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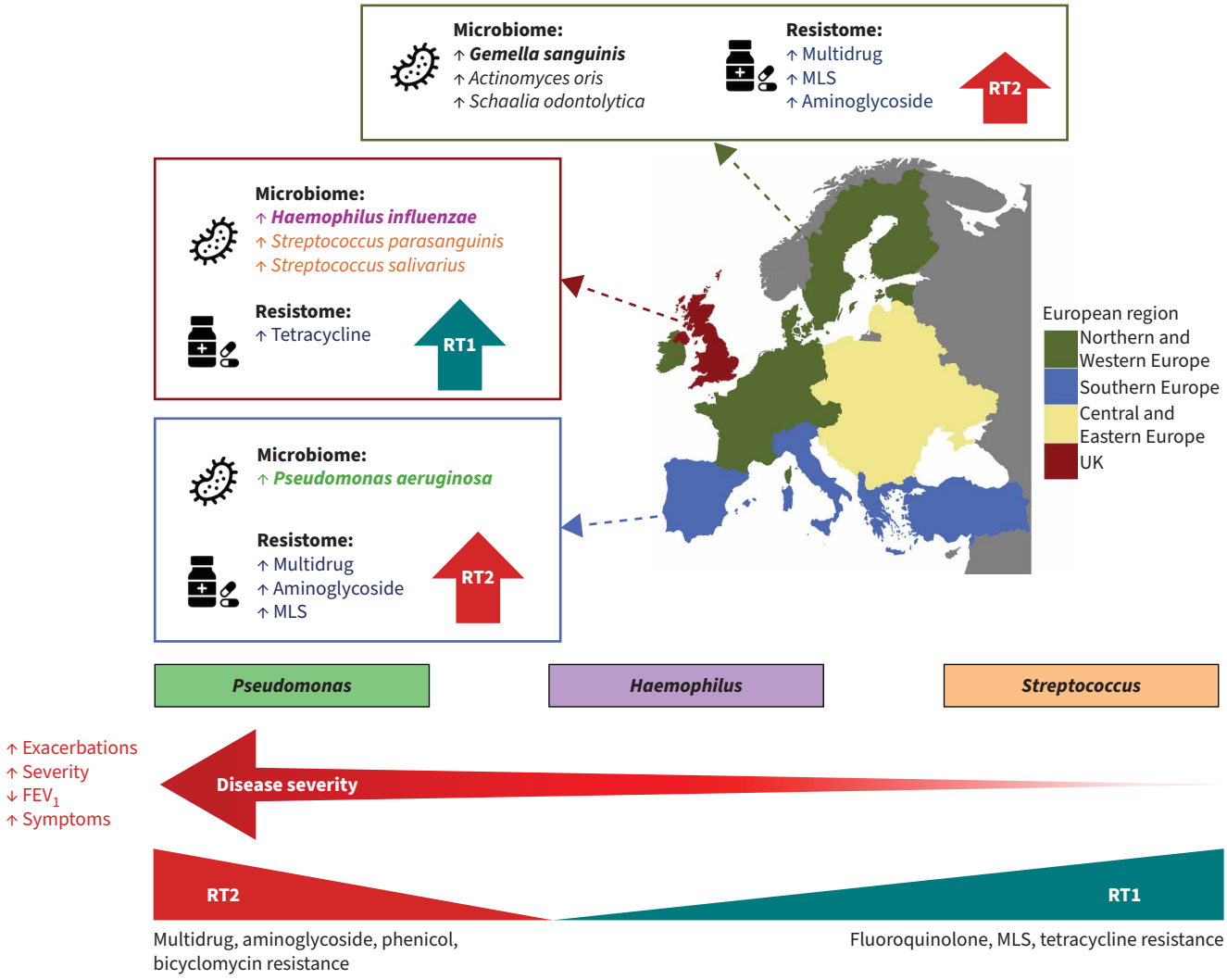

GRAPHICAL ABSTRACT Airway metagenomics in the EMBARC-BRIDGE study reveals pan-European variation in microbiome and resistome profiles in bronchiectasis. MLS: macrolide-lincosamide-streptogramin; RT: resistotype; FEV_1: forced expiratory volume in 1 s.

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Sputum metagenomics in bronchiectasis reveals pan-European variation: an EMBARC-BRIDGE study

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Sputum metagenomics reveals striking variation between different European countries in microbiome composition and resistome https://bit.ly/4daVUiW

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Abstract

Background The European Multicentre Bronchiectasis Audit and Research Collaboration (EMBARC) registry shows considerable variation in culturable microbes in sputum between different European countries. The additive role of next-generation metagenomic sequencing remains unexplored and the association with antimicrobial resistomes unknown.

Methods We used next-generation shotgun metagenomic sequencing to prospectively assess sputum from 349 individuals recruited into the EMBARC Bronchiectasis Research Involving Databases, Genomics and Endotyping (BRIDGE) study from three European regions: Northern and Western Europe, Southern Europe and the UK. Samples were included from eight European countries. Microbiome and resistome profiles were assessed in relation to clinical outcomes.

Results Next-generation metagenomic sequencing reproduced differences between countries in microbial profiles that were previously shown by culture in the EMBARC study. Metagenomics provided enhanced detection for some bronchiectasis pathogens, including *Pseudomonas aeruginosa*, *Haemophilus influenzae* and *Streptococcus pneumoniae*. Three metagenomic microbial clusters dominated by the genera *Pseudomonas*, *Streptococcus* and *Haemophilus* demonstrated pan-European but variable distribution. Diverse resistomes, linked to underlying microbiomes, were identified across Europe, with significantly higher diversity of resistance gene determinants in Southern Europe. Resistome composition significantly differed between regions, characterised by regionally contrasting multidrug-resistant profiles. The EMBARC-BRIDGE cohort validated established bronchiectasis resistotypes RT1 and RT2, which occur at varying frequency across regions. Despite geographic variation in microbiome and resistome profiles in

bronchiectasis across Europe, analogous antimicrobial resistance gene profiles associated with the key bronchiectasis genera *Pseudomonas*, *Streptococcus* and *Haemophilus*, independent of country or region.
Conclusion Sputum metagenomics confirms and extends prior observations of regional variation in bronchiectasis microbiology. Important variation in the distribution of pathogens and antimicrobial resistance genes has implications for antimicrobial practices across Europe.

Introduction

Bronchiectasis is a chronic respiratory disease characterised by progressive and irreversible airway dilatation. This is underpinned by a pathogenesis referred to as a "vicious vortex", in which infection, inflammation and epithelial and mucociliary dysfunction intersect concurrently rather than sequentially with structural airway damage. Patients with bronchiectasis often experience progressive symptomatic disease with a clinical course of recurrent airway infection and exacerbations [1, 2]. The prevalence and recognition of bronchiectasis is increasing globally and carries a significant socioeconomic burden on individuals and healthcare systems [2–5]. While there are currently no available licensed therapies, largely owing to disease heterogeneity, emerging approaches focusing on precision medicine and targeting inflammation are showing promise [6–13].

Because infection remains a key aspect of the vicious vortex model of pathogenesis, a number of studies employing next-generation sequencing (NGS) have been performed to study the microbiome and better understand resident microbial communities [10, 14–18]. The microbiome in bronchiectasis reveals reduced diversity, and *Pseudomonas*-dominance is characterised by greater disease severity, more frequent exacerbations and higher mortality in contrast to other organisms [19, 20]. Critically, the established geographic variation in microbiology and microbiomes in bronchiectasis further underscores disease heterogeneity, significantly influencing endophenotypes, treatment responses and exacerbation-related differences [4, 21–24]. While microbial composition does not change during exacerbations, alterations in microbial interactions are observed that associate with exacerbation risk [22, 25–29]. The use of NGS has revolutionised our understanding of the microbiome, demonstrating high concordance with and greater sensitivity than conventional culture for pathogen detection across diagnostic settings [30, 31].

Use of long-term and inhaled antibiotics is linked to clinical and symptomatic benefit in bronchiectasis; however, long-term and/or repeated antibiotic use predisposes to the development of drug resistance [32–34]. A complex relationship therefore exists between dominant and residential microbial communities, particularly when placed under selective antibiotic pressure, which is further influenced by disease severity and clinical status. With the increasing use of shotgun metagenomics, a core macrolide resistome has been linked to underlying microbiomes across respiratory disease, including bronchiectasis [35]. Bronchiectasis-specific and therapeutically modifiable "resistotypes" (RTs) are described and associated with clinical outcomes [35, 36].

In 2023, the European Multicentre Bronchiectasis Audit and Research Collaboration (EMBARC) published registry findings in 16 963 individuals across Europe and reported geographic variation in sputum microbiology by traditional culture [4, 37]. *Pseudomonas* and *Haemophilus* were identified as the two most common pathogens, with *Pseudomonas* being most common in Southern Europe and *Haemophilus* being more common by culture in Northern Europe and the UK. These may be true differences, but the low sensitivity of culture combined with differences in laboratory practices mean that it remains uncertain whether there are clinically relevant differences in the pathogen profiles between European countries. Leveraging individuals from the EMBARC registry and enrolled into the Bronchiectasis Research Involving Databases, Genomics and Endotyping (BRIDGE) sub-study, we investigated the additive value of next-generation metagenomic sequencing to that provided by traditional culture, and explored geographic variation in microbiomes and resistomes across Europe and their implications on clinical outcomes [38, 39]. The failure of large-scale international clinical trials in bronchiectasis to replicate results underscores the inherent significant heterogeneity in bronchiectasis, even across Europe, where several countries have been participating sites [40–44]. While several studies by our group and others have previously assessed geographic variation of the microbiome on a broader (continental) scale, a distinct lack of comparative studies across regions such as Europe exists. This study addresses this gap, investigating within-Europe variation across multiple regions and countries, and providing fresh insights into the regional microbiome variation previously unexplored [29, 36, 45].

Materials and methods

Study population

We prospectively recruited a cohort of individuals (n=349) with stable bronchiectasis as part of the EMBARC-BRIDGE study (ClinicalTrials.gov Identifier: NCT03791086; supplementary methods). A total of 566 patients were initially enrolled up to December 2021. Of these, 424 patients provided a sputum sample suitable for analysis. DNA was successfully extracted from 417 of these samples, and after sequencing and quality control filtering, data from 349 patients were retained for the final analysis.

A detailed overview of the sample selection process, including exclusion at each stage, is provided in supplementary figure E1. Briefly, patients with a primary diagnosis of bronchiectasis, defined as the presence of radiological findings on high-resolution computed tomography consistent with bronchiectasis (*i.e.* bronchial dilatation with broncho-arterial ratio >1, lack of airway tapering and/or airway visibility within 1 cm of the pleura space [46, 47]) affecting one or more lobes, with clinical symptoms of cough, sputum production and/or recurrent respiratory infections were recruited [47]. Patients were clinically stable at recruitment, defined as the absence of antibiotic treatment for a pulmonary exacerbation in the preceding 4 weeks [37, 48]. Patients were excluded if they were unable to provide informed consent; were <18 years of age; or had active tuberculosis, bronchiectasis due to cystic fibrosis, solely traction bronchiectasis due to an alternate predominant respiratory disease or prior heart or lung transplantation [49]. Individuals were recruited from three European regions that included eight separate countries as follows: Northern and Western Europe (Belgium, n=21; Germany, n=13; Netherlands, n=5), Southern Europe (Greece, n=34; Italy n=34; Spain, n=106) and the UK (England, n=25; Scotland, n=111). Countries were grouped according to the modified EU EuroVoc classification [37]. All clinical data were collated for analysis in line with EMBARC-BRIDGE protocols and sample collection was performed as described. The study was approved by the research ethics committee in each participating country (18/LO/1935) and all patients gave written informed consent to participate. Details on clinical data and specimen collection are provided in the supplementary methods.

DNA extraction and metagenomic sequencing

DNA extraction was performed at the University of Dundee, Scotland. Extracted DNA was transported to Nanyang Technological University (NTU), Singapore, for metagenomic sequencing. Sputum DNA was extracted using the DNeasy PowerSoil Pro Kit (Qiagen, cat. no. 47016) as previously described [50]. Briefly, 100 mg sputum was homogenised by bead-beating for 15 min on the Vortex-Genie 2 (Scientific Industries), followed by centrifugation at 15 000 g for 1 min before loading onto the QIAcube connect (Qiagen) for processing according to manufacturers' instructions. Samples were processed in batches alongside 100 µL of molecular grade water (Sigma-Aldrich) as DNA extraction blanks. To ensure quality control during transport to Singapore, DNA samples were shipped on dry ice in temperature-controlled containers. The integrity and quantity of extracted sputum DNA was determined using a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) on arrival in Singapore. DNA was subjected to shotgun metagenomic sequencing using a HiSeq 2500 platform (Illumina) at the NTU core sequencing facility according to standard library preparation and DNA sequencing protocols, as previously described [29, 35, 51, 52]. Both DNA extraction blanks (n=3) and sequencing library blanks (n=3) were included to assess for experimental background contamination associated with DNA extraction and metagenomic sequencing, respectively (supplementary figure E2). All raw short-read metagenomics sequencing data used in this study have been submitted to the National Center for Biotechnology Information Sequence Read Archive under project accession number PRJNA1186390.

Statistical analysis

All categorical data were compared using the chi-squared or Fisher's exact test as appropriate. Non-parametric Wilcoxon or Kruskal–Wallis testing was applied for group comparisons of continuous variables, as appropriate. In cases of more than two groups, Dunn's *post hoc* test with Benjamini–Hochberg correction was applied to control for a false discovery rate arising from multiple comparisons. As a preprocessing step, all microbiome datasets were filtered to include only microbes with ⩾1% relative abundance in ⩾5% of patients (*i.e.* n=18). The resistome dataset was filtered to include resistance genes with ⩾0.1% relative abundance in ⩾1% of patients (*i.e.* n=4). Metagenomics-based microbial presence was defined as >1% relative abundance. Microbiome α-diversity was calculated using the Shannon diversity index, and differences in microbiome β-diversity between groups was assessed by permutational multivariate analysis of variance (PERMANOVA) based on Bray–Curtis dissimilarity using the R package "vegan" (version 2.6–4) [53]. Principle coordinate analysis based on Bray–Curtis dissimilarity of microbiome and resistome profiles was carried out using the R package "phyloseq" (version 1.46.0) [54]. Differential abundance analysis of microbiome and resistome profiles was performed by linear discriminant analysis effect size implemented using the R package "microbiomeMarker" (version 1.0.2) with default parameters [55]. Spectral clustering was implemented using the R package "SNFtool" (version 2.3.1) to cluster microbiome and resistome profiles based on the affinity matrix computed using Bray–Curtis dissimilarity [56]. The optimal number of clusters for microbiome (k=3) and resistome (k=2) profiles was determined using the eigengap method and cluster quality assessed by silhouette scores. Silhouette scores were 0.65 and 0.89 for microbiome and resistome clusters, respectively, indicating good cluster quality [29, 36, 56]. Unsupervised resistome clusters were subsequently validated against the original RTs [36] using a supervised nearest-neighbour approach. For each patient in the EMBARC-BRIDGE cohort, a predictive RT label (RT1 or RT2) was assigned based on Bray–Curtis

distance, by comparing their resistome profile to that of the closest match from the Cohort of Asian and Matched European Bronchiectasis 2 (CAMEB2) cohort, which served as the reference for the original RTs. All statistical analyses were performed using custom scripts written in R (version 4.1.3, www.r-project.org) and Python (version 3.10.6, www.python.org). Data visualisation was performed in R using "ggplot2" package (version 3.4.2). A p-value of <0.05 was considered statistically significant.

Further details on quality control, preprocessing, microbiome and antimicrobial gene assignment from metagenomics are provided in the supplementary methods.

Results

The lung microbiome in bronchiectasis demonstrates geographic variation across Europe

Sputum metagenomics of 349 individuals with bronchiectasis from the EMBARC-BRIDGE study were assessed. Patient demographics and clinical data are summarised in table 1. Geographic variation in sputum microbiology, by culture, was previously reported in the published EMBARC registry data [37]. Here, we illustrate that metagenomic sequencing broadly reproduces the regional variation in the prevalence of the most common isolated microorganisms by culture (figure 1a–d) [37]. While a higher prevalence of *Pseudomonas aeruginosa* was observed in Northern and Western Europe (NWE) (n=39) and Southern Europe (SE) (n=174) by metagenomics compared to culture, SE exhibits the highest relative abundance of this organism by metagenomics (figure 1a–d). Metagenomics revealed a higher prevalence and significantly greater relative abundance of *Haemophilus influenzae* in individuals from the UK (n=136) compared to SE (n=174). Median relative abundance was higher in the UK than in SE (0.4%, interquartile range (IQR) 0.1–17.0% *versus* 0.2%, IQR 0.1–8.7%; Kruskal–Wallis test p<0.05; Dunn's *post hoc* test with Benjamini–Hochberg correction) (figure 1a–d). Of note, *Streptococcus pneumoniae* demonstrated high detection by metagenomics (*versus* culture) across all regions, reinforcing established clinical challenges in isolating this *Streptococcus* species by culture-based methodologies (figure 1a) [57]. Metagenomic sequencing revealed significant differences in microbial diversity and composition between regions, with SE demonstrating the lowest α-diversity (figure 1d–f). Differential abundance analysis identified the microbes that were increased between regions as *Gemella sanguinis*, *Actinomyces oris* and *Schaalia odontolytica* (NWE); *P. aeruginosa* (SE); and *H. influenzae*, *Streptococcus parasanguinis* and *Streptococcus salivarius* (UK) (figure 1g). Importantly, the microbiome exhibited geographic variation across regions even within disease severity status groups, exacerbator status groups and among long-term antibiotic user groups (supplementary figures E3–E5).

Unsupervised clustering of sputum metagenomes in bronchiectasis identified three patient clusters with Pan-European distribution

We next performed unsupervised spectral clustering of sputum metagenomes and identified three clusters, discriminated by commonly identified bacterial species from the bronchiectasis airway: *P. aeruginosa* (n=71), *Streptococcus* spp. (n=224) and *H. influenzae* (n=54) (figures 2a–c). The highest α-diversity was observed in the *Streptococcus*-dominant cluster in comparison to the two other clusters (both p<0.001 *versus* the *Streptococcus*-dominant cluster) (figure 2d). A pan-European distribution was observed in all three clusters, differing significantly by regional frequency of occurrence (p<0.001) (figure 2e). The highest proportion of *Pseudomonas*-dominance occurred in SE, making up to one third of individuals (33%), while *Haemophilus*-dominance predominated in the UK, occurring in over one fifth of individuals (21%) (figure 2e).

The Pseudomonas*-dominant cluster associates with the poorest clinical outcomes*

In line with the established literature in bronchiectasis [58–61], the *Pseudomonas*-dominant cluster exhibited the greatest clinical severity, evidenced by a higher exacerbation frequency (median 2 events, IQR 1–4 events; p<0.01), increased disease severity (median Bronchiectasis Severity Index 10 points, IQR 8–13 points; p<0.001), poorest lung function (median forced expiratory volume in 1 s 55.5% predicted, IQR 41.0–69.9% predicted; p<0.001) and increased symptoms (median Medical Research Council dyspnoea scale 3, IQR 2–4; p<0.01) when compared to the other clusters (figure 3a–d). Clinical parameters were comparable between the *Streptococcus*-dominant and *Haemophilus*-dominant clusters.

The bronchiectasis resistome and associated resistotypes demonstrate geographic variation across Europe

The bronchiectasis resistome, encompassing the distinct repertoire of known antimicrobial resistance (AMR) genes detectable in the airway metagenomic analysis, is described in association with its underlying lung microbiome; however, geographic variation has not been examined [35, 36]. Having determined that geographic variation to lung microbiomes in bronchiectasis exists across Europe, we next assessed for variation in underlying resistomes and for the existence of previously established RTs [36].

TABLE 1 Summary of the EMBARC-BRIDGE bronchiectasis cohort including demographic and clinical variables

	EMBARC-BRIDGE	European region			
		Northern and Western Europe	Southern Europe	UK	p-value (across regions)
Subjects, n	349	39	174	136	
Age, years	68 (59–77)	54 (37–70)	69 (61–77)	70 (61–76)	<0.001***
Sex					NS
Male	177 (50.7)	15 (38.5)	83 (47.7)	79 (58.1)	
Female	172 (49.3)	24 (61.5)	91 (52.3)	57 (41.9)	
Aetiology					<0.001***
Idiopathic	199 (57.0)	19 (48.7)	88 (50.6)	92 (67.7)	
Post-infective	67 (19.2)	6 (15.4)	52 (29.9)	9 (6.6)	
Other	83 (23.8)	14 (35.9)	34 (19.5)	35 (25.7)	
Smoking status					<0.001***
Never	191 (54.7)	28 (71.8)	108 (62.1)	55 (40.4)	
Current	24 (6.9)	0 (0.0)	10 (5.7)	14 (10.3)	
Ex-smoker	134 (38.4)	11 (28.2)	56 (32.2)	67 (49.3)	
BSI status					0.023*
Mild	91 (26.1)	15 (38.5)	40 (23.0)	36 (26.5)	
Moderate	126 (36.1)	8 (20.5)	59 (33.9)	59 (43.4)	
Severe	132 (37.8)	16 (41.0)	75 (43.1)	41 (30.1)	
BMI, kg·m^{-2}	25 (22–29)	23 (21–27)	24 (22–27)	27 (24–31)	<0.001***
MRC dyspnoea score					0.013*
1–3	276 (79.1)	34 (87.2)	146 (83.9)	96 (70.6)	
4	44 (12.6)	5 (12.8)	17 (9.8)	22 (16.2)	
5	29 (8.3)	0 (0.0)	11 (6.3)	18 (13.2)	
FEV_1, % predicted	71 (53–92)	75 (56–92)	67 (49–84)	79 (55–97)	0.010*
Radiological severity					NS
1–2 lobes involved	131 (37.5)	14 (35.9)	66 (37.9)	51 (37.5)	
⩾3 lobes involved	218 (62.5)	25 (64.1)	108 (62.1)	85 (62.5)	
Exacerbator status					<0.001***
0	104 (29.8)	20 (51.3)	32 (18.4)	52 (38.3)	
1 to 2	121 (34.7)	10 (25.6)	73 (42.0)	38 (27.9)	
3 or more	124 (35.5)	9 (23.1)	69 (39.6)	46 (33.8)	
Hospital admissions in the year preceding recruitment					<0.001***
Yes	117 (33.5)	29 (74.4)	45 (25.9)	43 (31.6)	
No	232 (66.5)	10 (25.6)	129 (74.1)	93 (68.4)	
***Pseudomonas* colonisation**					<0.001***
Yes	77 (22.1)	16 (41.0)	50 (28.7)	11 (8.1)	
No	272 (77.9)	23 (59.0)	124 (71.3)	125 (91.9)	
Colonisation with other organisms					<0.001***
Yes	77 (22.1)	22 (56.4)	31 (17.8)	24 (17.6)	
No	272 (77.9)	17 (43.6)	143 (82.2)	112 (82.4)	
Bronchodilators					NS
Yes	214 (61.3)	26 (66.7)	108 (62.1)	80 (58.8)	
No	135 (38.7)	13 (33.3)	66 (37.9)	56 (41.2)	
Inhaled corticosteroids					NS
Yes	197 (56.4)	20 (51.3)	97 (55.7)	80 (58.8)	
No	152 (43.6)	19 (48.7)	77 (44.3)	56 (41.2)	
Mucolytics					<0.001***
Yes	80 (22.9)	27 (69.2)	4 (2.3)	49 (36.0)	
No	269 (77.1)	12 (30.8)	170 (97.7)	87 (64.0)	
Long-term antibiotics					NS
Yes	113 (32.4)	16 (41.0)	49 (28.2)	48 (35.3)	
No	236 (67.6)	23 (59.0)	125 (71.8)	88 (64.7)	

Data are presented as median (interquartile range) or n (%), unless otherwise indicated. BSI: Bronchiectasis Severity Index; BMI: body mass index; MRC: Medical Research Council; FEV_1: forced expiratory volume in 1 s; NS: nonsignificant. *: p<0.05; **: p<0.01; ***: p<0.001.

Sputum metagenomics revealed a diverse AMR gene profile in bronchiectasis across Europe (figure 4a, b), with significantly higher diversity in SE compared to NWE (p<0.05) and the UK (p<0.001) (figure 4c). Resistome composition significantly differed across regions (figure 4d) and discriminant analysis identified

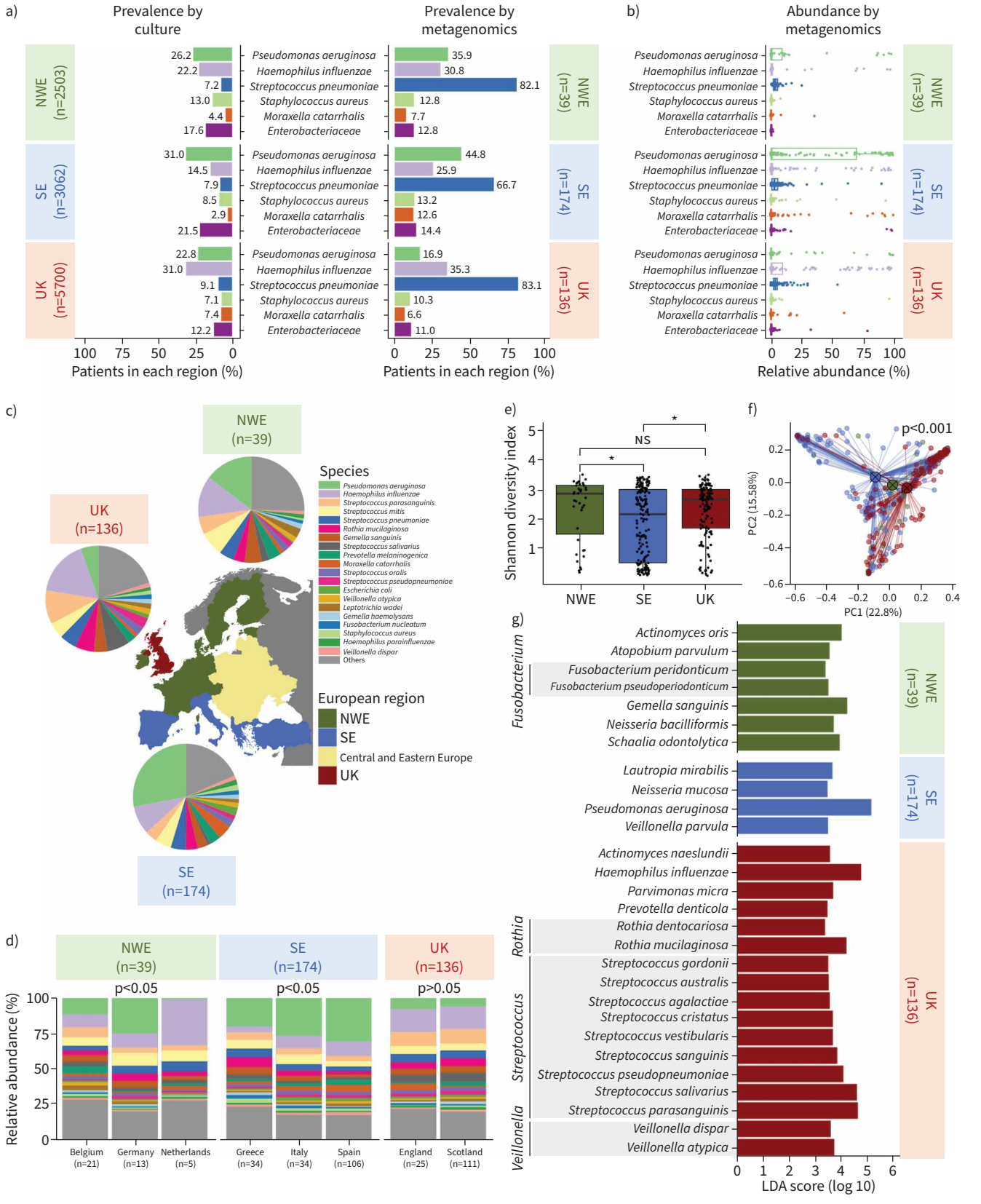

FIGURE 1 The lung microbiome in bronchiectasis exhibits geographic variation across Europe. a) Prevalence of microorganisms by sputum culture, defined as the isolation of a microorganism from sputum over the preceding year [37], in comparison to prevalence by shotgun metagenomic sequencing, where the microorganism is detected at >1% relative abundance. b) Relative abundance of the top six microorganisms detected in

bronchiectasis from Northern and Western Europe (NWE), Southern Europe (SE) and the UK. c) Pie charts and d) stacked bar plots illustrating lung microbiome profiles in bronchiectasis from NWE, SE and the UK. p-value determined by PERMANOVA. e) Differences in α-diversity (Shannon diversity index) of the lung microbiome across Europe. Data presented as median and interquartile ranges, with whiskers indicating range. f) Principal coordinate analysis (PCoA) plot, based on Bray–Curtis dissimilarity, illustrating differences in β-diversity of the lung microbiome across Europe. PCoA centroids of the individual regions are indicated with circles intersected by a cross. p-value determined by PERMANOVA. g) Linear discriminant analysis (LDA) effect size analysis illustrating differentially abundant microbial species across Europe. NS: nonsignificant; PC: principal coordinate. *: p<0.05.

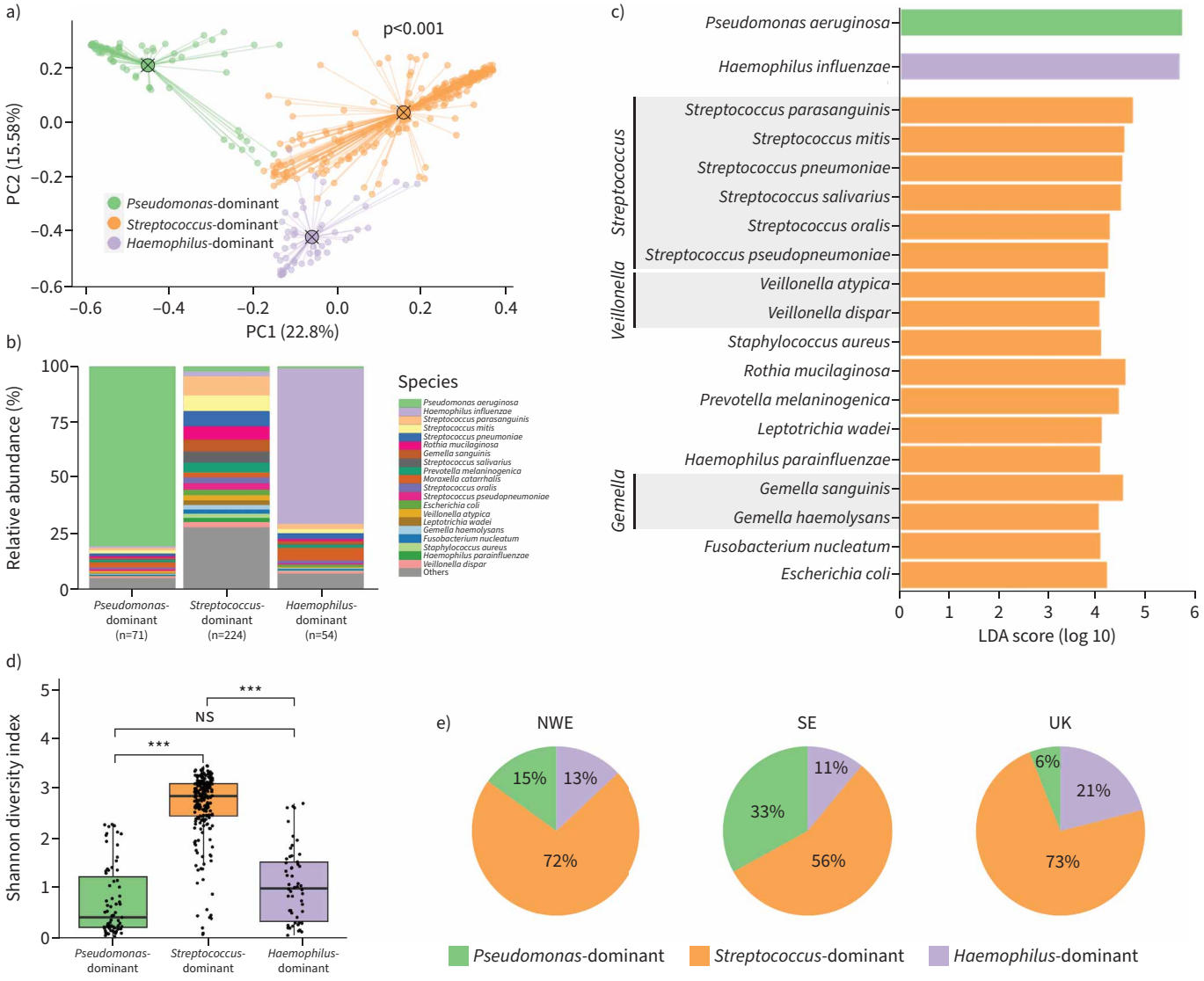

FIGURE 2 Unsupervised spectral clustering of the sputum metagenome in bronchiectasis reveals three clusters with pan-European distribution. a) Principal coordinate analysis (PCoA) plot, based on Bray–Curtis dissimilarity, illustrating three microbiome clusters: *Pseudomonas*-dominant (n=71; green), *Streptococcus*-dominant (n=224; orange) and *Haemophilus*-dominant (n=54; purple). Circles intersected by a cross indicate PCoA centroids of the identified clusters. p-value determined by PERMANOVA. b) Stacked bar plots illustrating microbiome composition of the three clusters. c) Linear discriminant analysis (LDA) effect size analysis illustrating the top 20 differentially abundant microbial species across the identified clusters. d) Differences in α-diversity (Shannon diversity index) of the lung microbiome across the identified clusters. Data presented as median and interquartile ranges, with whiskers indicating range. e) Distribution of the identified clusters across the respective regions in Northern and Western Europe (NWE) (n=39), Southern Europe (SE) (n=174) and the UK (n=136). NS: nonsignificant; PC: principal coordinate. ***: p<0.001.

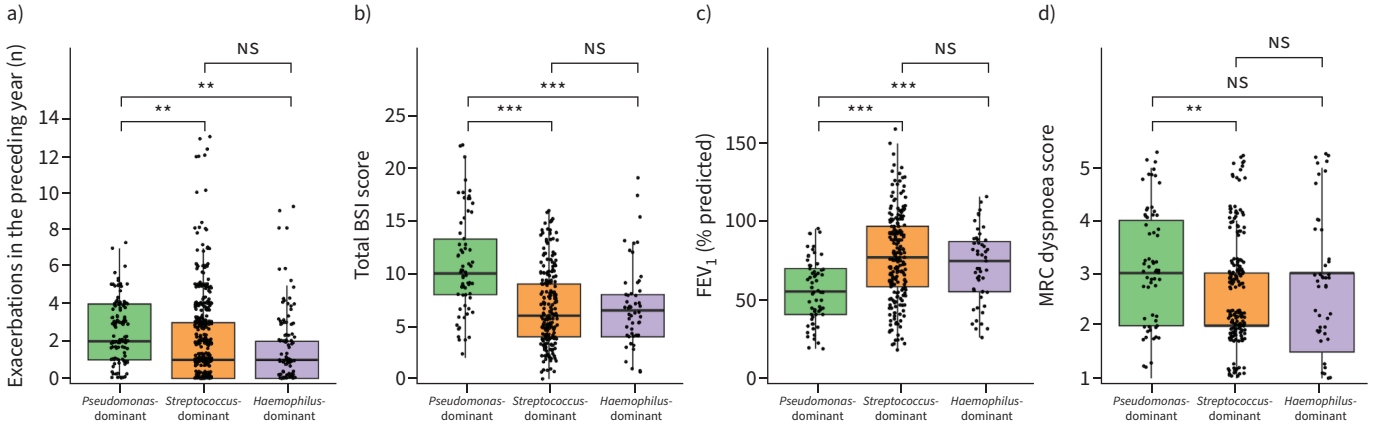

FIGURE 3 The *Pseudomonas*-dominant microbiome cluster is associated with increased exacerbations, greater disease severity and the poorest lung function in comparison to the *Haemophilus*-dominant or *Streptococcus*-dominant clusters. Box plots illustrating significant differences in a) exacerbation frequency, b) disease severity (recorded using the Bronchiectasis Severity Index (BSI)), c) lung function (as forced expiratory volume in 1 s (FEV_1) % predicted) and d) dyspnoea score (using Medical Research Council (MRC) scale) across the three clusters. Data presented as median and interquartile ranges, with whiskers indicating range. NS: nonsignificant. **: p<0.01; ***: p<0.001.

a range of differentially abundant AMR genes between regions, most notably belonging to drug classes of therapeutic antibiotics including aminoglycosides, β-lactams, fluoroquinolones, macrolide-lincosamide-streptogramin (MLS) and tetracyclines (figure 4e). NWE and SE were importantly discriminated by profiles of AMR genes conferring multidrug resistance (which further varied between these regions), and aminoglycoside and MLS resistance. In contrast, the UK exhibited less discriminant AMR genes, largely conferring tetracycline resistance (figure 4e).

Prior work by our group used the CAMEB2 to define two therapeutically modifiable RTs, RT1 and RT2, with the latter RT associated with poor clinical outcomes [36]. We next sought to validate these RTs in the EMBARC-BRIDGE cohort and assess their geographic distribution across regions. Unsupervised spectral clustering of the lung resistome re-capitulated the two RTs, RT1 and RT2 (figure 5a, left), demonstrating a high degree of concordance with that previously established with the CAMEB2 cohort (figure 5a, right). Resistotype concordance was further confirmed by cross-assigning individuals from the EMBARC-BRIDGE cohort with the nearest RT centroid established in the published CAMEB2 cohort, again demonstrating strong concordance: 100% in RT1 (n=235) and 93% in RT2 (n=83) (figure 5b). In the EMBARC-BRIDGE cohort, fluoroquinolone, MLS and tetracycline resistance genes discriminated RT1, while RT2 was characterised by multidrug, aminoglycoside, phenicol and bicyclomycin resistance genes (figure 5c) [36], re-capitulating 85% (17 out of 20 AMR genes) for RT1 and 89% (47 out of 53 AMR genes) for RT2 from the previously published CAMEB2 cohort [36]. Clinical correlates of the RTs were reproduced with RT2 (relative to RT1), demonstrating increased exacerbation frequency, greater disease severity, poorer lung function and increased symptoms (figure 5d). Importantly, RT occurrence exhibited significant geographic variation across Europe, with the highest prevalence of RT2 in SE (42%; n=67) followed by NWE (27%; n=10) (figure 5e).

Microbiome–resistome correlation networks across Europe identify analogous antimicrobial resistance gene profiles associated with key microbial genera

Because we established that bronchiectasis microbiomes and resistomes vary by region across Europe, we next sought to determine whether such variation is driven by variability in the key microbial genera: *Pseudomonas*, *Streptococcus* and *Haemophilus* spp (*c.f.* figure 2). Microbiome–resistome co-occurrence analyses identified the greatest association in SE among all regions (figure 6a), driven by the greater diversity of the resistance gene profile harboured by the microbiome in this region (figure 4c). *Pseudomonas* spp. associated with RT2 discriminant genes, while *Streptococcus* and *Haemophilus* spp. associated with RT1 discriminant genes, independent of geographic origin (figure 6b–d, supplementary tables E1 and E2). Importantly, the top discriminating RT2 resistance genes, including multidrug (*OprM*, *mexI*, *mexH*, *mexK*, *mexV*, *MexE*), MLS (*PA_CpxR*), aminoglycoside (*APH(3')-IIb*), phenicol (*PA_catB7*) and bicyclomycin (*bcr-1*), associated with *Pseudomonas* spp. comparably across all regions (figure 6b). Similarly, highly discriminating RT1 resistance genes, including fluoroquinolone (*patA*, *patB*, *pmrA*), MLS (*RlmA(II)*, *ErmB*, *ErmF*) and tetracycline (*tetB(46)*) associated with *Streptococcus* spp. comparably

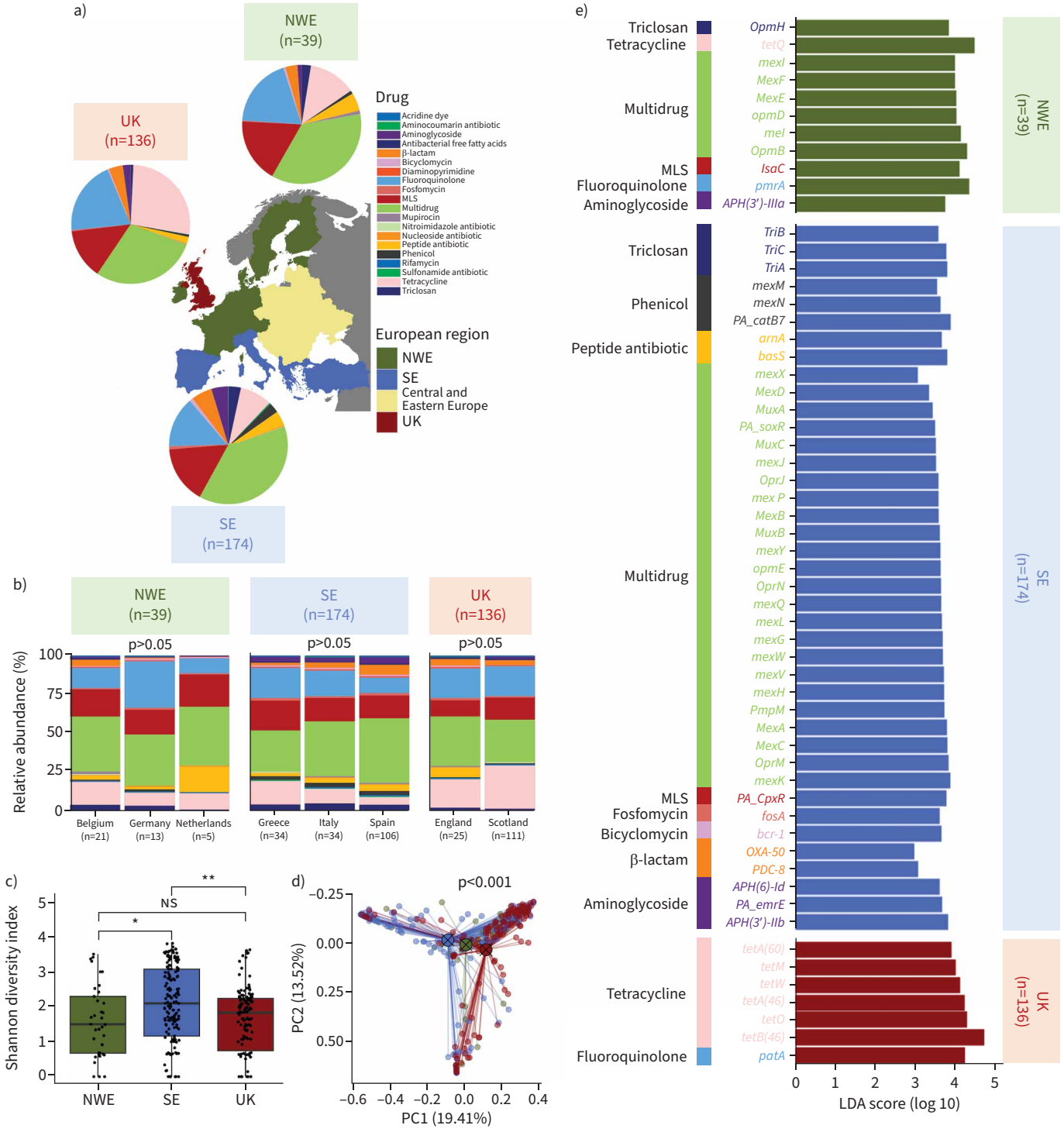

FIGURE 4 The resistome in bronchiectasis demonstrates geographic variation across Europe. a) Resistome profiles in bronchiectasis across Northern and Western Europe (NWE) (n=39), Southern Europe (SE) (n=174) and the UK (n=136) with resistance genes grouped and coloured according to drug class. b) Resistome profiles in NWE, SE and the UK. Resistance genes are grouped and coloured according to drug class. p-value determined by PERMANOVA. c) Differences in α-diversity (Shannon diversity index) of the resistome profiles across the respective European regions, presented as median and interquartile ranges, with whiskers indicating range. d) Principal coordinate analysis (PCoA) plot, based on Bray–Curtis dissimilarity, illustrating differences in β-diversity of the resistome across the respective European regions. PCoA centroids of the individual regions are indicated with circles intersected by a cross. p-value determined by PERMANOVA. e) Linear discriminant analysis (LDA) effect size analysis illustrating differentially abundant resistance gene determinants across the respective European regions. MLS: macrolide-lincosamide-streptogramin; NS: nonsignificant; PC: principal coordinate. *: p<0.05; **: p<0.01.

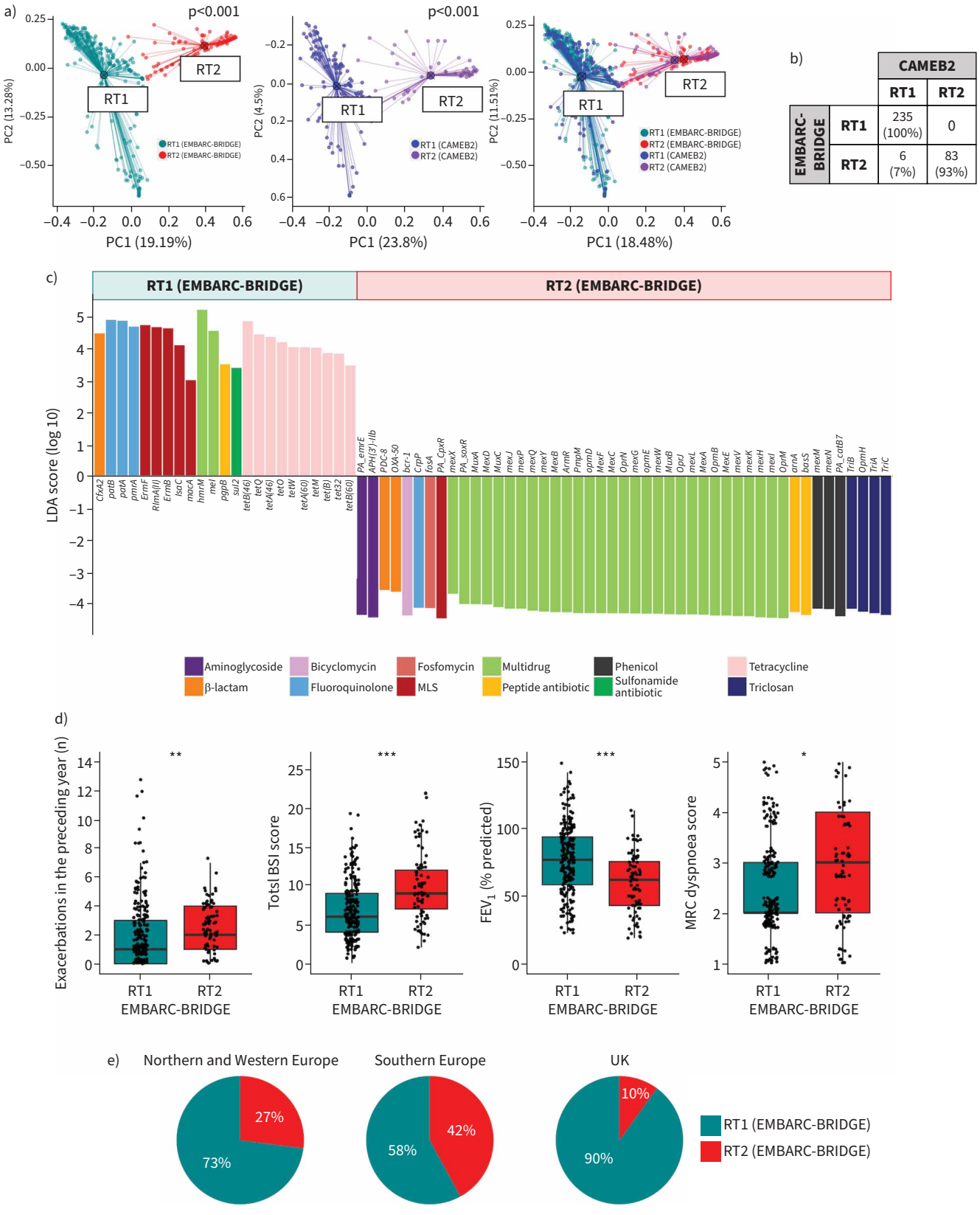

FIGURE 5 Resistotypes in bronchiectasis demonstrate geographic variation across Europe. a) Principal coordinate analysis (PCoA) plots of resistance gene profiles, based on Bray–Curtis dissimilarity, illustrating resistotype (RT) clusters in the EMBARC-BRIDGE cohort (left); the CAMEB2 cohort [36] (centre) and both cohorts combined (right), demonstrating high concordance. Circles intersected by a cross indicate PCoA centroids of the identified clusters. p-value determined by PERMANOVA. b) Concordance table illustrating the high degree of consensus in RT classifications

between the EMBARC-BRIDGE cohort and CAMEB2 cohort: 100% in RT1 (n=235) and 93% in RT2 (n=83). c) Linear discriminant analysis (LDA) effect size analysis of resistance gene determinants in the RT1 and RT2 resistotype clusters in the EMBARC-BRIDGE cohort. d) Differences in exacerbation frequency (far left), disease severity (recorded using the Bronchiectasis Severity Index (BSI)) (middle left), lung function (as forced expiratory volume in 1 s (FEV_1) % predicted) (middle right) and dyspnoea score (using Medical Research Council (MRC) scale) (far right) between RT1 (turquoise) and RT2 (red) in the EMBARC-BRIDGE cohort. e) Distribution of RTs across the respective regions in Europe. MLS: macrolide-lincosamide-streptogramin; NS: nonsignificant; PC: principal coordinate. *: p<0.05; **: p<0.01; ***: p<0.001.

across all regions (figure 6c). Meanwhile. *Haemophilus* spp. associated, across all regions, with *hmrM*, a multidrug-resistance gene linked to RT1 (figure 6d). We additionally assessed resistance profiles of other most abundant microbes not recognised as traditional pathogens, *i.e. Rothia*, *Gemella* and *Prevotella* spp. (supplementary figure E6a–c, supplementary tables E3–E5). Notably, the associated profiles of resistance genes comprised RT1-discriminant genes, with apparent geographic variability in antibiotic classes.

Discussion

Here, we show that the lung microbiome in bronchiectasis demonstrates geographic variation in diversity and composition across Europe, broadly reproducing results from traditional culture for common bronchiectasis organisms including *P. aeruginosa* and *H. influenzae*; however, an improved detection of "difficult to culture" organisms such as *S. pneumoniae* is gained by metagenomics. Culture positivity relates to the metagenomic abundance, suggesting that culture reports the most prevalent bacteria. Three metagenomically derived patient clusters dominated by *P. aeruginosa*, *H. influenzae* and *Streptococcus* spp., respectively, illustrates a pan-European distribution, but they differed in their frequency and occurrence by region. Comparably, the bronchiectasis resistome also demonstrated geographic variation across Europe, validating established bronchiectasis RTs despite a varying frequency of occurrence for RT1 and RT2 across regions [36]. While SE and NWE have the highest frequency of RT2, each was discriminated by AMR gene profiles conferring multidrug resistance, with their specific multidrug AMR genes varying by individual region. Interestingly, the variation in resistomes across Europe was not driven by the inherent microbial differences, but rather a consistent microbiome–resistome correlation was observed between key bronchiectasis genera (independent of European region) and their associated AMR gene pattern. This suggests that a targeted approach to microbial eradication based on metagenomics may hold therapeutic value.

A key finding of this work was that shotgun metagenomic sequencing largely reproduces broad regional prevalence and variation by culture across Europe for the commonest bronchiectasis pathogens, including *P. aeruginosa* and *H. influenzae* [37]. Metagenomics demonstrated increased detection for a number of other important potential pathogens, *e.g. S. pneumoniae* was detected at significantly higher frequencies although recovery from culture is described as challenging and often negative [62–65]. Notably, there is currently no gold standard for the diagnosis of *S. pneumoniae* lung infection [57] and sputum culture remains an imperfect measure of detection despite routine use in clinical diagnostics [66]. Despite intense efforts to develop molecular assays to detect *S. pneumoniae* from clinical specimens, their robustness and clinical utility remain uncertain, offering a potential role for metagenomics [67]. While the widespread implementation of metagenomics into routine clinical practice is at a very early stage, our findings support an important and potential role for respiratory pathogen surveillance in bronchiectasis and other respiratory diseases [68–70]. The association between culture and metagenomics, nonetheless, is not yet fully understood, exemplified by microbes such as nontuberculous mycobacteria [71, 72]. A limitation of most studies is that culturomics are not routinely performed, which may increase culture positivity rates. These approaches will have key roles in bridging the gap between microbiological culture-based work-up and airway metagenomic profiling, allowing greater insight into sensitivity and specificity and defining complex microbial culture conditions required for the growth of fastidious organisms. Upper airway microbes may outcompete and limit the growth of lower airway microbes in culture, potentially masking the presence of pathogens through bacterial persistence mechanisms, including "viable but non-culturable" states [73]. Notably, negative sputum culture is an interesting feature of bronchiectasis; the EMBARC registry reported 60–70% of patients having negative cultures [49]. This is possibly reflective of the respiratory "adaptive island model", whereby active bacterial elimination limits the number of viable bacteria detected at the time of sputum sampling [74, 75]. This suggests a sampling limitation rather than true lack of culture sensitivity. Nevertheless, emerging evidence supports the clinical utility of metagenomics for infection control, guiding antimicrobial treatment decisions and informing public health strategies [68, 76].

The environment influences microbiome composition and geographic variation is documented beyond the lung [77–79]. Distinct geographic microbiome features in bronchiectasis include taxonomic and functional

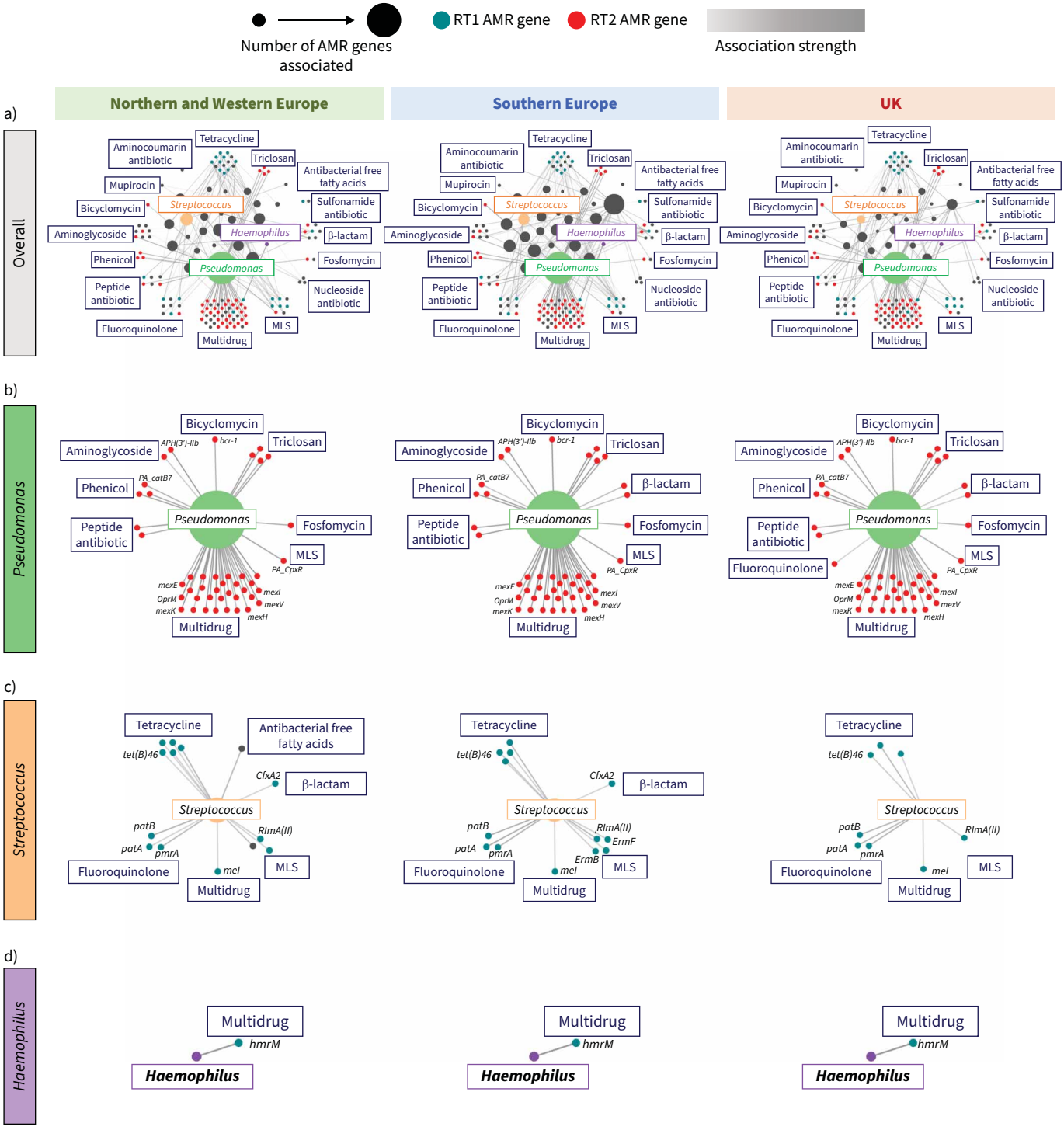

FIGURE 6 Microbiome–resistome correlation networks identify analogous antimicrobial resistance (AMR) gene profiles associated with key microbial genera (*Pseudomonas*, *Streptococcus* and *Haemophilus*) across Europe. a) Overall microbiome–resistome correlation networks classified by respective European region with the EMBARC-BRIDGE cohort. Nodes representing microbial genera are placed centrally, with nodes indicating AMR genes surrounding and grouped by drug class. The size of a microbial node reflects the number of AMR genes associated, with a bigger node indicating a higher gene number. Nodes representing *Pseudomonas*, *Streptococcus* and *Haemophilus* are coloured green, orange and purple, respectively, and represent key microbes. Nodes representing AMR gene determinants that discriminate resistotypes (RT) are coloured turquoise for RT1 and red for RT2. Grey lines indicate associations between microbes and respective AMR genes, with line transparency reflecting the strength of the association. AMR genes associated with b) *Pseudomonas*, c) *Streptococcus* and d) *Haemophilus* across the respective European regions are further detailed. Nodes representing the top 10 most discriminant AMR genes associated with RT1 (turquoise) and RT2 (red) are indicated. MLS: macrolide-lincosamide-streptogramin.

differences, which inform regional endophenotypes [21, 22, 36]. Our findings align with global observations and, for the first time, confirm geographic variation of the bronchiectasis microbiome across Europe. This has important clinical and research implications, especially for antibiotic-focused clinical trials, in which due consideration of the predominant microbiology is required to facilitate trial design, planning and recruitment. Because global differences in bronchiectasis are now recognised, intra-continental differences, illustrated by this study, may have an important role, especially considering the failure to reproduce key findings across "paired" antibiotic clinical trials such as the RESPIRE [40, 41] and ORBIT [42] programmes. These trials assessed different formulations of inhaled ciprofloxacin, a fluoroquinolone antibiotic, in patients with bronchiectasis. In our study, regional variation in genes encoding fluoroquinolone resistance was identified across Europe, where *pmrA* and *patA* associate with NWE and the UK, respectively. Importantly, resistance to ciprofloxacin is also encoded by various multidrug efflux pumps, including *MexC*, *MexD* and *OprJ* [80–83], all of which are enriched in SE and discriminant for RT2, in which *P. aeruginosa* predominates. These findings underscore geographic variation in microbial drivers of resistance, supporting the implementation of geographically stratified approaches in the design of future clinical trials.

Notwithstanding this, it is important to acknowledge that various other genetic mutations or single nucleotide variants may emerge during antibiotic therapy (*e.g.* mutations in the *nfxB* gene that lead to efflux overexpression of *MexCD-OprJ* in *P. aeruginosa* [83]) that are not readily identified by the protein-based marker detection of resistance genes employed in our metagenomic analysis. This may contribute to emerging resistance over the course of clinical trials. Further, the mobilisation of pumps such a *MexCD-OprJ* highlights their role in mediating resistance beyond host species such as *P. aeruginosa* [84], adding further complexity to the interpretation of resistome data. The AIR-BX trials [43] of inhaled aztreonam (AIR-BX1 and AIR-BX2) also demonstrated discordant results, mirroring challenges seen in the RESPIRE and ORBIT programmes. Several mutational variants in negative transcriptional regulators of the *mexAB-OprM* efflux system have been implicated in aztreonam resistance in *P. aeruginosa*, and could result in resistance to structurally unrelated antipseudomonal antibiotics [85–87]. Interestingly, we identified consistent associations between *P. aeruginosa* and *mexAB-OprM* resistance genes across Europe. Environmental differences across Europe, however, may potentially promote geographic variants of mutations in negative regulators of the *mexAB-OprM* efflux system, culminating in a common end-point of resistance to aztreonam in *P. aeruginosa*. The emergence of such variants may not be captured in the Comprehensive Antibiotic Resistance Database (CARD) database, as used in this study. Geographic variation has been shown to influence outcomes in other disease states, including inflammatory bowel disease, and previously in bronchiectasis, where Asian and European patients were compared, reiterating the importance of location in determining microbiology, clinical presentation and, potentially, treatment response [22, 36, 45, 88]. Furthermore, even within the same country, an extrapolation of gut microbiome-based metabolic models across regions proved unsuccessful, adding further evidence to the importance of geographic variation in microbiome-based disease models [89]. Microbiome-related differences by geography can be attributed to a variety of host and environmental factors, including genetics, immunity, lifestyle, environmental exposures and dietary influences [90–94]. Taken together, understanding microbiome-related differences within regions, countries and continents, evidenced here from a European perspective, is critical to achieve the precision-based approach to endophenotyping, clinical care and treatment required in bronchiectasis.

Commensal organisms are generally unidentified or unreported by routine microbiology laboratories. Differential abundance analysis of bronchiectasis metagenomes across Europe, however, identifies an increased abundance of commensal organisms, including *Streptococcus* spp. and *Rothia mucilaginosa* in the UK and *A. oris*, *Neisseria bacilliformis*, *G. sanguinis* and *S. odontolytica* in NWE. The role of commensals in bronchiectasis remains an important area of ongoing research. For some of these organisms, a spectrum of pathogenesis ranging from commensal to pathobiont to pathogen is increasingly recognised [45, 95]. Oral commensals, thought to originate solely from the oropharyngeal compartment, have now been identified in the lower airway, with recent work demonstrating roles in modulating host immune responses [96–98]. *R. mucilaginosa* and *Aggregatibacter* inhibit inflammation, *P. melaninogenica* improves *S. pneumoniae* lung clearance, and a protective role for *Streptococcus mitis* in combination with *P. melaninogenica* and *Veillonella parvula* has been demonstrated in murine *S. pneumonia* challenge models through a MyD88-dependent priming of the Th17 response [99–103]. Conversely, *Neisseria subflava* represents a pathobiont in bronchiectasis, promoting airway damage and unfavourable metabo-lipidomic responses [45]. The emerging and varied roles of commensals in bronchiectasis should therefore be recognised, and metagenomics may play an important role in determining the relevance, differences and balance between beneficial commensals and harmful pathobionts under contrasting conditions and geographic location.

The resistome, a collection of AMR gene determinants, exhibits less geographic variability compared to microbiomes and, even in health, exhibits core macrolide resistance [35]. We observed key differences in the bronchiectasis resistome across European regions (*i.e.* UK, SE, NWE); yet, resistomes were largely homogenous across countries within regions, despite microbial differences. Several genes were linked to non-clinical antimicrobials (*e.g.* triclosan, phenicol), suggesting environmental influences on regional resistance profiles. Bronchiectasis RT1 and RT2 have been recently established, demonstrating clinical association and amenability to therapeutic intervention [36]. We validated these RTs with high accuracy across Europe, observing increased prevalence of RT2 in regions where microbiomes were dominated by *P. aeruginosa*, including SE. This correlation suggests that specific bronchiectasis-related genera associate with distinct AMR gene patterns across regions, independent of broader microbial diversity. Thus, in *Pseudomonas*-dominated regions (*e.g.* SE), antimicrobial strategies that prioritise treatments addressing *Pseudomonas*-associated resistance patterns may minimise broader microbiome disruption and avoid resistance amplification across other microbial communities. Similarly, in RT1-dominated areas, where *Streptococcus* and *Haemophilus* are prevalent, treatments could be tailored to high MLS and tetracycline resistance patterns. Notably, while RT2 was more prevalent in both SE and NWE, each region exhibited distinct AMR gene profiles within RT2 phenotypes, suggesting geographically specific variants related to this RT. These findings support a further-refined, geographically tailored eradication approach leveraging metagenomic insights to enhance antimicrobial strategy in bronchiectasis. Furthermore, considering the potential of prolonged antibiotic use to shape the microbiome [104] and drive antimicrobial drug resistance, understanding the resistome provides insight into microbial resistance dynamics and scope for a tailored personalised approach to managing bronchiectasis. Despite this, microbiomes also influence treatment response, and the resistome cannot be inferred solely from microbial taxa. Critically, the validation of previously established RTs reinforces their role in bronchiectasis endophenotyping to facilitate clinical translation.

While this work reveals important findings with potential clinical and research implications, it does have limitations. The cross-sectional study design precludes longitudinal evaluation of microbiome–resistome stability, and while geographic variation to microbiomes and resistomes are evident, our work has no coverage of Central and Eastern European regions, which are reported to have severe bronchiectasis and frequent exacerbations [37]. Our results are derived from the first patients enrolled in the pan-European EMBARC-BRIDGE study, which is now extending to further sites, including those in Eastern Europe. The recruitment of only bronchiectasis patients in this study precludes our assessment of regional variations in the "healthy baseline" microbiome as comparators; however, prior published work has already demonstrated this to differ significantly from bronchiectasis [35]. While metagenomic analysis provides an overview of resistomes across Europe, transmissibility and the nature of the resistance genes (*i.e.* chromosomal or encoded in mobile genetic elements such as plasmids, phages, transposons or integrons) were not assessed. Further, metagenomics faces limitations in capturing fine-scale, whole-genome resolution, potentially overlooking key mutations in resistance genes or regulatory proteins that play a key role in established pathogens [105]. This issue also applies to less-characterised commensal or pathobiont species, in which resistance mechanisms remain largely unexplored. Notably, the CARD database comprises only published and experimentally validated resistance genes [106]. Cryptic and silent resistance genes are hence not captured, thereby limiting evaluation of the "silent" resistome [107]. Our identification of resistance genes in association with bronchiectasis pathogens remains correlative and requires confirmatory evaluation by phenotypic assessment for AMR. Although observational, our study highlights RTs as clinically relevant microbial signatures associated with disease burden and AMR profiles. RT2 is indicative of an increased pathogen load and a higher AMR potential, underscoring the challenges in treating pathogen-dominant microbiomes. These insights may help refine antimicrobial strategies and support the development of more targeted treatment approaches. Our findings contribute to the broader understanding of microbiome-driven heterogeneity in bronchiectasis across Europe, which may inform future mechanistic studies and clinical trial design. Finally, while shotgun metagenomics permits taxonomic characterisation and functional annotation, challenges relating to the detection of fungi and antifungal resistance persists, given their overall relatively low abundance within microbiomes and the immaturity of robust bioinformatic pipelines to address this. The study of viral communities (the virome) with shotgun metagenomics is similarly plagued with challenges, including its minute abundance as well as incomplete viral databases and bioinformatic shortcomings impeding viral taxonomic identification and functional annotation [108–110]. The diverse architecture of viral genomes (linear or circular, DNA or RNA, single- or double-stranded) further impedes simultaneous assessment of the virome using metagenomics [109, 111, 112].

In conclusion, sputum metagenomes in bronchiectasis reproduce culture-based results with enhanced detection for some pathogens. Underlying resistomes demonstrate geographic variation and validate

established RTs. Our findings provide a basis to consider appropriate antibiotic usage, optimise antimicrobial trial design and perform geographic endo-phenotyping. Conserved resistance gene profiles of key microbial genera across regions provides a basis for targeted geographic-specific microbial eradication in bronchiectasis.

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