<td>154 (62%)</td>
<td>65 (70%)</td>
<td>57 (51%)</td>
<td></td>
</tr>
<tr>
<td>Hospital admission in the year preceding study recruitment: n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001 ***</td>
</tr>
<tr>
<td>Yes</td>
<td>-</td>
<td>98 (39%)</td>
<td>50 (52%)</td>
<td>30 (27%)</td>
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</tr>
<tr>
<td>No</td>
<td>-</td>
<td>153 (61%)</td>
<td>47 (48%)</td>
<td>82 (73%)</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas colonisation: n (%)</td>
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<td></td>
<td></td>
<td>ns</td>
</tr>
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<td>-</td>
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<td>14 (14%)</td>
<td>16 (14%)</td>
<td></td>
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<tr>
<td>No</td>
<td>-</td>
<td>216 (86%)</td>
<td>83 (86%)</td>
<td>96 (86%)</td>
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</tr>
<tr>
<td>Colonisation with other organisms: n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001 ***</td>
</tr>
<tr>
<td>Yes</td>
<td>-</td>
<td>89 (36%)</td>
<td>37 (38%)</td>
<td>15 (13%)</td>
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<tr>
<td>No</td>
<td>-</td>
<td>162 (64%)</td>
<td>60 (62%)</td>
<td>97 (87%)</td>
<td></td>
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<td>Bronchodilator use</td>
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<td>-</td>
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<td>47 (48%)</td>
<td>57 (51%)</td>
<td></td>
</tr>
<tr>
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<td>-</td>
<td>132 (53%)</td>
<td>50 (52%)</td>
<td>55 (49%)</td>
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<tr>
<td>Inhaled corticosteroids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001 ***</td>
</tr>
<tr>
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<td>-</td>
<td>99 (39%)</td>
<td>18 (19%)</td>
<td>66 (59%)</td>
<td></td>
</tr>
<tr>
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<td>-</td>
<td>152 (61%)</td>
<td>79 (81%)</td>
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<tr>
<td>Long-term antibiotics</td>
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<td></td>
<td></td>
<td>0.044 *</td>
</tr>
<tr>
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<td>-</td>
<td>61 (24%)</td>
<td>14 (14%)</td>
<td>30 (27%)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>-</td>
<td>190 (76%)</td>
<td>83 (86%)</td>
<td>82 (73%)</td>
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</tbody>
</table>
Table 1: Demographic characteristics of the CAMEB2 bronchiectasis study population including non-diseased controls (n=25). Stable bronchiectasis patients were recruited as part of the Cross sectional Asian and Matched European Bronchiectasis 2 (CAMEB2) cohort including patients recruited from Singapore (SG, n=95), Kuala Lumpur (KL, n=35), Dundee (DD, n=96) and Milan (MI, n=25). A subset of Asian patients (from Singapore and Kuala Lumpur; SG-KL) were matched to Europeans (from Dundee and Milan; DD-MI) respectively based on age, sex, exacerbation frequency and lung function. Characteristics of the matched subset (n=209) are illustrated separately with corresponding p-values indicated for relevant comparisons (SG-KL vs DD-MI). Demographic data is presented as median ± interquartile range (IQR) and/or patient numbers (n) with accompanying percentages (%) as appropriate. BMI: Body Mass Index, mMRC: modified Medical Research Council dyspnoea scale, BSI: Bronchiectasis Severity Index. ns: non-significant. *p<0.05, ** p<0.01, *** p<0.001.
FIGURE LEGENDS

Figure 1. Characterization of the Bronchiectasis Resistome. (a) Stacked bar plots representing the observed resistome profiles in bronchiectasis (n=251, CAMEB2 cohort) compared to a non-diseased comparator cohort (n=25). Stacked bars represent the relative abundance of resistance genes grouped according to drug class. (b) Beta-diversity of resistance gene profiles (i.e. Bray-Curtis distance) assessed by Principal Co-ordinate Analysis (PCoA) illustrating resistome diversity and coloured by cohort: non-diseased (blue) and bronchiectasis (red) (PERMANOVA, p<0.001). (c) Longitudinal resistome dynamics in stable bronchiectasis assessed at baseline (BL) and follow-up (FUP) time points across varying time intervals (left; 3 months, centre; 6 months, right; 7 months). (d-e) Prospective longitudinal analysis of resistome profiles during exacerbations of bronchiectasis (n=18, cohort 3 – ‘exacerbation’ cohort) with sampling at baseline (pre-exacerbation) [B], during exacerbation [E] and two-weeks post-exacerbation following antibiotic treatment [P]. (f-g) Prospective longitudinal analysis of resistome profiles in the bronchiectasis exacerbation cohort grouped by observed time to next exacerbation as <12 weeks or >12 weeks respectively. PCoA of resistome (beta-) diversity (i.e. Bray-Curtis distance) comparing (e) time points across an exacerbation and (g) time to next exacerbation are illustrated below their respective stacked bar plots (d and f). p-values for PERMANOVA analysis comparing groups are illustrated. Central points intersected by a cross indicate the centroid in PCoA visualizations.
**Figure 2. Association between resistome composition and clinical features in bronchiectasis.** Stacked bar plots representing resistome profiles in stable bronchiectasis recruited to the CAMEB2 cohort (matched and un-matched, n=251) stratified according to (a) exacerbation status i.e. number of exacerbations in the preceding year categorized as non-exacerbators (N.Ex; i.e. no exacerbations); exacerbators (Ex; 1-2 exacerbations) or frequent exacerbators (F.Ex; 3+ exacerbations), (b) lung function based on FEV1 % predicted as >70%, 50-70%, 30-50% and <30% predicted, (c) disease severity based on the bronchiectasis severity index (BSI) as mild (BSI = 0-4), moderate (5-9) or severe (>=9) and (d) bronchiectasis aetiology as idiopathic (IP), post-infection (PI), post-tuberculosis (PTB) or other. Assessment of inter-group differences in relative abundance of ARG classes was evaluated using the Kruskal Wallis test with FDR correction; * p < 0.05, ** p < 0.01.

**Figure 3. The Cross sectional Asian and Matched European Bronchiectasis 2 (CAMEB2) cohort and geographic variability in the bronchiectasis resistome.** (a) Schematic overview of the matching strategy employed for individuals included into the CAMEB2 cohort. Asian patients (Singapore; n = 95 and Kuala Lumpur, Malaysia; n = 35) were compared to Europeans (Dundee, Scotland; n = 96 and Milan, Italy; n = 25) to identify a cohort of n=209 patients matched for age, sex, exacerbation frequency and lung function while differing in their geographic origin. (b) Beta-diversity of resistance gene profiles (i.e. Bray-Curtis distance) assessed by Principal Coordinate Analysis (PCoA) illustrating resistome diversity and coloured by geographic origin assessing matched patients from CAMEB2: Singapore (red), Kuala Lumpur (yellow), Dundee (blue) and Milan (green) (PERMANOVA, p=0.006). (c) Beta-diversity of resistance gene profiles (i.e. Bray-Curtis distance) assessed by Principal Coordinate Analysis (PCoA) illustrating resistome diversity and coloured by aetiology assessing matched patients from CAMEB2: idiopathic (IP- purple), post-infection (PI- orange), post-tuberculosis
(PTB- blue) and others (grey). Central points intersected by a cross indicates the centroid in PCoA visualizations.

**Figure 4. Characterization of bronchiectasis resistotypes.** (a) Heat map illustrating CAMEB2 resistome profiles that associate with the identified resistance gene profile clusters (‘resistotypes’) indicated as RT1 (blue) and RT2 (purple). The heat map details the resistance gene composition of each resistotype cluster at the drug class level, expressed as log₂ percentage (%) relative abundance. (b) Principal Coordinate Analysis (PCoA) of gene level resistome profiles demonstrating the distinct resistotype RT1 and RT2 clusters as defined by beta-diversity of resistance gene profiles (i.e. Bray-Curtis distance). Circles intersected by a cross indicate PCoA centroids of compared groups, PERMANOVA p<0.001. (c) Linear Discriminant Analysis (LEfSe) highlighting resistance genes that distinguish the RT1 and RT2 resistotype clusters. (d-f) Analysis of clinical features associated with the resistotype clusters RT1 (blue) and RT2 (purple) including (d) exacerbation frequency in the year preceding recruitment, (e) lung function (as FEV₁ % predicted) and (f) disease severity (as bronchiectasis severity index; BSI). (g) Prevalence of the resistotypes across the recruited cohorts and respective geographic regions. SG: Singapore; KL: Kuala-Lumpur, Malaysia; DD: Dundee, Scotland and MI: Milan, Italy.

**Figure 5. Microbiome correlates of bronchiectasis resistotypes RT1 and RT2.** Stacked barplots illustrating the relative abundance of microbial taxa associated with (a) resistotypes RT1 and RT2 and stratified by (b) continent and (c) country of origin. (d) Analysis of resistance gene content among the top microbial taxa identified in the CAMEB2 cohort. Horizontal bars indicate the number of ARGs (x-axis) identified on contigs assigned to the indicated bacterial
taxa (y-axis) based on metagenomic assembly. Prevalent bacterial taxa ranked within the top-40 (by average relative abundance) are coloured, while lower abundance taxa are greyed. (e) Analysis of resistance gene distribution across bacterial taxa. Horizontal bars indicate the number of bacterial taxa (x-axis) expressing the respective indicated resistance genes (y-axis) based on metagenomic contig analysis. Resistance genes are differentiated by colour according to drug-class as indicated in y-axis labels (in parenthesis).

Figure 6. The effect of *P. aeruginosa* eradication therapy on bronchiectasis resistotypes.

(a) Microbiome composition of n=8 *P. aeruginosa* culture-positive bronchiectasis patients (P1-P8) receiving eradication therapy with 14 days oral ciprofloxacin followed by 3 months of nebulized colistin. Stacked bar plots illustrate the lung microbiome composition pre- and post-eradication therapy. (b) Resistome profiles of *P. aeruginosa* culture-positive bronchiectasis patients (P1-P8) receiving eradication therapy with 14 days oral ciprofloxacin followed by 3 months of nebulized colistin. Stacked bar plots illustrate resistome composition (at drug class level) pre- and post- eradication therapy. (c) Principal Coordinate Analysis (PCoA) plot illustrating resistome gene profiles incorporating patients undergoing *P. aeruginosa* eradication therapy (pre-eradication: green and post-eradication: red) orientated to their previously determined resistotype clusters: RT1 (blue) and RT2 (purple). A black arrow indicates the trajectory of the eradication therapy group centroids illustrating a shifting proximity from the initial RT2 (purple) to RT1 (blue) cluster in the pre- and post-eradication state respectively. (d) Stacked bar plots depicting antimicrobial resistome profiles of patients before and after antimicrobial treatment. The left section illustrates changes in these profiles following the administration of acute antibiotics for treating pulmonary exacerbations compared to the right illustrating changes observed after *P. aeruginosa* eradication therapy. * = p < 0.05, ns = p > 0.05 (paired PERMANOVA).
Figure 1

(a) Relative abundance (%) of bacteria in non-diseased and bronchiectasis patients. (b) Principal coordinate analysis (PCoA) plot showing the separation between non-diseased and bronchiectasis samples. (c) Relative abundance (%) of bacteria over time points 3, 6, and 7 months post-baseline (BL) follow-up (FUP). (d) Exacerbation timepoint analysis showing relative abundance of bacteria. (e) PCoA plot for exacerbation timepoints with PERMANOVA p = 0.786. (f) >12 weeks exacerbation compared to <12 weeks exacerbation. (g) PCoA plot for >12 weeks vs. <12 weeks exacerbation with PERMANOVA p = 0.026.
Figure 2

(a) Exacerbations **

(b) FEV1 (% predicted) **

(c) Severity

(d) Aetiology *

[Graphs and data showing relative abundance of exacerbations, FEV1, severity, and aetiology categories.]

- **Exacerbations**: Relative abundance of exacerbation categories.
- **FEV1 (% predicted)**: Distribution of FEV1 values.
- **Severity**: Severity levels of exacerbations.
- **Aetiology**: Categories of aetiology contributing to exacerbations.

Legend:
- Acidine dye
- Antibacterial fatty acids
- Aminoglycoside
- Aminocoumarin antibiotic
- Beta lactam
- Bicyclomycin
- Diaminopyrimidine
- Fusidic acid
- Mupirocin
- Peptide antibiotic
- Sulfonamide antibiotic
- Fluoroquinolone
- MLS
- Nitroimidazole antibiotic
- Phenicol
- Tetracycline
- Multidrug
- Nucleoside antibiotic
- Rifampicin
- Tridosal
Figure 4

(a) Resistotype RT1

(b) Resistotype RT2

(c) Drug class

<table>
<thead>
<tr>
<th>Drug class</th>
<th>Aminoglycoside</th>
<th>Tetracycline</th>
<th>Phenicol</th>
<th>MLS</th>
<th>Peptide antibiotic</th>
<th>Nitroamidazole</th>
<th>Fluoroquinolone</th>
<th>Aminocoumarin</th>
<th>Beta-lactam</th>
<th>Antibacterial free FA</th>
<th>Triclosan</th>
<th>Fosfomycin</th>
<th>Diaminopyrimidine</th>
<th>Sulfonamide</th>
<th>Rifampicin</th>
<th>Mupirocin</th>
<th>Bicyclomycin</th>
<th>Acridine dye</th>
<th>Fusidic acid</th>
</tr>
</thead>
</table>

(d) Prevalence

**(a)** (b) (c) (d) (e) (f) (g)
Acute (N=18) Erd. (N=8)

PERMANOVA, p = 0.0156

Figure 6
Airway ‘Resistotypes’ and Clinical Outcomes in Bronchiectasis


*These authors contributed equally
SUPPLEMENTARY MATERIALS AND METHODS

Ethical approval. The study was approved by the IRBs of all participating institutions as follows: CIRB 2016/2073 and CIRB 2017/2109 (mutually recognized by DSRB), NTU IRB-2016-01-031, NTU IRB-2017-07-023, NTU IRB-2017-12-010 NTU IRB-2017-05-035 (Singapore); UMMC 2018725-6524 (Malaysia); NHD 12/ES/0059, NHD 16/NW/0101 (Dundee, Scotland), 255_2020 (Comitato Etico Milano Area 2, Milan, Italy) and TR000642 (Greece). All patients gave written informed consent to participate.

Clinical data and specimen collection. All patients underwent clinical, radiological, and functional evaluation and had relevant demographic and clinical data recorded (summarized in Tables 1, E1, and E2). Exacerbation history was recorded in accordance with established consensus and patients were assigned to relevant exacerbation groups based on number of exacerbations in the year preceding recruitment (0 – ‘non-exacerbator’, 1-2 ‘exacerbator’, 3+ ‘frequent exacerbator’) (1, 2). Bronchiectasis severity was assigned according to the bronchiectasis severity index (BSI) and further divided into ‘mild’ (BSI; 0-4), ‘moderate’ (BSI; 5-8) or ‘severe’ (BSI; 9 and above) (3). All other clinical data necessary for computing BSI including age, sex, Body Mass Index (BMI), Medical Research Council (MRC) dyspnoea score, FEV1 % predicted values, radiological severity, number of hospitalizations in the preceding year, and microbial colonization was recorded for each individual, as was data on disease etiology and smoking status. Data on use of bronchodilators, inhaled corticosteroids, mucolytics and long-term prophylactic antibiotics was collated. In contrast to the original CAMEB cohort which matched patients on age, sex and disease severity (i.e. BSI) (4-7), exacerbation status (as opposed to disease severity) and lung function (as FEV1 % predicted) served as the new basis for matching in CAMEB 2 in addition to age and sex (Table 1).
Spontaneously expectorated ‘representative’ sputum from a deep cough with the assistance of a chest physiotherapist and/or induction protocol (where appropriate) was collected into sterile containers and transported (on ice) for evaluation (8). All specimens from clinical sites were transported promptly, appropriately and processed centrally at a single site to ensure consistency and standardization of all experimental work and downstream analysis. Samples from the Singaporean hospitals were transported on ice by courier to Nanyang Technological University (within 4 hours of collection). To ensure quality control of materials transported from sites outside Singapore, specimens were shipped on dry ice in temperature-controlled containers and their integrity checked on arrival before experimental use as previously described (7, 9).

**DNA extraction and metagenomic sequencing.** DNA was extracted from a 250mg sample of thawed sputum using methods previously described (7, 9, 10). Samples were homogenized in bead mill tubes (VWR) containing 1mm sterile glass beads (Sigma-Aldrich) using a bead mill homogenizer (VWR). DNA was purified using the Roche High-pure PCR Template Preparation Kit (Roche) according to manufacturers’ instructions. The integrity of extracted sputum DNA was confirmed using the Qubit dsDNA High Sensitivity (HS) Assay Kit (Invitrogen). DNA was sequenced on a HiSeq 2500 platform (Illumina, USA) at the NTU core sequencing facility according to standard library preparation and DNA sequencing methods as previously established (7, 9, 10). Both blank DNA extraction controls (n=9) and sequencing library blanks (n=3) were included and were processed simultaneously to assess levels of experimental background contamination associated with DNA extraction and sequencing respectively (Supplementary Figure E1), with blanks exhibiting significantly lower sequencing depth than samples; median = 0.01 (IQR: 0.01-0.06) vs 3.8 (IQR: 3.5-4.2) million reads per sample.
Short-read metagenomic sequencing data from this study have been uploaded to the National Center for Biotechnology Information (NCBI) sequence read archives under project accession numbers PRJNA595703, PRJNA590225 and PRJNA982297. The accessions of specific samples and cohorts to which they relate are indicated in the open access online repository: https://github.com/RespiratoryMicrobiome/BE-Resistome

Reviewer link: https://dataview-ncbi-nlm-nih-gov.elib.tcd.ie/object/PRJNA982297?reviewer=ftn4p37ipaofonfsnmp869tibs

**Quality control of metagenomic sequencing data.** Trimmomatic (version 0.39) was used to clip Illumina adapters from the paired-end raw sequencing reads, remove low-quality bases at both ends, and remove reads <30 bp in length after trimming (parameters ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:10:1:TRUE LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:30) (11). FastQC (version 0.11.8) was used to check the quality of original and trimmed reads (12) and Bowtie2 (version 2.3.5.1) used to align the quality-filtered reads to the human reference genome (hg38) (13). Unmapped, non-human reads were separated and sorted from human reads using samtools (version 1.9) (14). FASTA files containing these reads, used for various downstream analyses, were obtained using bedtools (version 2.28) (15) Processed sequenced reads generated from shotgun metagenomic sequencing of sputum were classified to determine the relative abundance of antimicrobial resistance genes (ARGs) and microbial taxa (including DNA viruses) as described below.

**Antimicrobial resistance gene (ARG) identification.** Non-host reads were subjected to antimicrobial resistance (AMR) gene identification to determine the airway resistome. The abundance of AMR genes present in the metagenomic data were quantified using ShortBRED
(16) as previously described (10). Briefly, the ‘shortbred_identify.py’ script was used to create unique protein markers with a 95% cluster identity using AMR gene sequences from the Comprehensive Antibiotic Resistance Database (CARD) and UniRef90 protein databases (17, 18). The ‘shortbred_quantify.py’ script was subsequently used to calculate the relative abundance of AMR genes within the metagenomic data. Drug class assignments of the AMR genes was based on CARD assignment. (17) Antimicrobial ‘resistotypes’ – i.e., distinct ARG profiles observed among specific patient groups - were delineated by spectral clustering of Bray-Curtis distances, based on maximal silhouette score. The mean misclassification ratio (based on 100 bootstrap iterations) was determined to assess cluster robustness (7).

**Microbial assignment of metagenomic sequenced reads.** Taxonomic assignment of shotgun metagenomic analysis was achieved using the Kaiju taxonomic classifier as previously described (version 1.7.2) (9, 19). Here, taxonomic calls from the Kaiju classifier were further scrutinized by BLAST confirmatory analysis of raw sequence data performed by considering only species whose relative abundance is >1% in at-least a single sample. A sub-sample of reads (assigned by kaiju) were randomly subjected to BLASTN analysis (using the megablast function and a specified E-value of 10) against the corresponding database sequences for that species. Sequence similarity of $\geq 50\%$ was as considered as a significant hit and were used for further analysis, while taxa failing to reach this cut off were excluded from analysis (9). Taxonomic composition was further interrogated by analysis of microbial interaction networks. Graphs were derived using an ensemble (Spearman and Pearson correlation) based network inference coupled to ‘ReBoot’, adapted from our previous work (20). Edge weights were corrected for multiple comparisons using the Benjamini and Hochberg FDR (False Discovery Rate) procedure and only edges with a corrected $p$-value $< 0.001$ and a total correlation (Spearman + Pearson’s) score above 0.5 were considered. The network was visualised using
cystoscope (version 3.10.1) (21). In parallel, the composition of viromes consisting of prokaryotic phages and eukaryotic viruses was estimated through a pipeline using Megahit (version 1.2.9) (22), VirFinder (version 1.1) (23), bowtie2 (version 2.3.3.1) (13), samtools (version 1.3.1) (14), CONCOCT (version 1.1.0) (24), and Demovir scripts (https://github.com/feargalr/Demovir) as previously described (7). In parallel, viral contigs identified at the order and family levels by the Demovir script were further assessed using BALSTN against the NCBI database of viruses (taxid: 10239) to identify the top database sequences matching the viral contigs. A schematic overview of the viral contig analysis pipeline is illustrated in supplementary Figure E5.

Quantitative PCR (qPCR)-based assessment of microbial burden

Fluorescence-based detection of total bacterial 16S rRNA

Quantitative PCR assay for 16s r-RNA gene (V1-V3 regions) was performed in duplicate using the SYBR Green-based detection method (KAPA SYBR® FAST (KK4602)) on an Applied Biosystems StepOne Plus Real Time PCR System. Primers were purchased from Integrated DNA Technologies (Table E3). Assays were carried out in a total volume of 20 μL, comprising 10 μL of PCR mastermix, 6.8 μL of nuclease-free water, 0.8 μL of primers, and 2 μL of nucleic acid extract. Rox reference dye (high) was used as per manufacturer’s instructions. DNA extracted from a Neisseria subflava clinical strain SG0010 (ASM2363508v1) isolated from a sputum sample from a bronchiectasis patient was used in five successive serial dilutions to create the standard curve (5). Cycle parameters were initial activation of DNA polymerase at 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds (denaturation) & 60°C for 45s (annealing and extension).

Fluorescence-based detection of P. aeruginosa and H. influenzae
Quantitative PCR (qPCR) assays for *P. aeruginosa* (PA) & *H. influenzae* (HI) were performed in duplicate using QuantiNova Probe PCR Master Mix (Qiagen, 208254) on an Applied Biosystems StepOne Plus Real Time PCR System. Primers were purchased from Integrated DNA Technologies and TaqMan MGB probes from Thermo Fisher Scientific (Table E3). Assays were carried out in a total volume of 20 μL, comprising 10 μL of PCR mastermix (Qiagen), 2.2 μL of nuclease-free water, 3.6 μL of oligonucleotide mixture, 2 μL of nucleic acid extract and, 1μL of the 10X EXO-IPC Mix from TaqMan Exogenous Internal Positive Control Reagents commercial kit (Applied Biosystems) containing a set of primers and probe (VIC/TAMRA) targeting the synthetic EXO-IPC DNA. Rox reference dye was used in a 1:20 dilution as per manufacturer’s instructions. A PAO1 laboratory strain & *H.influenzae* ATCC strain 49247 was used in five successive serial dilutions to create standard curves respectively for the PA & HI assays. Cycle parameters were initial activation of DNA polymerase at 95°C for 2 minutes, followed by 40 cycles of 95°C for 0.05 seconds (denaturation) & 60°C for 10 seconds (annealing and extension).

**Metagenomic contig assembly and analysis.** To further define the genetic context of identified resistance genes, metagenomic contig assembly was performed. Contigs were assembled from paired-end sequencing reads (in fasta format with host reads removed) using Megahit (version 1.2.9) (22). Reads were cross-assembled into contigs of which only contigs >1kb in length were considered in subsequent analysis and contigs >10 kb in length cut into sequences of 10-20 kb using CONCOCT (version 1.1.0) (24). BLASTX against the CARD database (applying cut-off e-values of <1e-50) were used to determine whether contigs were associated with AMR genes. Contigs carrying ARGs were identified and classified as ‘plasmid-associated’ or ‘phage-related’ by applying the PlasForest or Demovir packages respectively (7, 27). The microbial origins of the identified chromosomal contigs containing resistance genes
were determined by comparing these contigs to Genbank sequences of airway associated taxa using BLASTN (e-value <1e-50). Using this approach, the number of resistance genes associated with major bacterial taxa of the airway including their distribution and prevalence was determined.
REFERENCES


SUPPLEMENTARY TABLES AND FIGURE LEGENDS

Table E1. Demographic table summarizing characteristics of longitudinal study subjects with bronchiectasis.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Bronchiectasis; (n=21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age: median (IQR)</td>
<td>71 (68-76)</td>
</tr>
<tr>
<td>Gender: n (%)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>15 (71%)</td>
</tr>
<tr>
<td>Male</td>
<td>6 (29%)</td>
</tr>
<tr>
<td>Etiology n (%)</td>
<td></td>
</tr>
<tr>
<td>Idiopathic</td>
<td>15 (71%)</td>
</tr>
<tr>
<td>Post-infectious</td>
<td>1 (5%)</td>
</tr>
<tr>
<td>Other</td>
<td>5 (24%)</td>
</tr>
<tr>
<td>BSI status: n (%)</td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>5 (24%)</td>
</tr>
<tr>
<td>Severe</td>
<td>13 (62%)</td>
</tr>
<tr>
<td>BSI score: median (IQR)</td>
<td>10 (6-14)</td>
</tr>
<tr>
<td>No. of exacerbations in previous year: n (%)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>1-2</td>
<td>4 (19%)</td>
</tr>
<tr>
<td>3 or more</td>
<td>15 (71%)</td>
</tr>
<tr>
<td>≥ 1 hospital admission in the preceding year: n (%)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>6 (71%)</td>
</tr>
<tr>
<td>No</td>
<td>15 (29%)</td>
</tr>
<tr>
<td>FEV₁ % predicted: median (IQR)</td>
<td>70 (53-78)</td>
</tr>
<tr>
<td>Antibiotic treatment</td>
<td></td>
</tr>
<tr>
<td>β-lactam</td>
<td>12 (57.1%)</td>
</tr>
<tr>
<td>Fluoroquinolone</td>
<td>3 (14.1%)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>3 (14.1%)</td>
</tr>
<tr>
<td>none</td>
<td>3 (14.1%)</td>
</tr>
</tbody>
</table>

Patient characteristics in the longitudinal exacerbation series (n=21). Demographic data is presented as the median value ± interquartile range (IQR) and/or patient numbers (n) with percentages (%) as appropriate. BMI: Body Mass Index, mMRC: Modified medical research council dyspnea scale, BSI: Bronchiectasis Severity Index.
Table E2. Demographic table detailing the characteristics of bronchiectasis patients undergoing *P. aeruginosa* eradication therapy.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>72 (69-75)</td>
</tr>
<tr>
<td>Sex (Female)</td>
<td>2/8 (20%)</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>24 (23-29)</td>
</tr>
<tr>
<td>mMRC</td>
<td>1 (1-2)</td>
</tr>
<tr>
<td>FEV1 % predicted</td>
<td>56 (41-72)</td>
</tr>
<tr>
<td>No. of exacerbations in previous year</td>
<td>3 (2-4)</td>
</tr>
</tbody>
</table>

Patient characteristics in the longitudinal *P. aeruginosa* eradication arm (n=8). Demographic data is presented for all *P. aeruginosa*-colonized patients receiving eradication therapy including age, sex, BMI (kg/m2), mMRC, FEV1 % predicted and no. of exacerbations in the previous year (collated at time of recruitment). BMI: Body Mass Index, mMRC: Modified medical research council dyspnoea scale.
<table>
<thead>
<tr>
<th>Assay</th>
<th>Target region</th>
<th>Oligonucleotide</th>
<th>Concentration [uM]</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-PCR</td>
<td>16S V1-V3 region</td>
<td>Forward: TCCTACGGGAGGCAGCAGT&lt;br&gt;Reverse: GGACTACCAGGGTATCTAATCCTGTT</td>
<td>0.4 uM</td>
<td>(25)</td>
</tr>
<tr>
<td>(Total 16S)</td>
<td></td>
<td></td>
<td>0.4 uM</td>
<td></td>
</tr>
<tr>
<td>RT-PCR</td>
<td>DNA gyrase subunit B (gyrB)</td>
<td>Forward: CCTGACCATCCGTCGCCACAAC&lt;br&gt;Reverse: CGCAGCAGGAAGCCGACGCC&lt;br&gt;Probe: FAM-CCGTGGTGTAGACCTGTCCAGACC-QSY</td>
<td>0.8 uM&lt;br&gt;0.8 uM&lt;br&gt;2 uM</td>
<td>(26)</td>
</tr>
<tr>
<td>(PA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT-PCR</td>
<td>L-fucokinase (fucK)</td>
<td>Forward: ATGGCGGGAACATCAATGA&lt;br&gt;Reverse: ACGCATAGGAGGGAAATGGTT&lt;br&gt;Probe: FAM-CGGTAATGGGATCCAT-MGB</td>
<td>0.8 uM&lt;br&gt;0.8 uM&lt;br&gt;2 uM</td>
<td>(26)</td>
</tr>
<tr>
<td>(HI)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure E1. Schematic overview of study cohorts and experimental design.

The primary aim of this study was to evaluate the variability of the resistome and its clinical implications in bronchiectasis. Our multifaceted approach is delineated through four distinct cohorts. These include cross-sectional (upper rows) and longitudinal arms (lower rows) of the study. Cohort 1: the 'Non-diseased' cohort, comprises healthy individuals with no recorded respiratory disease, serving as a baseline for resistome profiles. Cohort 2, the ‘CAMEB2’ Bronchiectasis (cross-sectional) cohort', allows for a comparative analysis against the backdrop of the 'non-diseased' cohort, emphasizing notable differences in resistome composition associated with bronchiectasis. Temporal shifts in the resistome are explored through Cohort 3, the 'bronchiectasis longitudinal ('Exacerbation') cohort. Serial sampling from patients during bronchiectasis exacerbations and subsequent therapy facilitated an assessment of the resistome temporal dynamics. Cohort 4, the longitudinal \( P. \ aeruginosa \) ‘Eradication’ cohort, includes patients subjected to targeted \( P. \ aeruginosa \) eradication therapy. The analysis of paired longitudinal samples, pre- and post-therapy (n=8, t=2), provided insights into resistome relationships with \( P. \ aeruginosa \), Collectively, these cohorts form the framework of our investigation into the resistome's role as a marker of clinical relevance in bronchiectasis. SG-Singapore, KL – Kuala Lumpur, DD – Dundee, MI – Milan.

Supplementary Figure E2. Assessment of background microbial DNA contamination in negative control samples. Stacked bar plots represents the whole genome shotgun (WGS) metagenomic derived taxonomic profiles for sputum samples (“Samples”, n =344) DNA extraction negative controls (“Blanks”, n=9) and sequencing blanks (“Blanks-seq”, n=3). Aggregated relative abundance data is presented.
Supplementary Figure E3. Assessment of the therapeutic effects of inhaled corticosteroids (ICS) and long-term macrolide use on the antimicrobial resistome. Stacked bar plots illustrate the composition of the antimicrobial resistome in (a) patients receiving inhaled corticosteroids (ICS) versus those without and (b) patients on long-term macrolide therapy versus those without. Corresponding PCoA of resistome beta-diversity (Bray-Curtis) distance comparing (c) effects of ICS use and (d) long-term macrolide use in stable bronchiectasis patients are illustrated. PCoA centroids of respective comparison groups are indicated by a circle with cross.

Supplementary Figure E4. Gene-level geographic differences in the bronchiectasis resistome. A LeFSe linear discriminant analysis plot illustrating resistance genes from the antimicrobial resistome between Asian (Singapore and Kuala Lumpur; SG-KL) and European (Dundee and Milan; DD-MI) cohorts.

Supplementary Figure E5. Taxonomic geographic differences in the bronchiectasis microbiome. Taxonomic analysis of microbiome composition assessed across (a-b) geographic origin and (c-d) bronchiectasis aetiology. (a) Stacked bar charts represent the relative abundance of bacterial species identified in matched patients from the CAMEB2 cohort by geographic origin. (b) Beta-diversity of taxonomic profiles (i.e. Bray-Curtis distance) assessed by Principal Coordinate Analysis (PCoA) illustrating taxonomic diversity and coloured by geographic origin assessing matched patients from the CAMEB2 cohort: Singapore (red), Kuala Lumpur (yellow), Dundee (blue) and Milan (green) (PERMANOVA, p=0.006). (c) Stacked bar charts represent the relative abundance of bacterial species identified in matched patients from the CAMEB2 cohort by bronchiectasis aetiology and (d) Beta-diversity of resistance gene profiles (i.e. Bray-Curtis distance) assessed by Principal
Coordinate Analysis (PCoA) illustrating resistome diversity and coloured by aetiology assessing matched patients from the CAMEB2 cohort: idiopathic (purple), post-infection (orange), post-tuberculosis (blue) and others (grey). Circles intersected by a cross indicate centroids in PCoA visualizations.

**Supplementary Figure E6. Metagenomic analysis pipeline for the discovery and annotation of viruses in the airway microbiome.** (a) Schematic overview of the applied bioinformatic pipeline. Total sequencing reads were subjected to QC trimming and human read removal before contig assembly and processing by the megahit and CONCOCT packages. The Virfinder package was used to identify viral contigs that were used as an input to the Demovir taxonomic classifier. The final reference database of 1,663 identified viral contigs was then compared to viral reference sequences in the NCBI database (taxid: 10239) to further identify specific hits against contigs identified in the prior steps of the pipeline. (b) Bar graph illustrating metrics for outputs at each stage of the applied pipeline.

**Supplementary Figure E7. Bacteriophage profiling of the bronchiectasis airway.** (a) Bacteriophage relative abundance among n=251 stable bronchiectasis patients from the CAMBE2 cohort. Stacked bar plots represent the relative abundance of the 13 viral families identified. (b) Analysis of the prevalence of the viral families identified in bronchiectasis. (c) BLAST analysis and identification of metagenomic contigs classified according to their associated microorganism. (d) Viral profiles in supervised analysis of various clinical variables of interest including exacerbation status i.e. number of exacerbations in the preceding year categorized as non-exacerbators (N.Ex; i.e. no exacerbations); exacerbators (Ex; 1-2 exacerbations) or frequent exacerbators (F.Ex; 3+ exacerbations); lung function based on FEV1 % predicted as >70%, 50-70%, 30-50% and <30% predicted; disease severity based on the bronchiectasis severity index (BSI) as mild (BSI = 0-4), moderate (5-9) or severe (>=9)
and bronchiectasis aetiology as idiopathic (IP), post-infection (PI), post-tuberculosis (PTB) or other. ns = non-significant (all p>0.05 by Kruskal Wallis with FDR correction). (e) Taxonomic analysis of bacteriophageome composition assessed across geographic origin as Singapore (SG), Kuala Lumpur, Malaysia (KL), Dundee, Scotland (DD) and Milan, Italy (MI) with corresponding (f) Beta-diversity of bacteriophage profiles (i.e. Bray-Curtis distance) assessed by Principal Coordinate Analysis (PCoA) illustrating bacteriophage diversity and coloured by geographic origin assessing matched patients from CAMEB2: Singapore (red), Kuala Lumpur (yellow), Dundee (blue) and Milan (green) (PERMANOVA; p=0.008). Circles intersected by a cross indicate centroids in PCoA visualizations.

Supplementary Figure E8. Assessment of microbial read depth effects on taxonomic and ARG profiles. Scatter plots illustrate the correlation between log-transformed non-human reads and calculated Shannon diversity index (SDI) of (a) microbial species and (b) ARGs. Data points, representing individual samples, are colour-coded by sequencing depth (red – shallow <100,000, green – Deep > 100,000). A black line represents a LOESS regression, illustrating the trend in the data with surrounding grey shading indicating a 95% confidence interval. Pearson correlation coefficients and p-values are indicated. Boxplots with overlaid points display the distribution of (c) log-transformed non-human reads and (d) microbial reads (non-human as a percentage of total reads) across resistotypes RT1 (blue) and RT2 (purple). P-values associated with the comparison of measures across RT1 and RT2 using the Wilcoxon test are indicated.

Supplementary Figure E9. Quantitative PCR analysis of microbial burden in the RT1 and RT2 resistotypes. Box and whiskers plots illustrate the bacterial burden observed in (a) the RT1 and (b) the RT2 resistotypes, as assessed by qPCR-quantification of total bacterial 16S
rRNA gene copies (grey), *H. influenzae* *fucK* gene copies (‘HI’ - purple) and *P. aeruginosa gyrB* gene copies (‘PA’ - green). *** = p <0.001. (c) The proportion of patients exhibiting *H. influenzae*-dominant (HI 16S gene copies > PA 16S gene copies) and *P. aeruginosa*-dominant (PA 16S gene copies > HI 16S gene copies) in each RT group are presented.

**Supplementary Figure E10. Sub analysis of resistotype clinical correlates in *P. aeruginosa* culture-negative patients.** Box-and-whisker plots illustrating the association between clinical features and resistotype clusters RT1 (blue) and RT2 (purple) including (a) exacerbation frequency in the year preceding recruitment, (b) lung function (as FEV₁ % predicted) and (c) disease severity (as bronchiectasis severity index; BSI). Statistical significance of differences between RT1 and RT2 after exclusion of *P. aeruginosa*-positive patients (Wilcoxin test) are indicated; ns = ‘not significant’, * = p <0.05, ** p <0.01.

**Supplementary Figure E11. Characterisation of the microbial interactome associated with identified resistotypes.** Co-occurrence network maps of (a) RT2 vs (b) RT1 resistotypes. Interactions between microbes (nodes) are represented by connecting lines (edges). Airway microbiome interactions are represented by green (positive) and red (negative) lines. Microbes (nodes) are defined according to three key network metrics; degree (measure of numbers of direct interactions with others, i.e. ‘Busy’), betweenness centrality (measure of level of influence over other microbes, i.e. ‘Influential’) and stress centrality (importance to network integrity, i.e. ‘Critical’) as represented in the legend. Circle size (degree), Circle border thickness (stress centrality) for each microbe (termed ‘critical’), while color depth reflects the betweenness centrality (the ‘influence’) of the microbe in the network.
Supplementary Figure E12. Quantitative PCR Analysis of *P. aeruginosa* Burden Pre- and Post-Eradication. Box-and-whisker plot illustrating *P. aeruginosa* 16S rRNA gene copy numbers in pre- and post-eradication samples. Individual data points are overlaid on the plot to provide a granular view of the distribution. A line connects paired samples across the two groups, visually representing changes in bacterial burden for each subject before and after the eradication treatment. Statistical significance of differences between pre- and post-eradication groups (paired two-tailed Wilcoxin test) is indicated on the plot. * = p <0.05.
### Study design overview

<table>
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<tr>
<th>Cohort</th>
<th>Details</th>
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| **1. Non-diseased comparator (n=25)** | • Normal spirometry  
• Free of chronic respiratory disease  
• Recruited in SG | • Representative (un-matched) snapshot of the airway resistome in a healthy population.  
• Baseline comparator. |
| **2. CAMEB2 (n=251, matched =209)** | • Stable bronchiectasis patients  
• Recruited in across four geographically distinct regions (SG, KL, DD, MI).  
• Cross-regional matching (Age, Sex, Exacerbation and FEV1). | • Characterisation of the bronchiectasis airway resistome  
• Determine clinical correlates of the resistome.  
• Assess contribution of geographic variability in disease-matched patients.  
• Assess microbiome-resistome associations. |
| **3. Bronchiectasis Exacerbation (n=21, 2-3 timepoints)** | • Recruited in DD (exacerbation arm) and SG (non-exacerbators).  
• Sample at baseline (DD/SG), during exacerbation (DD-only) and post exacerbation (DD-only) / following period without reported exacerbation (SG-only). | • Quantify temporal resistome variability in response to acute antimicrobial treatment of bronchiectasis exacerbation. |
| **4. *P. aeruginosa* Eradication (n=8, 2 timepoints)** | • Patients with newly documented PsA positive culture.  
• Sampling at baseline and post-eradication therapy follow up.  
• Recruited in Greece. | • Quantify temporal resistome variability in response to targeted *P. aeruginosa* eradication therapy. |
Supplementary Figure E3

(a) Inhaled corticosteroid use

(c) Long-term macrolide use

Relative abundance (%)

(b) (d)

PERMANOVA = 0.984

PERMANOVA = 0.346
Supplementary Figure E6

(a) 986m reads (247 Gb) → QC trimming → 971m reads (243 Gb)
971m reads (243 Gb) → Human read removal → 70m reads (17.5 Gb)
70m reads (17.5 Gb) → Megahit / concoct → 176481 contigs
176481 contigs → Virfinder → 20003 contigs
20003 contigs → Demovir → 1663 contigs
1663 contigs → BLASTN → 336 unique hits (778 total)

(b) Bar chart showing classified contigs:
- Virfinder: 20003 contigs
- Demovir: 1663 contigs
- BLAST: 442 contigs

986m reads (247 Gb) → 70m reads (17.5 Gb) → 176481 contigs → 20003 contigs → 1663 contigs → 336 unique hits (778 total)
Supplementary Figure E8

(a) Non-Human reads vs Species diversity

(b) Non-Human reads vs ARG diversity

(c) Non-Human reads RT1 vs RT2

(d) Non-Human reads RT1 vs RT2
Supplementary Figure E9
Supplementary Figure E10

(a) Exacerbations

\[ \text{ns} \quad (p = 0.104) \]

(b) FEV1 (% predicted)

(c) Severity (BSI)
Supplementary Figure E12

The figure shows a comparison of log10 (16S P. aeruginosa rRNA gene copies/μL) before and after treatment. The data points indicate a statistically significant decrease in the post-treatment period, as indicated by the * symbol. The green bars represent the range of values, with the black lines connecting the data points for each individual sample.