Airway dysbiosis: *Haemophilus influenzae* and *Tropheryma* in poorly controlled asthma

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ABSTRACT Asthma is a chronic inflammatory disorder of the airways where bacteria may act as protagonists of chronic inflammation. Little is known about the relation of airway inflammation to the presence of specific bacterial taxa. We sought to describe the sputum microbiome in adults with poorly controlled asthma.

DNA was extracted from induced sputum and microbial communities were profiled using 16S rRNA pyrosequencing. Bacterial species were characterised, and the relationship between microbial populations, asthma inflammatory subtypes and other covariates was explored. Real-time PCR was used to identify *Tropheryma whipplei* and *Haemophilus influenzae* in sputum.

Adults with neutrophilic asthma had reduced bacterial diversity and species richness. *Tropheryma* was identified and confirmed with real-time PCR in 12 (40%) participants. *Haemophilus* occurred most often in a group of younger atopic males with an increased proportion of neutrophils. PCR confirmed the presence of *H. influenzae* in 35 (76%) participants with poorly controlled asthma.

There are phenotype-specific alterations to the airway microbiome in asthma. Reduced bacterial diversity combined with a high prevalence of *H. influenzae* was observed in neutrophilic asthma, whereas eosinophilic asthma had abundant *T. whipplei*.

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Introduction

Asthma is a chronic heterogeneous inflammatory disorder of the airways involving eosinophilic and noneosinophilic inflammatory phenotypes. The mechanisms driving noneosinophilic airway inflammation and, in particular, neutrophilic inflammation are poorly understood.

A growing body of evidence supports a role for colonising bacteria in perpetuating inflammatory conditions in the lower airways [1, 2]. The airway inflammatory response in neutrophilic asthma suggests bacterial activation with significantly upregulated Toll-like receptor 2 expression and increased interleukin (IL)-8 and -1β levels compared with other inflammatory subtypes of asthma [3]. Patients with neutrophilic bronchitis are commonly culture-positive for *Haemophilus influenzae* [1, 2]. These data suggest a potential role for the presence of lower airway bacteria, particularly *H. influenzae*, in the persistence of airway inflammation observed in neutrophilic asthma.

Technological advances such as culture-independent microbial community profiling make a detailed assessment of airway bacterial colonisation possible [4]. There may be an altered microbiome in the airways of patients with asthma, where bacteria of the phylum Proteobacteria (which include *Haemophilus* species) are more prevalent in asthma compared with healthy controls [4, 5], particularly those with corticosteroid-resistant asthma [6]. The functional significance of these changes is unclear; however, associating bacterial colonisation with a typical host inflammatory response would provide stronger evidence for the role of airway dysbiosis in asthma. Little is known about the relationship between the airway inflammatory pattern that is specifically associated with the presence of specific bacterial taxa. In the present study, we used culture-independent microbial community profiling to characterise the lower airway microbiome in patients with and without asthma. Using these profiles, we explored the relationship between resident microbial communities and airway inflammatory phenotype. We hypothesised that patients with neutrophilic asthma would be characterised by a distinctive microbiome dominated by *Haemophilus* species compared with eosinophilic and paucigranulocytic asthma.

Methods

Participants

30 study participants (the initial cohort) were assessed with a full microbiome analysis (16S pyrosequencing) from four centres across Australia (Newcastle, New South Wales; Perth, Western Australia; and two centres in Brisbane, Queensland). An additional 16 adults with poorly controlled neutrophilic asthma were subsequently included in order to confirm the presence of *H. influenzae* using real-time PCR.

This study was conducted in accordance with the amended Declaration of Helsinki. Local institutional review boards approved the protocol (details in the online supplementary material) and written informed consent was obtained from all patients.

Asthma diagnosis was established using American Thoracic Society guidelines based on current episodic respiratory symptoms, doctor's diagnosis and evidence of variable airflow obstruction [7]. Participants with asthma were stable but symptomatic, despite being prescribed maintenance inhaled corticosteroid (ICS) treatment with an Asthma Control Questionnaire 6 (ACQ6) score >0.7 [8]. Participants had no reported exacerbations, infections or alterations in respiratory medications in the previous 4 weeks. Exclusion criteria and patient assessment details can be found in the online supplementary material. Nonsmoking healthy controls had normal lung function and no diagnosis of airway disease.

Design

All participants attended a single visit with pre- and post-bronchodilator spirometry and documentation of asthma symptoms, asthma-specific quality of life [9], medication use and smoking status assessed, and sputum induction performed.

Sample collection

Sputum induction with hypertonic saline (4.5%) was performed as previously described [10]. Selected sputum was stored for DNA extraction or dispersed using dithiothreitol for sputum cell count assessment and inflammatory subtype determination [3].

DNA preparation, 16S rRNA PCR, amplicon pyrosequencing and real-time PCR

Details of bacterial DNA preparation from samples, sequencing and sequencing data analysis along with the confirmatory real-time PCR methods are presented in the supplementary material.

Data analysis

Clinical data were analysed using Stata 11 (Stata, College Station, TX, USA) using standard methods as described in the online supplementary material.

Results

Clinical, inflammatory and typical culture profile

Participants with neutrophilic asthma were slightly younger and commonly male, with similar lung function, asthma control score and asthma symptoms compared with those with non-neutrophilic asthma (table 1). Sputum inflammatory cell counts revealed significantly more total cells with an increased proportion of neutrophils and a reduced proportion of eosinophils, macrophages and columnar epithelial cells compared with the non-neutrophilic asthma group (table 1).

Sputum microbiome

Sputum samples from participants with neutrophilic asthma were less diverse and dominated by a small number of species (low richness and evenness), whereas the non-neutrophilic asthma participants had a diverse array of species and they were more evenly distributed in the community (high richness and evenness). This was shown by a significantly lower Shannon index, richness and Simpson index in adults with neutrophilic asthma (table 2). This difference was maintained after correction for age, gender, previous smoking and ICS dose (ANCOVA, p=0.035).

Bacteria from the phyla Proteobacteria and Firmicutes (dominated by *Streptococcus* species) were most common, accounting for >90% of the operational taxonomic units (OTUs) in the neutrophilic asthma group (table 2 and figure 1). There was a significantly lower proportion of OTUs for Actinobacteria and Firmicutes, and significantly more Proteobacteria in those with neutrophilic compared with non-neutrophilic asthma (table 2). The reduced richness and diversity in the neutrophilic asthma group appeared to be driven by a group of four participants with a high number of sequences for *Haemophilus* with very few other taxa, as shown in figure 1. There was no difference in the Shannon or Simpson index according to the study site

TABLE 1 Clinical and inflammatory cell parameters of participants with neutrophilic and non-neutrophilic asthma

	Neutrophilic	Non-neutrophilic	p-value
Subjects	7	20	
Age years	50.7±9.7	60.3±10.8	0.049
Male	15 (71)	6 (30)	0.071
Atopic	6 (86)	16 (80)	0.713
Ex-smoker	2 (29)	11 (55)	0.224
Smoking pack-years	1 and 24	16.8 (2.2, 56.5)	0.621
FEV_1 % predicted	84.2 (17.0)	74.3 (17.8)	0.213
FEV_1/FVC %	74.8±7.5	66.8±10.0	0.064
ACQ6 score	1.0±0.7	1.4±1.0	0.342
GINA treatment step			0.562
1	0 (0)	1 (5)	
2	0 (0)	0 (0)	
3	1 (17)	1 (5)	
4	5 (83)	18 (90)	
Taking ICS	6 (86)	18 (90)	0.610
ICS dose µg	2000 (800, 2000)	2000 (1000, 2000)	0.933
VAS symptom scores			
Breathlessness	4.1 (1.1, 23)	7.2 (4.8, 10.0) (n=19)	0.341
Wheeze	1.1 (0.1, 7.0)	3.5 (1.0, 6.4) (n=19)	0.469
Cough	4.5 (2.2, 51)	7.5 (2.8, 10.0) (n=19)	0.665
Sputum production	3.0 (0.7, 14.0)	5.9 (0.7, 10.0) (n=19)	0.729
Inflammatory cell counts			
Total cell count $\times 10^6$ mL^{-1}	7.2 (6.7, 9.1)	3.2 (2.1, 5.0)	0.015
Viability %	84 (81, 94)	78 (73, 83)	0.064
Neutrophils %	74.4±12.2	30.0±16.7	<0.001
Eosinophils %	0.3 (0.0, 1.0)	2.1 (0.9, 4.4)	0.016
Macrophages %	21.5 (14.8, 34.8)	52.7 (41.6, 68.9)	<0.001
Lymphocytes %	0.5 (0.0, 1.75)	1.0 (0.3, 1.1)	0.538
Columnar epithelial cells %	0.3 (0.0, 1.0)	1.9 (0.9, 4.9)	<0.001
Squamous cells %	1.2 (0.0, 11.3)	4.7 (1.4, 8.6)	

Data are presented as n, mean±SD, n (%) or median (quartile 1, quartile 3), unless otherwise stated. FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; ACQ6: Asthma Control Questionnaire 6; GINA: Global Initiative for Asthma; ICS: inhaled corticosteroid; VAS: visual analogue scale.

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TABLE 2 Sputum sample bacterial diversity and phyla for adults with neutrophilic and non-neutrophilic asthma

	Neutrophilic	Non-neutrophilic	p-value
Subjects	7	20	
Shannon index	1.1 (0.9, 4.2)	3.8 (3.4, 4.2)	0.010
Simpson index	0.3 (0.3, 0.9)	0.9 (0.8, 0.9)	0.035
Bacteria phyla			
Actinobacteria	3.8 (0.2, 3.1)	4.0 (2.1, 6.7)	0.019
Bacteroidetes	2.3 (1.1, 26.7)	14.9 (8.4, 22.8)	0.092
Firmicutes	7.8 (3.1, 25.7)	35.8 (27.5, 41.8)	0.009
Fusobacteria	0.5 (0.3, 3.8)	3.7 (2.3, 7.8)	0.103
Proteobacteria	88.0 (25.1, 92.0)	38.0 (21.1, 43.1)	0.046

Data are presented as n or median (quartile 1, quartile 3), unless otherwise stated.

where the sample was collected (online supplementary table S1). Dominant members of the microbial community included *Prevotella*, *Streptococcus*, *Neisseriaceae* and *Haemophilus* (figure 1).

Smoking

16 (53%) participants had smoked in the past; this group tended to have a higher prevalence of sequences for bacteria from the phyla Actinobacteria and Bacteroidetes, and a lower prevalence of bacteria from phylum Proteobacteria compared with never-smokers. Bacteria from the phylum Fusobacteria were significantly more prevalent in ex-smokers compared with never-smokers. There was also a significant elevation in both the Shannon and Simpson indices in ex-smokers compared with never-smokers, indicating increased diversity in this group (table 3).

Smoking pack-years were significantly associated with the prevalence of Actinobacteria, (r=0.420 p=0.021), but not with any of the other bacterial phyla. There was no association between smoking pack-years with either the Shannon or Simpson indices.

Tropheryma whipplei

Seven (23%) participants had sequences corresponding to the genus *Tropheryma* (phylum Actinobacteria) in their induced sputum. Participants with *Tropheryma* sequences had similar age, lung function, ACQ6 score, visual analogue scale symptom score and sputum cell differential as those without OTUs for *Tropheryma* (table 4). In the seven participants where *Tropheryma* was identified, there was a significant negative association between *Tropheryma* OTU and wheeze scores (Spearman r=−0.821, p=0.023) (data not shown). Of note, five of the seven participants with *Tropheryma* also had eosinophilic airway inflammation.

Using PCR to screen for *Tropheryma whipplei*, 12 (40%) participants were found to be positive: six participants identified from the original pyrosequencing analysis and an additional six newly identified samples. These data confirmed that the *Tropheryma* OTUs were in fact *T. whipplei*.

Haemophilus influenzae

Figure 2 shows the principal component analysis (PCA) and identified *Haemophilus2* (OTU4) as a dominant microbe in a group of asthma participants. The clinical and inflammatory features of those with a high frequency of *Haemophilus* sequences (*Haemophilus* high) were then examined more closely. *Haemophilus* high participants were typically younger atopic males, who had never smoked, commonly of the neutrophilic asthma inflammatory subtype (table 5). Using univariate linear regression to examine other possible determinants of neutrophil proportion, there was no significant association between age, smoking pack-years, gender or dose of ICS. Participants in the *Haemophilus* high group had a 20% higher sputum neutrophil proportion than other participants (p=0.074).

Using logistic regression, we asked which clinical and inflammatory outcomes were associated with a participant being in the *Haemophilus* high group. Age, sex and sputum neutrophil proportion were each independent predictors of participants being in the *Haemophilus* high group using univariate analysis. Smoking pack-years and dose of ICS were not associated with the *Haemophilus* high group.

Multivariate logistic regression revealed that a model including age, sex and sputum neutrophil proportion together predicted inclusion in the *Haemophilus* high group (p=0.0004; R^2=0.599). None of the three

FIGURE 1 Heatmap showing the relative abundances of the dominant bacterial operational taxonomical units. f_: family; c_: class.

variables on their own predicted inclusion in the *Haemophilus* high group; however, this may be a power issue.

Given four of the six samples with a high abundance of *Haemophilus* OTU4 in the PCA were from a neutrophilic inflammatory subtype, we sought to enrich the number of neutrophilic asthma participants and determine if the species of *Haemophilus* was indeed *H. influenzae*. We identified a further 16 participants with poorly controlled neutrophilic asthma. Real-time PCR analysis was undertaken on all 46 participants (the initial cohort of 30 and the additional 16 with neutrophilic asthma) to identify *H. influenzae*. The clinical and inflammatory profiles of the further 16 participants are shown in online supplementary table S2.

24 of the 30 initial participants and 11 of the additional 16 neutrophilic asthma participants were PCR-positive for *H. influenzae*. The detection rate of *H. influenzae* was not different in those with neutrophilic and non-neutrophilic asthma (81% compared with 75%, p=0.453). There were no significant

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TABLE 3 Sputum sample bacterial diversity and phyla for ex-smokers and never-smokers

	Ex-smoker	Never-smoker	p-value
Subjects	16	14	
Shannon index	3.8 (3.5, 4.4)	2.8 (1.1, 4.1)	0.010
Simpson index	0.9 (0.8, 0.9)	0.7 (0.3, 0.9)	0.040
Bacteria phyla			
Actinobacteria	4.0 (2.0, 7.5)	2.6 (0.3, 4.5)	0.077
Bacteroidetes	14.5 (8.4, 23.9)	9.1 (1.1, 22.9)	0.299
Firmicutes	32.4 (26.3, 37.2)	21.2 (5.4, 40.7)	0.271
Fusobacteria	4.0 (3.4, 9.3)	2.1 (0.4, 5.0)	0.028
Proteobacteria	41.5 (22.6, 43.1)	60.3 (21.3, 91.4)	0.360

Data are presented as n or median (quartile 1, quartile 3), unless otherwise stated.

differences in age, gender, atopy, lung function, ICS dose or smoking history between those who were PCR-positive or -negative for *H. influenzae* (data not shown). The ACQ6 score was significantly higher in those who were PCR-positive compared with those who were PCR-negative, mean±SD ACQ6 1.9±1.1 compared with 1.1±0.9, p=0.037.

Discussion

In the present study, adults with asthma had reduced bacterial diversity with the lowest diversity demonstrated in those with neutrophilic asthma. Dominant taxa were identified within specific inflammatory phenotypes. We identified a group of adult males with high abundance of *Haemophilus* sequences with predominantly neutrophilic airway inflammation. We also identified a group of participants with asthma who had *Tropheryma* sequences which we confirmed as *T. whipplei*. These participants most commonly exhibited an eosinophilic asthma subtype.

TABLE 4 Clinical and inflammatory characteristics of participants with and without *Tropheryma* sequences

	Tropheryma present	*Tropheryma* absent	p-value
Subjects	7	23	
Age years	53±14	60±9	0.175
Male	2 (29)	10 (43)	0.403
Atopic	5 (71)	19 (87)	0.347
Ex-smoker	4 (57)	12 (52)	0.581
Smoking pack-years	16.7 (7.7, 47.8)	14.4 (1.3, 40.3)	0.505
FEV_1 % predicted	81±24	76±15	0.577
FEV_1/FVC %	71±10	68±10	0.517
ICS dose µg	2000 (2000, 4000)	2000 (1000, 2000)	0.150
ACQ6 score	1.0±0.7	1.5±1.0	0.250
VAS symptom scores			
Breathlessness	6.5 (2.1, 23.0)	7.0 (4.1, 10.0)	1.000
Wheeze	3.5 (1.0, 4.6)	3.2 (0.8, 6.7)	0.683
Cough	6.0 (3.7, 7.2)	7.4 (2.2, 10.0)	0.386
Sputum production	3.8 (0.0, 5.9)	4.7 (1.8, 10.0)	0.740
Inflammatory cell counts			
Total cell count $\times 10^6$ mL^{-1}	5.2 (3.3, 7.2)	4.6 (2.2, 8.9)	0.713
Viability %	85 (74, 94)	81 (75, 84)	0.328
Neutrophils %	40.9±25.5	41.8±25.7	0.942
Eosinophils %	1.8 (1.0, 7.0)	1.5 (0.3, 4.0)	0.769
Macrophages %	45 (35, 77)	41 (21, 61)	0.419
Lymphocytes %	1.0 (0.3, 2.5)	0.5 (0.3, 1.0)	0.457
Columnar epithelial cells %	0.5 (0.3, 2.5)	1.3 (0.3, 3.0)	0.475
Squamous cells %	6.8 (1.5, 9.5)	3.6 (0.5, 6.3)	0.154

Data are presented as n, mean±SD, n (%) or median (quartile 1, quartile 3), unless otherwise stated. FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; ACQ6: Asthma Control Questionnaire 6; ICS: inhaled corticosteroid; VAS: visual analogue scale.

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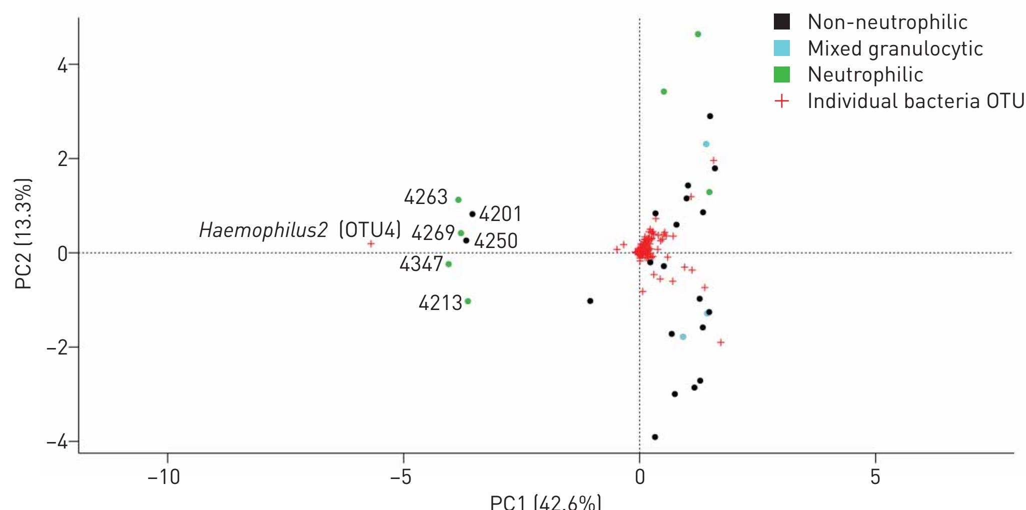

FIGURE 2 Principal component analysis plot. The axes represent each of the principal components (PC1 and PC2) and the proportion of the variation explained is shown in brackets. OTU: operational taxonomic unit.

Our data show reduced bacterial diversity in neutrophilic asthma. This is the first examination of bacterial diversity within asthma inflammatory subtypes. Diversity has been reported to be increased [5, 11] or similar [6] between patients with asthma and healthy controls. Similar variation in diversity has been reported in patients with chronic obstructive pulmonary disease (COPD) where bacterial diversity has been reported as increased [12] or reduced [13] compared with controls. As the majority of participants

TABLE 5 Clinical and inflammatory characteristics of participants with and without a high abundance of *Haemophilus* sequences (*Haemophilus* high and *Haemophilus* low groups)

	***Haemophilus* high**	***Haemophilus* low**	**p-value**
Subjects	6	24	
Age years	45±14	62±7	<0.001
Male	5 (83)	7 (29)	0.026
Atopic	6 (100)	18 (78)	0.283
Ex-smoker	0 (0)	16 (67)	0.005
Smoking pack-years		14.4 (1.9, 40.3)	
FEV_1 % predicted	82.2±14.2	76.2±17.9	0.452
FEV_1/FVC %	73.2±8.8)	67.6±10.5	0.241
ICS dose µg	2000 (2000, 2000)	2000 (900, 2000)	0.393
ACQ6 score	1.19±0.68	1.44±1.00	0.571
VAS symptom scores			
Breathlessness	2.8 (1.1, 8.0)	7.0 (4.8, 18.0)	0.077
Wheeze	1.1 (0.8, 2.0)	4.0 (1.1, 7.0)	0.063
Cough	5.0 (3.0, 6.0)	7.2 (1.8, 10.0)	0.706
Sputum production	2.7 (0.7, 3.3)	5.9 (1.8, 10.0)	0.215
Inflammatory cell counts			
Neutrophilic asthma	4 (67)	3 (15)	0.024
Total cell count $\times 10^6$ mL^{-1}	7.0 (3.5, 8.4)	4.4 (2.5, 8.6)	0.568
Viability %	82 (75, 84)	82 (76, 90)	0.484
Neutrophils %	61.2±33.6	35.9±19.8	0.027
Eosinophils %	1.3 (0.3, 4.3)	1.6 (0.5, 3.6)	0.917
Macrophages %	23.8 (14.8, 42.3)	46.3 (34.8, 62.5)	0.087
Lymphocytes %	1.5 (0.5, 2.5)	0.5 (0.3, 1.0)	0.104
Columnar epithelial cells %	0.4 (0.3, 1.0)	1.8 (0.4, 3.3)	0.152
Squamous cells %	5.6 (1.0, 9.9)	3.7 (1.1, 7.0)	0.622

Data are presented as n, mean±SD, n (%) or median (quartile 1, quartile 3), unless otherwise stated. FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; ACQ6: Asthma Control Questionnaire 6; ICS: inhaled corticosteroid; VAS: visual analogue scale.

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were taking ICS, we are unable to determine if the use of ICS influences bacterial diversity. We corrected the diversity analysis for the dose of ICS, and found the difference in both Simpson and Shannon index was maintained after correction for ICS dose.

Studies of asthma in both adults and children report an increased frequency of sequences from the phylum Proteobacteria [4–6, 14]. Huang *et al.* [14], in their study of 30 adults with severe asthma, observed that a worsening ACQ score was associated with an abundance of Proteobacteria and an association between T-helper 17-related gene expression (including CXCL8 and its receptors) with Proteobacteria taxa. We extend this to show this increase in Proteobacteria taxa is most prominent in those with neutrophilic asthma. We also observed significantly fewer OTUs from bacteria in the phyla Firmicutes and Actinobacteria in neutrophilic asthma. The anaerobic bacterial phylum Fusobacteria was also less frequent in neutrophilic asthma, although not statistically different. This phylum has been associated with corticosteroid-resistant asthma [15] which is known to be noneosinophilic in nature and suggests the absence of common oral anaerobes may be an important difference between asthma subtypes.

Currently, the impact of long-term ICS use on bacterial diversity is unknown. Early studies suggest that ICS use may influence the airway microbiome, resulting in a unique cluster of COPD patients taking ICS compared with those not taking ICS [12]. Similarly there was a unique cluster of taxa among those with corticosteroid-resistant asthma, including the genera *Haemophilus* and *Tropheryma* [15]. In this study, where the majority of participants were taking high doses of ICS, we also show a high prevalence of *T. whipplei* and *H. influenzae* in poorly controlled asthma. Little is understood about the impact of inhaled medications on oral flora. ICS may promote adverse oral effects; the most commonly reported of these is a higher rate of oral *Candida* species detection [16] and there is some evidence of mucosal damage in children with asthma [17].

This study is limited in its cross-sectional nature as it is not possible to distinguish between cause and effect. The strengths of this study include the well-characterised study population combining detailed clinical assessment with assessment of both airway inflammation and the airway microbiome. Induced sputum has been used frequently to study the airway microbiome [18–20]. We have attempted to reduce oral contamination by selecting the lower airway portions of the sample from the salivary material; however, oral contamination cannot be excluded. Other research suggests that while lower airway samples do contain an abundance of oral microbiota, there is a lung-specific signal where some OTUs are disproportionately found in bronchoalveolar lavage compared with oral wash, particularly *Haemophilus* species [21].

We identified seven patients with asthma who had sequences belonging to the genus *Tropheryma* (phylum Actinobacteria). The only named species of this genus, *T. whipplei*, causes Whipple's disease, a gastrointestinal disorder characterised by arthritis, diarrhoea and weight loss [22]. Increasing evidence supports a role for *T. whipplei* in airway disease with identification in interstitial lung disease [23], pneumonia [24, 25] and recently in corticosteroid-resistant asthma [6]. Pulmonary symptoms may precede gastrointestinal symptoms, including intermittent fever, dry cough and shortness of breath, which suggests that airway colonisation or infection may contribute to the symptoms and exacerbations observed in asthma. Our PCR confirmation resulted in a further five participants having evidence of *T. whipplei*, with a PCR detection rate of 40%. *Tropheryma* has also been reported in asymptomatic HIV infection (13%), specifically in the lung rather than the mouth [26], and this is further evidence that our identification of *Tropheryma* in samples of induced sputum may originate in the lower airway. The participants with *Tropheryma* tended to be taking a higher dose of ICS; however, this was not statistically different. While further research with longitudinal follow-up is needed to determine if this group of patients will develop Whipple's disease, identification of *Tropheryma* suggests previously unidentified microorganisms may be present in the airways, possibly contributing to asthma symptoms and airway inflammation.

H. influenzae is the most common species from the phylum Proteobacteria resulting in airway infection, and has been isolated from the airways of both adults and children with asthma [1, 27, 28]. In neutrophilic asthma, there is a high incidence of *H. influenzae* identified using traditional culture methods, compared with other inflammatory phenotypes [2]. In this study, we observed a group of adults with asthma with a predominance of *Haemophilus* OTUs who were younger, atopic, nonsmoking males who tended to have higher proportions of neutrophils. Since the sensitivity of the pyrosequencing method does not allow the exact species to be determined, we conducted real-time PCR using specific probes and found that 80% of participants with poorly controlled asthma were positive for *H. influenzae*. While PCR does not distinguish between the presence of dead and live organisms, it is important to recognise that even dead or degraded bacteria can elicit an immune response, *e.g.* bacteria endotoxin can induce significant inflammatory and immune signals. We have previously described high levels of sputum endotoxin in patients with neutrophilic asthma and, in the same group, a high incidence of positive culture for *H. influenzae* [2].

In conclusion, we have been able to link differences in the airway microbiome to altered host inflammatory response in asthma. There are phenotype-specific alterations to the airway microbiome in

asthma. Reduced bacterial diversity combined with a high prevalence of *H. influenzae* was observed in neutrophilic asthma, whereas eosinophilic asthma had abundant *T. whipplei*. These changes may modulate local airway inflammation and immune responses. Understanding the relationships between these events will contribute to the development of more specific treatment strategies.

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