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Evaluation of the airway microbiome in nontuberculous mycobacteria disease

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16S rRNA gene sequencing is not sensitive to detect *Mycobacterium* but identifies microbiota signatures associated with inflammation http://ow.ly/opXm30ldtQH

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ABSTRACT Aspiration is associated with nontuberculous mycobacterial (NTM) pulmonary disease and airway dysbiosis is associated with increased inflammation. We examined whether NTM disease was associated with a distinct airway microbiota and immune profile.

297 oral wash and induced sputum samples were collected from 106 participants with respiratory symptoms and imaging abnormalities compatible with NTM. Lower airway samples were obtained in 20 participants undergoing bronchoscopy. 16S rRNA gene and nested mycobacteriome sequencing approaches characterised microbiota composition. In addition, inflammatory profiles of lower airway samples were examined.

The prevalence of NTM^+ cultures was 58%. Few changes were noted in microbiota characteristics or composition in oral wash and sputum samples among groups. Among NTM^+ samples, 27% of the lower airway samples were enriched with *Mycobacterium*. A mycobacteriome approach identified *Mycobacterium* in a greater percentage of samples, including some nonpathogenic strains. In NTM^+ lower airway samples, taxa identified as oral commensals were associated with increased inflammatory biomarkers.

The 16S rRNA gene sequencing approach is not sensitive in identifying NTM among airway samples that are culture-positive. However, associations between lower airway inflammation and microbiota signatures suggest a potential role for these microbes in the inflammatory process in NTM disease.

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Introduction

Nontuberculosis *Mycobacterium* (NTM) disease has an estimated incidence of 1.0–7.2 cases per 100 000 persons per year, and its incidence is increasing for unknown reasons [1]. Despite widespread exposure to these organisms, only a minority of exposed individuals will acquire NTM, and an even smaller subgroup will develop clinically evident disease. Importantly, effectiveness of treatments for active NTM disease has been limited due to an absence of antimicrobial agents with low toxicity and good *in vivo* activity against the organism. Thus, treatment for NTM is not recommended for everyone, as the clinical course of the pulmonary disease is variable [2].

The currently clinically available methods of culturing airway samples are not able to represent interactions that NTM may have with other bacterial organisms present in a complex microbial environment. With improvements in culture-independent techniques, the microbiota–host immune interaction can be examined in further detail [3]. There have been several studies examining the airway microbiota in non-cystic fibrosis bronchiectasis [4–10] using sputum obtained from cohorts where NTM is not prevalent. However, description of the lower airway microbiome has been challenging because the bacterial burden in the lung is approximately a million-fold lower than in the gut and a hundred-fold lower than in the upper airway [11, 12]. The presence of supraglottic microbes such as *Veillonella* or *Prevotella* in the lower airway is common [11–18], and they are associated with increased inflammation [11, 19], supporting the idea that microbiota changes are linked to the host immune phenotype in the airway mucosa. Therefore, we seek to identify microbial signatures associated with culture-positive NTM that may affect the host immune phenotype. Here, we utilised a 16S rRNA gene high-throughput sequencing approach in parallel with a modified "mycobacteriome" sequencing approach on a large cohort of subjects with non-cystic fibrosis bronchiectasis and high prevalence of NTM.

Material and methods

Study subjects

This was a prospective observational study of non-HIV-infected patients (n=106) with a diagnosis of non-cystic fibrosis bronchiectasis enrolled over a 2-year period at New York University (NYU) as part of a USA multicentre bronchiectasis cohort (Bronchiectasis Research Registry). All participants signed informed consent and the protocol was approved by the NYU institutional review boards (S14–01400). See the online supplementary methods for details on patient selection.

Procedures

Oral wash and induced sputum samples were collected from every patient at enrolment and again when sputum was clinically indicated over the 2-year study period (online supplementary table S1). In addition, for every induced sputum we collected an oral wash sample prior to sputum induction. A portion of this sputum sample was sent to the clinical laboratory for culture and, based on the epithelial cell count, 90.5% of induced sputum samples met criteria for good quality [20]. Aliquots of oral wash and induced sputum were frozen at −80°C for bacterial DNA sequencing. In order to investigate how reliable induced sputum was to evaluate the lower airway microbiota and to evaluate the inflammatory status of the lower airway mucosae, a subset of patients (n=20) underwent bronchoscopy (clinically indicated in 14 cases, while the remaining six were undertaken for research-only purposes). Sampling included equipment background controls (sterile saline, Yankauer and bronchoscope), supraglottic (sampled using Yankauer) and two bronchoalveolar lavage (BAL) samples: one from an involved lung segment (predefined based on computed tomography (CT) scan) and the other from a non-involved lung segment. No samples were obtained from participants during an acute exacerbation or recent antibiotic use (<1 month). Whole BAL fluid aliquots were frozen at −80°C for bacterial 16S rRNA gene sequencing as well as 16S quantitative (q)PCR.

Details of DNA sequencing are described in the online supplementary methods. In addition to 16S rRNA gene sequencing performed using Illumina MiSeq (San Diego, CA, USA), we used a nested PCR approach in parallel to enrich for *Mycobacterium* DNA coding for the 16S rRNA gene prior to library preparation for sequencing in order to describe the mycobacteriome, as previously published [21]. The obtained 16S rRNA gene sequences were analysed using the Quantitative Insights into Microbial Ecology (QIIME 1.9) package [22].

Immune profiling was performed for all BAL samples from the 20 patients who were part of the bronchoscopy subgroup. *In vivo* inflammation was assessed from acellular bronchoscopy samples by cell count differential and cytokines using Luminex 200 (Austin, TX, USA), as previously described [23, 24]. *Ex vivo* cytokine production of BAL cells was evaluated during toll-like receptor (TLR)4 stimulation (online supplementary methods).

Statistical analysis

For association with discrete factors, we used nonparametric tests (Mann–Whitney or Kruskal–Wallis ANOVA). We used the *ade4* package in R to construct principal coordinate analysis (PCoA) based on weighted UniFrac distances [25, 26]. To cluster microbiome communities into exclusive "metacommunities" we used a Dirichlet multinomial mixture model with the R package *DirichletMultinomial* [27, 28]. To evaluate differences between groups of 16S data or inferred metagenomes, we used linear discriminant analysis (LDA) effect size (LEfSe) [29]. For tests of association with continuous variables, we used nonparametric Spearman correlation tests. Co-occurrence between most abundant bacterial genera (>1% relative abundance in at least one sample) were assessed using SparCC [30] with 20 iterations and 500 bootstrap replicates to eliminate correlations where significance was driven by outliers and visualised using Cytoscape v3.0.2 [31]. Only biomarkers that passed false discovery rate correction were used for this analysis [32]. All data are publicly available in the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) under accession number PRJNA418131. All codes used for the analysis included in this manuscript are available at https://github.com/segalmicrobiomelab/ntm_bronchiectasis_microbiome.

Results

Participants

Table 1 shows demographics and clinical characteristics of 106 patients. All participants had imaging abnormalities. Culture data show that 61 (58%) out of 106 participants had positive NTM sputum cultures at baseline. Body mass index was lower among NTM^+ participants ($p<0.01$). Importantly, patients had variable prevalence of symptoms and radiographic findings.

Comparing sputum and oral wash microbiome

To evaluate the airway microbiome, we utilised all obtained oral wash and sputum samples (n=297). Oral wash samples had higher α-diversity than sputum (Shannon index; online supplementary figure S1a). Furthermore, β-diversity analysis showed significant differences between these two sample types (PERMANOVA $p<0.001$; online supplementary figure S1b), although there was a greater degree of similarity between samples from the same subject than between subjects (online supplementary figure S1c). LEfSe analysis showed that sputum samples were enriched with *Prevotella*, *Veillonella* and *Corynebacterium*, while oral wash samples were enriched with *Streptococcus*, *Rothia* and *Actinomyces* (online supplementary figure S1d).

Comparison of airway microbiota in NTM^+ versus NTM^- using sputum and oral wash samples

Next, we compared differences in microbiota for each of these sample types based on NTM status. The NTM culture status was based on the culture result of the specimen that was sequenced. Figure 1 and online supplementary figure S2 evaluate differences based on NTM culture status at the time of sample collection. In oral wash samples, there was no significant difference in α-diversity, but a significant difference in β-diversity between NTM^- and NTM^+ samples (PERMANOVA $p=0.043$). In sputum samples, there were no statistically significant differences in either α-diversity ($p=0.05$) or β-diversity ($p=0.08$) between NTM^- and NTM^+ samples. Similar negative results were seen when comparing NTM status based on American Thoracic Society/Infectious Diseases Society of America diagnostic criteria (NTM culture positivity in at least two sputum samples or one BAL sample) [33] or when only baseline sample were considered (data not shown).

Interestingly, *Mycobacterium* was not found to be enriched in NTM^+ samples. Indeed, this genus was only found present in a very small percentage of oral and sputum samples. Therefore, we investigated whether more differences in microbiota could be identified by sampling the lower airway.

Microbiota comparison across the airways using bronchoscopic samples

A subgroup of 20 participants from this cohort (40% with culture-positive NTM) underwent bronchoscopy (online supplementary table S2 and figure S3). First, we compared how representative the sputum was of the lower airway microbiota (where we sampled involved and non-involved lung segments based on CT). Quantification of 16S rRNA copies using qPCR showed that sputum had approximately $\log_2$ higher bacterial load compared to BAL samples (online supplementary figure S4). The high bacterial load in sputum was comparable with the bacterial load present in oral wash and supraglottic samples. Figure 2 displays the α- and β-diversity for all bronchoscopy related samples. There were significant differences in β-diversity ($p<0.01$). The degree of similarity between upper airway, sputum and BAL samples was calculated as weighted UniFrac distance. Interestingly, sputum was more similar to oral wash or to supraglottic samples than to BAL (true for both involved or non-involved lung segments; figure 2c). These data suggest that sputum cannot be used as a surrogate for the lower airways to study the airway microbiota in this cohort.

TABLE 1 Baseline demographic and clinical information of all patients recruited

	All patients	NTM status		p-value
		−	+	
Subjects	106	45	61	
Age years	67.5±10.7	67.6±10.1	67.3±11.3	0.88
Female	95 (89)	37 (82)	58 (95)	0.71
Caucasian	92 (87)	34 (76)	58 (95)	0.39
BMI kg·m^{-2}	22.7±4.3	24.2±5.2	21.5±3.1	<0.01
Smoking packs per day	1.3±2.2	1.2±1.0	1.4±3.0	0.85
Smoking years	19.2±13.1	22.9±14.5	15.1±9.9	0.04
Symptoms				
Cough	63 (59)	22 (49)	41 (67)	0.07
Sputum	47 (44)	14 (31)	33 (54)	0.15
Haemoptysis	8 (8)	3 (7)	5 (8)	0.76
Shortness of breath	42 (39)	17 (38)	25 (41)	0.92
Wheeze	14 (13)	7 (16)	7 (11)	0.6
Fatigue	26 (25)	9 (20)	17 (28)	0.6
Post-nasal drip	36 (34)	19 (42)	17 (28)	0.03
Sinusitis	4 (4)	4 (9)	0 (0)	0.03
Current NTM culture				
MAC	56 (53)	0 (0)	56 (92)	<0.001
M. abscessus	5 (5)	0 (0)	5 (8)	<0.05
Current bacterial culture				
Oropharyngeal flora	15 (14)	4 (9.5)	11 (18)	0.13
P. aeruginosa	6 (6)	1 (2)	5 (8)	0.19
MSSA	5 (5)	2 (4)	3 (5)	0.91
Other	10 (9)	4 (9.5)	6 (10)	0.89
Negative culture	17 (16)	9 (20)	8 (13)	0.34
Lung physiology[#]				
FVC % pred	92.7±20.1	92.6±20.4	92.8±20.0	0.97
FEV_1 % pred	83.7±21.8	83.4±22.1	84.0±21.8	0.91
FEV_1/FVC %	70.2±10.6	69.2±10.7	71.0±10.6	0.44
RV % pred	121.8±31.7	118.4±32.8	124.5±31.0	0.41
TLC % pred	106.4±18.5	105.6±16.2	106.9±20.2	0.76
D_{LCO} % pred	87.4±20.7	90.7±23.7	85.4±18.7	0.35
HRCT thorax				
Bronchiectasis	79 (75)	34 (76)	45 (78)	0.45
Thickened airways	52 (49)	23 (51)	29 (59)	0.73
Mucoid impaction	54 (51)	20 (44)	34 (74)	0.57
Nodules	70 (66)	29 (64)	41 (67)	0.29
Tree-in-bud	30 (28)	11 (24)	19 (31)	0.41
Ground-glass opacification	31 (29)	17 (38)	14 (23)	0.16
Questionnaire data[¶]				
SGRQ total score	27.5±19.3	28.4±21.1	26.8±18.1	0.68
RSI total	11.9±9.2	13.7±9.4	10.7±8.9	0.12
FSSG total	8.5±8.5	9.8±9.7	7.6±7.5	0.22
EAT-10 total	3.0±5.8	3.0±5.6	40.8±20.0	0.97

Data are presented as n, mean±SD or n (%), unless otherwise stated. NTM: nontuberculous mycobacteria; BMI: body mass index; MAC: *Mycobacterium avium* complex; *M. abcessus*: *Mycobacterium abscessus*; *P. aeruginosa*: *Pseudomonas aeruginosa*; MSSA: methicillin-sensitive *Staphylococcus aureus*; FVC: forced vital capacity; FEV_1: forced expiratory volume in 1 s; RV: residual volume; TLC: total lung capacity; D_{LCO}: diffusion capacity of the lung for carbon monoxide; HRCT: high-resolution computed tomography; SGRQ: St George's respiratory questionnaire; RSI: reflux symptom index; FSSG: frequency scale for the symptoms of gastro-oesophageal reflux disease; EAT-10: eating assessment tool. [#]: n=89; [¶]: n=101.

Comparison of airway microbiota in NTM$^+$ versus *NTM$^-$ using bronchoscopic samples*

Of the patients that underwent bronchoscopy, 12 (60%) out of 20 were NTM^- and eight (40%) out of 20 were NTM^+. There were no significant differences in the bacterial load of NTM^+ *versus* NTM^- lower airway samples (p=nonsignificant). Online supplementary figure S5 shows no statistically significant differences in α- or β-diversity in BAL samples when categorised based on NTM culture status. Similarly, no differences were noted between BAL samples obtained as part of clinically indicated bronchoscopy as

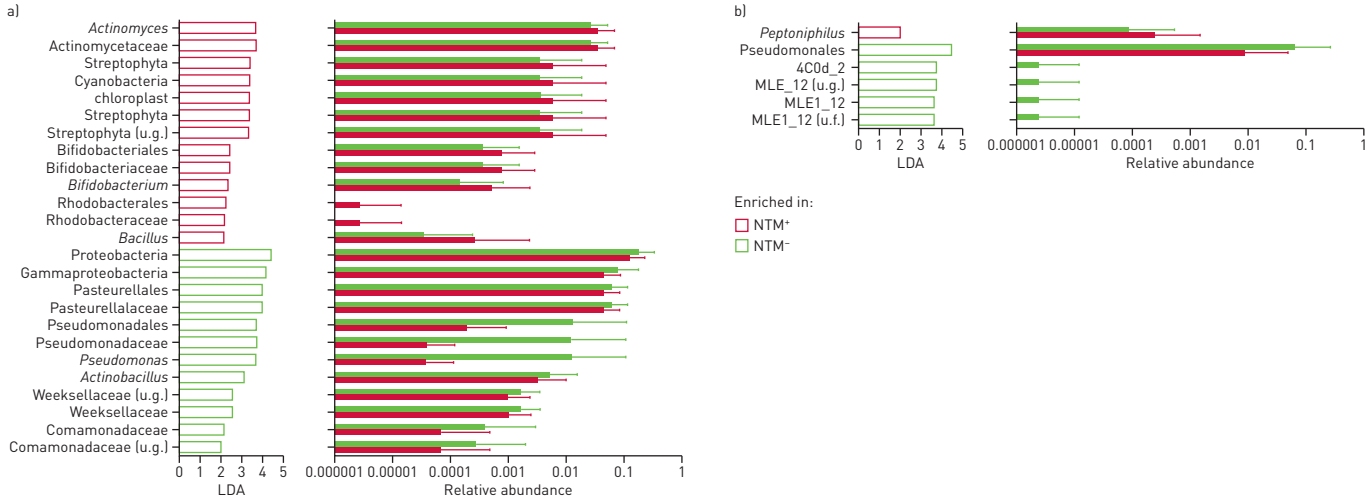

FIGURE 1 Taxonomic differences between all oral wash and sputum samples for the nontuberculous mycobacteria (NTM)$^+$ and NTM$^-$ groups. a) For oral wash samples, linear discriminant analysis (LDA) effect size (LEfSe) identified significant taxonomic differences in microbiome enrichment based on NTM status, but there was no enrichment with *Mycobacterium* in NTM$^+$ oral wash samples; b) for sputum, LEfSe detected few taxonomic differences and there was no enrichment with *Mycobacterium* in NTM$^+$ sputum samples. u.g.: undetermined genus; u.f.: undetermined family.

compared with research bronchoscopy (data not shown). Even though *Mycobacterium* was enriched in NTM$^+$ BAL samples (figure 3), this taxon was only present in 27% of these culture positive samples (median relative abundance 0 (0–0.014)). These data suggested that this sequencing approach was not able to detect the "pathogen" identified as responsible for the disease process and is consistent with prior literature that has shown poor accuracy of 16S rRNA gene sequencing methods to detect *Mycobacterium* [21].

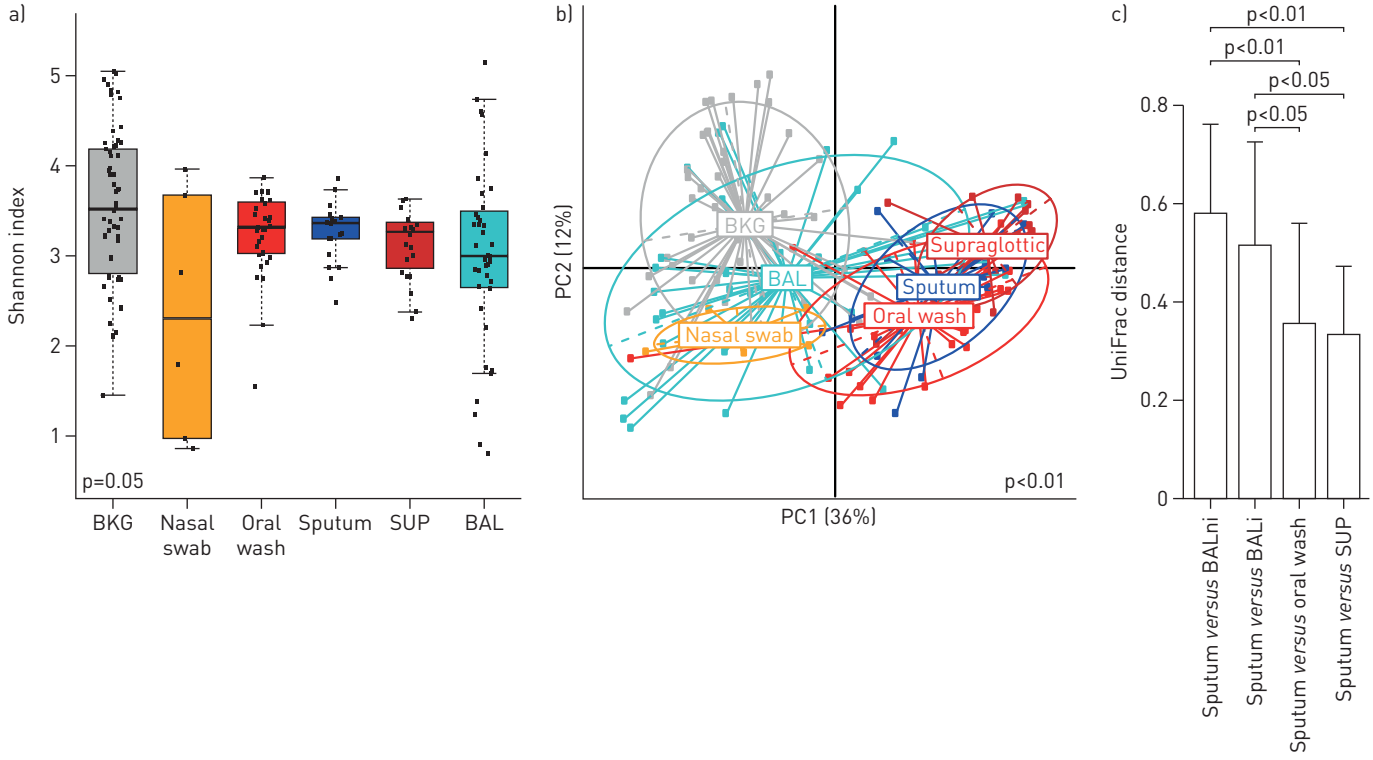

FIGURE 2 Evaluation of the lower airway microbiota using bronchoscopic samples. Bronchoscopy samples included background (BKG), nasal swab, oral wash, sputum, supraglottic (SUP) and bronchoalveolar lavage (BAL). a) There were significant differences in α-diversity (Shannon diversity index) between all samples; b) β-diversity (based on weighted UniFrac) showed differential clustering based on sample type (PERMANOVA p<0.01); c) comparison of the degrees of similarity between upper and lower airway samples based on UniFrac distance. Results showed that the microbiota in sputum were more similar to the microbiota of supraglottic and oral wash samples than to the microbiota in BAL samples (both involved (BALi) and non-involved (BALni)).

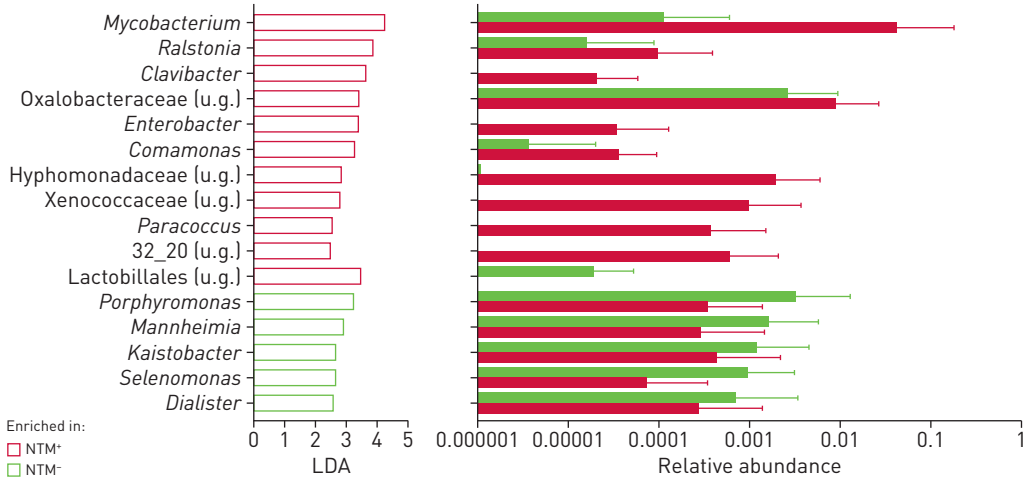

FIGURE 3 Taxonomic differences between nontuberculous mycobacteria (NTM)$^+$ and NTM$^-$ in lower airway microbiota. Linear discriminant analysis (LDA) effect size analysis showed significant differences in lower airway microbiota composition enrichment based on NTM status. Unlike sputum samples, *Mycobacterium* was enriched in NTM$^+$ bronchoalveolar lavage samples.

Evaluation of the mycobacteriome

Therefore, we used an optimised protocol to enrich for *Mycobacterium* DNA coding for the 16S rRNA gene using a recently published DNA isolation method [34] and a nested PCR approach [35] (online supplementary material). First, we used a mock community of *Mycobacteriun fortuitum* and *Streptococcus* in order to establish the limit of detection for *Mycobacterium* with this approach (online supplementary results and online supplementary figure S6 for further details). We then used this nested mycobacteriome approach in all samples from the participants who underwent bronchoscopy and created PCoA plots comparing standard 16S rRNA gene sequencing and nested mycobacteriome approaches of BAL, sputum and supraglottic samples. Figure 4 shows significant overlap for a large proportion of samples, but identifies compositional differences in others. In addition, histograms in figure 4 show the relative abundance of different *Mycobacterium* operational taxonomic units (OTUs) obtained with both nested mycobacteriome and 16S rRNA gene sequencing approaches. In BAL samples, *Mycobacterium* was detected in four (27%) out of 15 NTM$^+$ samples with standard 16S rRNA gene sequencing approaches, but with the nested mycobacteriome approach *Mycobacterium* was detected in all four samples plus three other NTM$^+$ BAL samples (47%). Blast analysis demonstrate that these sequences matched *Mycobacterium avium*. Furthermore, this nested mycobacteriome approach identified one (5%) out of 21 NTM$^-$ samples with *Mycobacterium*. Blast analysis of this OTU was annotated to *Mycobacterium houstonense* (an environmental *Mycobacterium* not known to be pathogenic). Use of this nested mycobacteriome approach on background control samples detected a significant amount of *Mycobacterium* reads (>5% relative abundance) in only one control background sample, and fewer reads in only two out of 19 background equipment samples. In addition, blast analyses of *Mycobacterium* reads found in background equipment samples were annotated to a nonpathogenic strain (online supplementary figure S7). Similar results were found when this approach was utilised in oral and sputum samples from those subjects who did not undergo bronchoscopy (online supplementary material).

Lower airway immunological profiling

To evaluate the association of microbial signatures in NTM disease with a distinct mucosal immune phenotype we examined BAL cell differentials, *in vivo* cytokine levels and *ex vivo* cytokine production. In NTM$^+$ participants, BAL samples from involved lung segments had significantly higher neutrophils and fewer macrophages when compared to non-involved lung segments (table 2). In contrast, in NTM$^-$ participants, BAL samples from involved lung segments had significantly higher lymphocytes. *In vivo* cytokine levels measured in BAL also showed a different inflammatory profile for NTM$^+$. In NTM$^+$ participants, BAL samples from involved lung segments had significantly higher levels of interferon (IFN)-γ, interleukin (IL)-8, IL-12p70, IFN-γ-inducible T-cell α chemoattractant (ITAC), macrophage inflammatory protein (MIP)1α and MIP1Bβ, as compared with non-involved lung segments. This pattern was not present in BAL samples from NTM$^-$ participants, where involved lung segments had lower levels of MIP3α and IL-17A. Similarly, a distinct inflammatory pattern was observed during *ex vivo* Toll-like receptor (TLR)-4 stimulation of BAL cells (online supplementary table S3). In NTM$^+$ participants, BAL

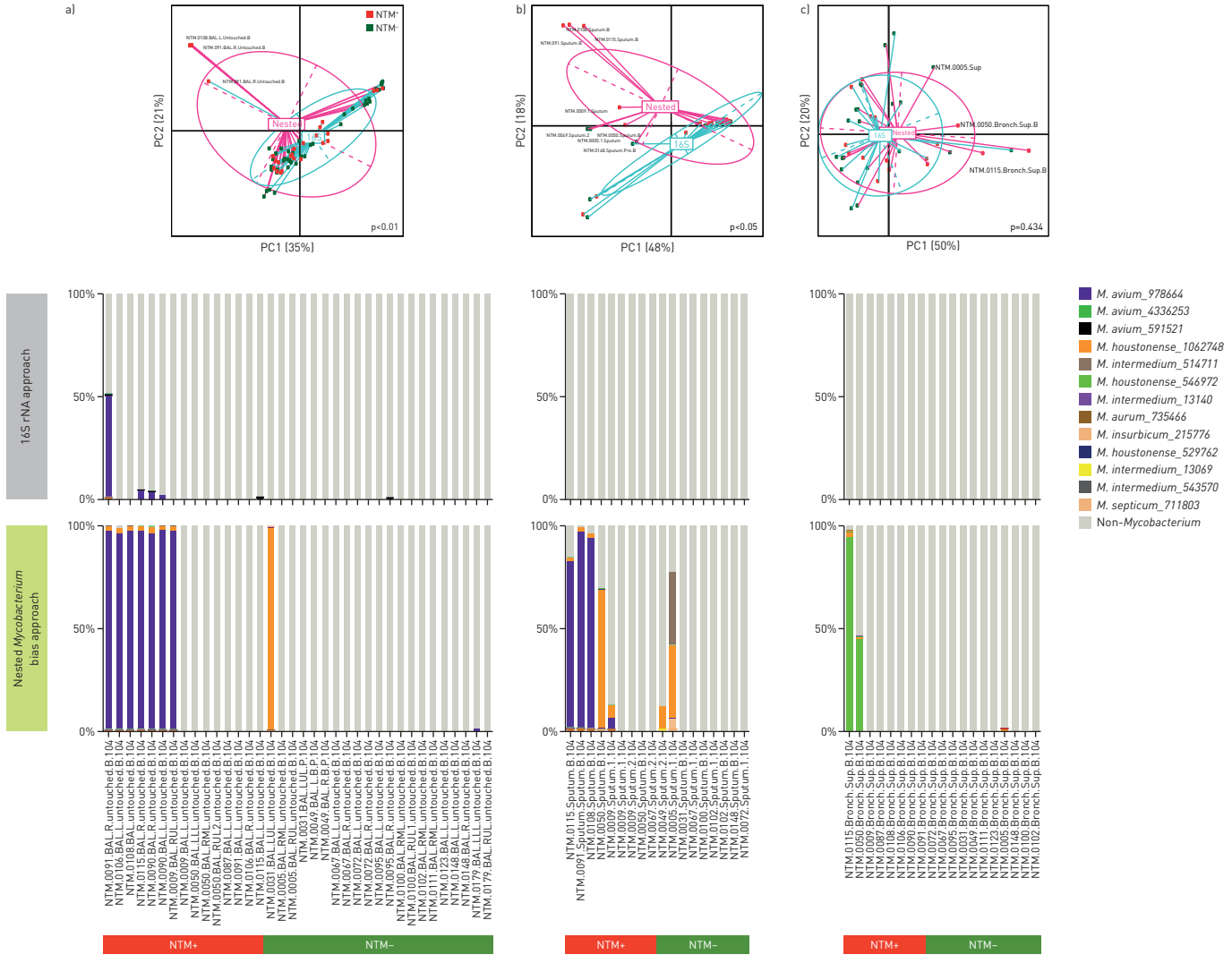

FIGURE 4 Comparison between sequence data obtained using an unbiased 16S rRNA approach and a biased mycobacteriome approach. Top panels show differences in β-diversity (based on weighted UniFrac distance) between samples processed with unbiased 16S *versus* biased mycobacteriome approach. Bar charts in the bottom panels show relative abundance of operational taxonomic units (OTUs) annotated to *Mycobacterium* and their annotation based on BLAST on the two datasets. a) In bronchoalveolar lavage (BAL) there were significant differences in β-diversity between BAL samples processed with unbiased 16S *versus* biased mycobacteriome approach (PERMANOVA p<0.01). With the biased mycobacteriome approach, *Mycobacterium avium* was identified in seven of the nontuberculous mycobacteria (NTM)$^+$ culture samples, while one NTM$^-$ sample had a *Mycobacterium* OTU annotated to *M. houstonense* (a strain not known to be pathogenic); b) in sputum there were significant differences in β-diversity between the sputum samples processed by unbiased 16S *versus* biased mycobacteriome approach (PERMANOVA p<0.01). With the biased mycobacteriome approach, *M. avium* was identified in three of the NTM$^+$ culture samples; c) in supraglottic samples there was no significant difference in β-diversity between samples processed by unbiased 16S *versus* biased mycobacteriome approach. In two samples, a *Mycobacterium* annotated to a nonpathogenic strain was identified.

cells from involved lung segments had blunted production of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IFN-γ. These differences were not noted in NTM$^-$ participants.

Next, we evaluated the microbiome signatures associated with these inflammatory biomarkers in NTM$^+$ samples and NTM$^-$ samples. For this, we used a network approach to evaluate taxa that trend to co-occur and were identified as distinct clusters based on Dirichlet multinomial model clustering (online supplementary material and figure S9).

In BAL of NTM$^+$ participants, oral commensals such as *Prevotella*, *Veillonella* and *Leptotrichia* tended to co-occur and had significant correlations with neutrophils and several cytokines including IL-6, IL-17, IL-23 and fractalkine (figure 5). Interestingly, *Mycobacterium* was in a separate co-occurrence cluster and had no significant correlation with inflammatory biomarkers. In BAL of NTM$^-$ samples, the relative abundance of oral commensals in the lower airway samples had fewer significant correlations with cytokines and BAL cells (online supplementary figure S10).

TABLE 2 Cell count, and *in vivo* cytokine levels in bronchoalveolar lavage of 20 patients from the bronchoscopy cohort

	NTM$^-$		p-value$^{\#}$	NTM$^+$		p-value$^{\#}$
	Involved	Non-involved		Involved	Non-involved	
Subjects n	12			8		
Cell count						
Macrophages	44.2 (28.2–75.7)	75.4 (51.7–80.4)	NS	19.6 (15.75–39.65)	75.4 (66.0–85.0)	0.02$^+$
Neutrophils	52.8 (13.4–69.8)	13.2 (10.5–35.05)	NS	79.6 (58.2–82.75)	17.1 (6.5–31.2)	0.02$^+$
Lymphocytes	2.8 (2.6–10.9)	11.4 (2.95–12.7)	0.02	2.2 (1.7–2.45)	1.8 (0.5–3.8)	NS
Eosinophils	0 (0–0)	0 (0–0)	NS	0 (0–0)	0 (0–0)	NS
***In vivo* cytokine level pg·mL^{-1}**						
ITAC	19.39 (19.4–224.4)¶	224.4 (135.6–274.9)	NS	426.4 (372.5–1135.8)¶	469.73 (352.8–489.7)	0.02§
MIP1β	64.875 (60.7–114.2)	83.545 (61.8–109.2)	NS	138.08 (134.1–188.8)	111.83 (71.8–141.4)	0.02§
IFN-γ	21.36 (6.4–30)	8.46 (6.7–13.8)	NS	63.085 (24.8–158.6)	22.095 (17.6–26.5)	0.03§
IL-8	423.565 (216.9–486.8)¶	351.12 (240.7–1810.2)	NS	5927.82 (5927.8–5927.8)¶	411.345 (242.8–586.8)	0.03§
IL-12 p70	3.95 (3.7–4.1)¶	3.715 (3.5–4.3)	NS	5.155 (4.2–6.6)¶	4.605 (4.3–4.6)	0.06
MIP1α	116.88 (66.2–179.7)	95.855 (72.4–128.3)	NS	268.83 (143.4–489.8)	117.975 (78.5–143.1)	0.06
MIP3α	49.105 (51.9–86.3)¶	86.26 (75.6–254.4)	0.03	356.01 (300.8–443.1)¶	201.49 (141.7–228.2)	NS
IL-17A	9.65 (10.1–18.2)	18.18 (15–20.7)	0.06	11.02 (10.5–13.2)	15.44 (10.4–19.9)	NS
IL-13	7.445 (8.3–19.3)	13.97 (11.3–17.9)	NS	9.73 (8.6–12)	70.87 (9.4–145.9)	NS
IL-2	2.75 (2.8–4.7)¶	4.68 (3.6–5.5)	NS	5.07 (3.7–8.3)¶	2.80 (2.8–4.3)	NS
Fractalkine	364.12 (364.1–391.3)¶	391.255 (360.4–449.6)	NS	488.115 (455.5–544.1)¶	558.94 (403.9–558.9)	NS
IL-1β	22.27 (1.9–11.1)	3.94 (2–6.5)	NS	34.345 (3.8–72.4)	2.405 (2.1–65.3)	NS
IL-6	11.275 (10.1–104)¶	104.025 (58.9–154.3)	NS	82.735 (60.9–133.4)¶	114.99 (69.7–147.3)	NS
IL-21	4.825 (4.5–6.5)	5.95 (4.7–7.3)	NS	6.655 (6–8.8)	7.45 (5.4–9.17)	NS
IL-7	15.935 (15.2–38.4)	24.97 (16.1–36.4)	NS	27.365 (26.7–31.6)	29.545 (22.8–33.6)	NS
IL-5	32.475 (13.4–40.3)	17.03 (12.1–23.2)	NS	26.395 (23.9–31.4)	56.18 (56.2–18.4)	NS
IL-23	158.025 (88.4–160.6)	118.66 (88.4–149.9)	NS	153.865 (126.4–173.7)	252.9 (165.4–359.9)	NS
TNF-α	208.485 (36.1–231.5)	44.63 (37.5–105.5)	NS	90.83 (67.3–130.3)	54.845 (39.4–105.2)	NS
GM-CSF	19.225 (18.1–41.2)	29.935 (19.6–40)	NS	35.795 (32.1–42.2)	27.215 (22.5–37.0)	NS
IL-4	29.09 (22–30.5)	25.42 (22.9–32.6)	NS	39.855 (31–50.2)	46.31 (37.1–49.0)	NS
IL-10	61.015 (50.8–84.6)	66.315 (55.1–74)	NS	45.74 (32.1–68.6)	83.5 (68.2–89.1)	NS

Data are presented as median (interquartile range), unless otherwise stated. NTM: nontuberculous mycobacteria; ITAC: interferon (IFN)-γ-inducible T-cell α chemoattractant; MIP: macrophage inflammatory protein; IL: interleukin; TNF: tumour necrosis factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; NS: nonsignificant; FDR: false discovery rate. $^{\#}$: Mann–Whitney U-test; ¶: comparing involved sites by NTM status; $^+$: FDR <0.05; §: FDR <0.2.

Discussion

The purpose of this investigation was to evaluate the airway microbiota using culture-independent techniques in a prospective cohort of patients suspected of having NTM disease. Our analysis showed that using sputum samples, few changes in microbiota composition could be identified between samples with and without NTM identified by culture. Using upper and lower airway samples from a subgroup of participants who underwent bronchoscopy we showed that induced sputum offers a poor representation of the lower airway microbiota in this patient population and more accurately reflects the composition of the oral cavity. Furthermore, the culture-independent approach did not find *Mycobacterium* in a large percentage of samples. We expanded these observations with a *Mycobacterium*-biased nested sequence approach to confirm that in the majority of NTM$^+$ participants the abundance for this organism was low or not detected in contrast with the many other microbes identified. These data demonstrate the limited sensitivity of these culture-independent methods to detect *Mycobacterium* and exemplify an unrecognised limitation of current universal sequencing methods to study pathogens present at low abundance. Finally, the lower airways of NTM$^+$ participants had a distinct immunological phenotype, in which levels of several inflammatory biomarkers correlated with the relative abundance of microbes identified as oral taxa and not with the relative abundance of *Mycobacterium*. These data suggest that micro-aspiration and/or failure to clear aspirated oral microbes may contribute to the inflammatory endotype in NTM disease.

Culture-independent techniques have demonstrated that the airways harbour a complex microbiota that has a significant impact on the host immune response [3, 11, 23, 36, 37]. In a recent study involving 76 non-cystic fibrosis bronchiectasis patients from a multicentre European cohort, *Haemophilus influenzae*, *Pseudomonas aeruginosa* and *Streptococcus* were found to be the most abundant species in sputum samples [4]. However, this cohort is characterised by a low prevalence of NTM. In the United States,

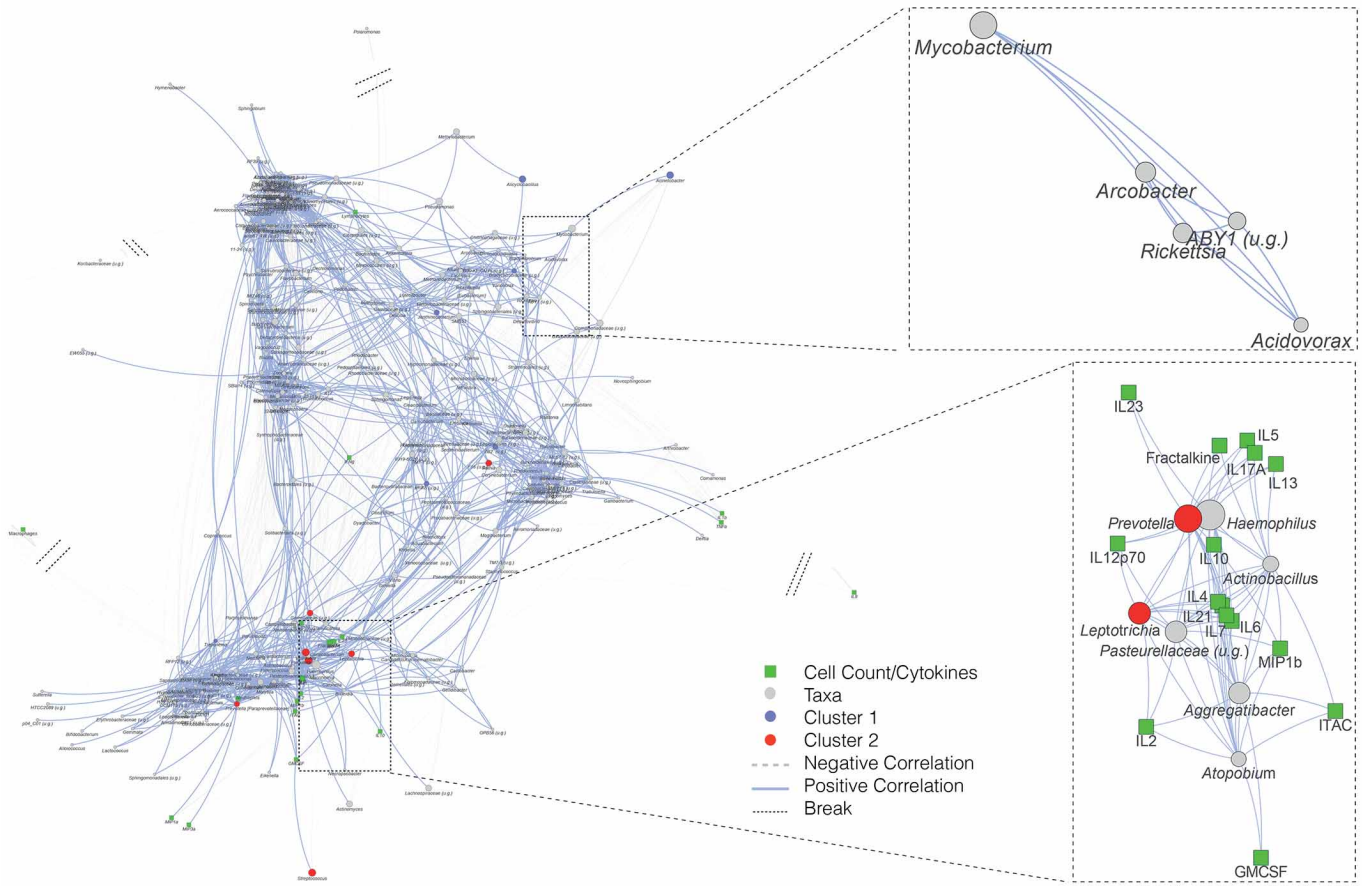

FIGURE 5 Associations between taxa and inflammatory biomarkers in the lower airways for nontuberculous mycobacteria (NTM)$^{+}$ samples. A network analysis using SparCC was constructed, to remove compositional and sparsity effects common in microbiome data, in order to identify correlations between taxa at a genus level and both inflammatory biomarkers. Each node represents a genus, with the size of nodes indicating the log-relative abundance from large (high) to small (low). In addition, taxa identified as markers for lower airway microbiota clusters (based on DMM analysis shown in online supplementary figure S5) were colour coded. Edges between nodes represent significant correlations, with the length of the edge representing the correlation coefficient strength (shorter edges indicating higher positive correlation). u.g.: undetermined genus.

NTM is a frequent cause of non-cystic fibrosis bronchiectasis, as recently shown by Aksamit *et al.* [38] and multiple strains of NTM are associated with bronchiectasis [39]. In our study, the prevalence of NTM was 58%, similar to the US Bronchiectasis Research Registry [38]. Diagnosis of NTM disease is commonly based on induced sputum. Therefore, we examined microbiota differences in induced sputum as well as oral wash in our cohort. Both sputum and oral wash samples showed nonsignificant differences in diversity metrics based on NTM status. Moreover, in NTM^{+} patients, *Mycobacterium* was not enriched in these samples.

To further characterise the lower airway microbiota in NTM disease a subset of patients underwent bronchoscopy with BAL and upper airway (oropharyngeal) sampling. Differences among oropharyngeal microbiota included enrichment with *Streptococcus* and *Rothia* in oral wash and enrichment with *Prevotella* and *Veillonella* in supraglottic samples. Importantly, comparison between induced sputum and both upper and lower airway samples showed that induced sputum is compositionally more similar to the upper airway microbiota (either oral wash or supraglottic) than to the lower airway microbiota. This supports that induced sputum is predominantly influenced by the composition of the upper airway microbiota and offers a poor representation of the lower airway microbiota. Similar to our findings, the use of induced sputum to evaluate the airway microbiota in a cohort of patients with asthma provided an incomplete reflection of the lower airways, mostly influenced by the oral microbiota [40]. This has implications for our limited understanding of the lower airway microbiota using non-invasive samples. In our cohort, BAL samples from NTM^{+} participants were enriched with *Mycobacterium* and Oxalobacteraceae, while BAL samples from NTM^{-} participants were enriched with *Porphyromonas*. However, similar to induced sputum, *Mycobacterium* was frequently not identified using 16S rRNA gene sequencing in samples with positive cultures for this organism. This is consistent with prior published

observations [21, 34]. As NTM tends to have only one or two 16S rRNA genes per genome, they can be underrepresented in the context of other taxa with more 16S rRNA genes per bacteria using standard methods of 16S sequencing [33, 41]. In BAL, *Mycobacterium* was identified only in 27% of the NTM^+ samples using standard 16S rRNA gene sequencing. For this investigation we seek to use a universal sequencing approach that allows us to characterise broadly the bacterial composition of the airway microbiota. Since *Mycobacterium* was rarely present among NTM^+ cases using the now broadly accepted 16S rRNA gene sequencing, we attempted to improve our sensitivity in identifying this genus by applying a nested amplification approach where the first PCR targeted a *Mycobacterium*-specific region that contains the V4 region of the 16S rRNA gene [21, 42–44]. It is possible that other *Mycobacterium*-specific primers would have a better yield at identifying this organism. Nonetheless, with this method of sequencing, we were able to identify *Mycobacterium* in 47% of the BAL samples where NTM grew in culture. In contrast, *Mycobacterium* was only identified in 17% of NTM^+ sputum samples, which is probably also related to how representative the sputum is of the lower airway microbiota.

Our bronchoscopic sampling also allowed us to compare the inflammatory phenotype of NTM^+ and NTM^- participants. It has previously been shown that with mycobacterial infection, through induction of IFN-γ, activated macrophages upregulate the expression of pro-inflammatory cytokines to help protect against mycobacterial infection. These cytokines include IL-6, IL-1β, IL-12, tumour necrosis factor-α and nitric oxide [45, 46]. In NTM^+ BAL samples obtained from involved sites, BAL cells stimulated with lippolysaccharide (LPS) showed significantly blunted IFN-γ and GM-CSF levels, suggesting important impaired innate immune responses. In a co-occurrence network analysis, significant associations between taxa identified as oral commensals (*e.g. Prevotella*, *Veillonella* and *Leptotrichia*) and T-helper-17 cytokines were also seen in NTM^+ BAL samples. The relative abundance of *Mycobacterium* was not significantly correlated with levels of inflammatory biomarkers suggesting the importance of other microbes on the lower airway inflammatory tone in NTM disease.

There were several limitations in this study. The patients enrolled in this cohort had a mild form of NTM. A large proportion of patients with NTM grew *Mycobacterium avium* complex, consistent with the US Bronchiectasis Research Registry [33, 38]. It is possible that different strains of *Mycobacterium* and different degrees of disease severity would have different airway microbiota and inflammatory signatures than that observed in our study. In this investigation, a relatively small proportion of patients underwent bronchoscopy allowing us to evaluate the lower airways [47] and most of these investigations were undertaken for clinical reasons. Although similar to our larger cohort, these patients may represent a different disease phenotype. However, given the limited representation of the lower airways provided by the microbiota present in sputum samples, further investigation is warranted in a larger cohort to uncover microbiota host interactions that might be relevant in this disease and to define which microbiota signatures present in induced sputum could be used to explore the lower airway microbiota. Furthermore, even though we identified some significant associations between lower airway microbiota signatures and inflammatory biomarkers in the NTM^+ group, we considered these results as exploratory and hypothesis-generating. A larger cohort of patients where bronchoscopic samples are obtained will be needed to confirm and expand these findings. Finally, the purpose of this study was to evaluate the microbiome community of patients with NTM-related bronchiectasis. Therefore, we did not evaluate the change in microbiome composition with exacerbations nor the effects of treatment for NTM. Importantly, patients on treatment for NTM were excluded from this analysis to avoid this potential confounder to the analysis of differences between NTM^+ *versus* NTM^- groups.

In summary, we identified the limitations of current unbiased culture-independent techniques to identify *Mycobacterium* in patients with culture positivity for NTM, which highlights the need for technical improvements in these methods. In addition, we describe how patients with NTM disease have a distinct inflammatory environment in the lower airways that may be associated with some of the components of the lower airway microbiota including taxa commonly identified as oral commensals. These data suggest a possible role of micro-aspiration or failure to clear upper airway microbes from the lower airways and may explain some of the heterogeneity in presentation and disease progression among participants with culture-positive NTM disease. The contribution of the lower airway microbiota to the pathophysiological inflammatory process in NTM disease warrants further investigation in a larger cohort and may have potential therapeutic implications.

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