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IMMUNOLOGICAL COROLLARY OF THE PULMONARY MYCOBIOME IN BRONCHIECTASIS: THE CAMEB STUDY

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ABSTRACT

Introduction: Understanding the composition and clinical importance of the fungal mycobiome was recently identified as a key topic in a "research priorities" consensus statement for bronchiectasis **Methods:** Patients were recruited as part of the CAMEB study: an international multicentre cross-sectional Cohort of Asian and Matched European Bronchiectasis patients. The mycobiome was determined in 238 patients by targeted amplicon shotgun sequencing of the 18S-28S rRNA internally transcribed spacer regions ITS1 and ITS2. Specific qPCR for detection of and conidial quantification for a range of airway *Aspergillus* species was performed. Sputum galactomannan, *Aspergillus*-specific IgE, IgG and Thymus and Activation Regulated Chemokine levels were measured systemically and associated to clinical outcomes. **Results:** The bronchiectasis mycobiome is distinct, and characterised by specific fungal genera including *Aspergillus*, *Cryptococcus,* and *Clavispora*. *A. fumigatus* (in Singapore/Kuala Lumpur) and *A. terreus* (in Dundee) dominated profiles, the latter associating with exacerbations. High frequencies of *Aspergillus*-associated disease including sensitization and allergic bronchopulmonary aspergillosis were detected. Each revealed distinct mycobiome profiles and associated with more severe disease, poorer pulmonary function and increased exacerbations. **Conclusion:** The pulmonary mycobiome is of clinical relevance in bronchiectasis. Screening for *Aspergillus*-associated disease should be considered even in apparently stable patients.

Key words: Fungi, *Aspergillus*, microbiome, mycobiome, bronchiectasis

Abbreviations:

AC: Aspergillus colonized

AS: Aspergillus sensitized

BMI: Body mass index

BSI: Bronchiectasis severity index

CAMEB: Cohort of Asian and Matched European Bronchiectasis

COPD: Chronic obstructive pulmonary disease

DD: Dundee

GM: Galactomannan

HRCT: High resolution computed tomography

ND: Non-diseased

OTU: Operational taxonomic unit

qPCR: Quantitative polymerase chain reaction

sABPA: Serological allergic bronchopulmonary aspergillosis

SAFS: Severe asthma with fungal sensitization

sCPA: Suspected chronic pulmonary aspergillosis

SG-KL: Singapore-Kuala Lumpur

sIgE: Specific immunoglobulin E

TARC: Thymus and activation regulated chemokine

INTRODUCTION

Bronchiectasis is a chronic respiratory disease characterised by progressive bronchial dilatation. To date, no therapy has been licensed for its treatment. Geographic regions illustrate preponderance for particular aetiologies, for instance post-tuberculous disease in countries of endemic infection [1]. Incipient infection in bronchiectasis incites deleterious host inflammatory responses and disease progression. A 'vicious cycle' of impaired mucociliary clearance, recurrent infection and chronic inflammation, with established links to bacteria, lead to progressive disease however, the role of fungi is poorly understood.

The pulmonary microbiome and its association with chronic respiratory disease is an emerging area of research. Culture-independent airway sequencing has revealed novel associations between the airway microbiome and lung disease [2]. To date, most airway microbiome studies have a bacterial focus; an approach that provides disease insight into pathogenesis and prognosis[3, 4]. In contrast however, sequencing of the fungal microbiome (the mycobiome) has lagged behind and is applied in relatively few studies and, to our knowledge none in bronchiectasis [5].

Fungal spores are environmentally ubiquitous hence their inhalation is an inevitable consequence of breathing [6]. While prompt mucociliary and phagocytic clearance occurs in the healthy lung, anatomically abnormal and immunocompromised airways such as in bronchiectasis are at higher risk of fungal acquisition, colonization and potential disease [6]. Our group and others have performed extensive work characterising fungi in cystic fibrosis (CF) related bronchiectasis where they colonize and act as pathogens associated with poorer clinical outcome [7-10].

Fungi, particularly *Aspergillus spp.* cause a range of pulmonary consequences including allergic, chronic and/or invasive disease [11, 12]. An overzealous host response to *Aspergillus* for instance is clinically important in severe asthma with fungal sensitization (SAFS), chronic obstructive pulmonary disease (COPD) and CF [13-15]. The role of fungi in bronchiectasis not due to CF is, however, less well defined. *Aspergillus* sensitization and/or allergic bronchopulmonary aspergillosis (ABPA) is a cause (~10%) and importantly a consequence of bronchiectasis [6, 10, 16]. The role of the mycobiome in bronchiectasis is yet to be comprehensively investigated and its importance and relevance is clearly highlighted as a priority research area in this field [17].

We characterise for the first time, the mycobiome in bronchiectasis assessing its clinical relevance in two geographically distinct cohorts from the CAMEB study, encompassing four Asian sites across Singapore and Kuala Lumpur and a single European site in Dundee.

METHODS

Study Population: Patients with stable bronchiectasis were recruited across three countries as part of the CAMEB study; a cross-sectional Cohort of Asian and Matched European Bronchiectasis. Recruitment included three sites in Singapore (Singapore General Hospital, Changi General Hospital and Tan Tock Seng Hospital; n=124), one Malaysian site (UKM Medical Centre, Kuala Lumpur; n=14) and an age-, sex- and disease-severity matched group from a single European site (Ninewells Hospital, Dundee, UK; n=100) and was conducted between March 2016 and July 2017. Full details on patient inclusion and exclusion criteria, non-diseased controls and the CAMEB population are provided in the supplementary methods. Clinical characteristics, bronchiectasis aetiology and patient demographics are shown in Table 1. The study was approved by the institutional review boards of all participating institutes and all patients gave written informed consent to participate. Further details are in the supplementary methods.

Full details on clinical data and specimen collection, statistical analysis, molecular methods including mycobiome analyses, sputum qPCR detection of fungi and immunological bioassays are provided in the supplementary methods.

RESULTS

The pulmonary mycobiome in bronchiectasis is distinct: Culture-independent analysis of the pulmonary mycobiome in bronchiectasis reveals that Ascomycota dominate the airway at the phylum level ($p < 0.01$, Mann-Whitney U-test) (Figure 1A). Fungal diversity was significantly lower in bronchiectasis, as measured by the number of identified taxa ($p < 0.01$) and the Shannon Diversity Index; SDI ($p < 0.05$) (Mann-Whitney U-test) (Figure 1A). Limitations of fungal ITS databases may account for the lower classification percentages observed (95.3%) compared to bacteria where genus-level OTU classification of ~99% can be achieved [18]. Despite Ascomycota dominating overall mycobiome profiles, a significant number of bronchiectasis patients (49%, n=116) harboured some degree of airway Basidiomycota. Ten percent (n=23) had Basidiomycota making up at least one quarter of their overall profile while 4% (n=9) exhibited Basidiomycota-dominant profiles (Figure 1B). *Candida*, *Saccharomyces* and *Penicillium* were the most frequently detected genera in both groups while differentially abundant, bronchiectasis-associated genera included *Aspergillus*, *Cryptococcus, Clavispora, Botrytis* and *Alternaria* (Figures 1C and 2A).

In the design of the CAMEB study, n=100 Asian patients (Singapore / Kuala Lumpur) were matched individually to patients from a European cohort (Dundee) by age; gender and total bronchiectasis severity index (BSI) score (Table 1). This allowed assessment of findings in two geographically distinct cohorts as well as providing insight into potential regional differences in the bronchiectasis mycobiome. Geographic variation in mycobiome signature was detected (Figure 2A). Patients from Singapore and Kuala Lumpur exhibited significant and higher average relative abundance of *Simplicillium*, *Trichosporon* and *Aspergillus* while patients from Dundee were distinguished by higher abundances of *Wickerhamomyces*, *Clavispora* and *Cryptococcus* ($p < 0.05$) following assessment of group differences using

metastats statistical analysis[19]. *Candida,* by far the most frequently observed fungal genera, was observed across both cohorts at equal frequency while patients from Dundee exhibited higher *Saccharomyces, Penicillium, Cryptococcus, Clavispora,* and *Botrytis* in particular. The only fungal genus in the top ten with higher prevalence in Singapore and Kuala Lumpur was *Aspergillus*, although this was detected at all the sampled sites (Figure 2B). Given our identification of *Aspergillus* as an exclusive bronchiectasis-associated fungal genus with high airway abundance; and its established pathogenic role in other chronic respiratory diseases [10, 12, 13, 15], we pursued it for further investigation.

***A. fumigatus* and *A. terreus* are identified in bronchiectasis and associate with exacerbations:** We next further characterised the presence of specific *Aspergillus* species in the airway by qPCR using established protocols published by our group and others [7, 20]. We assessed four major *Aspergillus* species: *fumigatus, terreus, flavus* and *niger* and found that non-diseased individuals had no detectable airway *Aspergillus*, in agreement with our mycobiome analysis (Figure 3A). In contrast, high proportions of bronchiectasis patients (from all sites) had detectable *A. fumigatus* and/or *A. terreus* with no patient demonstrating *A. flavus* or *A. niger* (Figure 3A). Interestingly, similar proportions of those recruited from an Asian site had either *A. fumigatus* and/or *A. terreus* whilst patients from Dundee demonstrated higher *A. terreus* (Figure 3A) which associates with increased exacerbations (Figures 3B). Importantly, significant numbers of patients (40%, n=96) harboured both species concurrently. Of these, an equal distribution between groups recruited from Singapore/Kuala Lumpur and Dundee respectively was observed (44% vs 52%, $p = 0.22$) (Figure 3C). Patients from Singapore and Kuala Lumpur were equally likely to have none, either or both species in their airway in contrast to patients from Dundee who exhibit a higher likelihood for *A. terreus* (Figure 3C). Greater exacerbations are seen in patients with *A.*

terreus alone or where both species were present compared to those without any fungi or *A. fumigatus* alone (Figure 3D).

Quantification of *A. fumigatus* to *A. terreus* conidial burden ratio and associated exacerbations: We next quantified *A. fumigatus* and *A. terreus* conidial burden using established protocols published by our group [7]. We then classified the detected loads as low (<500), intermediate (500-2000 or high (>2000) based on the number of conidia per gram of sputum. Measured conidial burden varied among *Aspergillus*-positive patients and was comparable between patients recruited from Singapore/Kuala Lumpur and Dundee respectively (Figure 4A). Interestingly, where patients harboured both species concurrently, their proportionality varied: patients from sites in Singapore and Kuala Lumpur exhibited higher proportions of *A. fumigatus* (median = 69% of conidial load) whilst the Dundee cohort had a higher *A. terreus* burden (median = 89% of total conidial load) (Figure 4B). This agrees with our earlier observation of regional variation; where cohorts in Singapore/Kuala Lumpur tended toward more *A. fumigatus* and the Dundee cohort greater *A. terreus* either when the fungi exist alone or together. When patients were grouped by detectable *Aspergillus* species and accompanying conidial burden (using high load as cut-off); a significant association existed between high concurrent conidial burdens of both *Aspergillus* species and greater exacerbations (Figure 4C). Specifically, however, when a high conidial burden of *A. terreus* was identified, either alone or in combination with *A. fumigatus*, significantly more exacerbations also occured (Figure 4C).

A high frequency of clinically relevant *Aspergillus*-associated disease occurs in bronchiectasis: Prior work from our group and others highlights the association of *Aspergillus*-associated disease (Aspergillosis) with adverse clinical outcomes across a range

of chronic respiratory diseases [7, 13, 21]. Given the high frequencies of *Aspergillus* detected, we next evaluated the occurrence of Aspergillosis and its clinical relevance in bronchiectasis. To do this, we used a modified immunological classification system developed for CF (Table E4) [14]. In addition to the criteria described by Baxter *et al.*, our modified version includes incorporation of potential *A. terreus*-associated disease (by inclusion of *A. terreus* sIgE and qPCR) along with other minor modifications detailed in Table E4. Of note; total IgE although measured, was not used in our classification of patients because it is described that ABPA may occur at IgE concentrations below the described cut-offs in the established criteria [22]. Patients were grouped into the following five categories: non-diseased (ND); *Aspergillus*-colonized (AC); *Aspergillus*-sensitized (AS); serological allergic bronchopulmonary aspergillosis (sABPA) and suspected chronic pulmonary aspergillosis (sCPA). Significant numbers in the CAMEB cohort met criteria for an *Aspergillus*-associated disease state (ND; 1.7%, AC; 3.4%, AS; 76.5%, sABPA; 18.1%, sCPA; 0.3%) (Figure 5). Measured total serum IgE, *Aspergillus*-specific IgG and sputum galactomannan varied between patient groups (Figure E1). Specific-IgE against crude antigens of *A. fumigatus* and *A. terreus* respectively were highest in AS (0.77 and 0.57 kU/L) and sABPA (0.92 and 0.93 kU/L) ($p < 0.001$) (Figures 5A-B). Thymus and Activation Regulated Chemokine (TARC); previously identified as a marker of CF-ABPA was also assessed [23]. We found TARC to be a poor indicator of AS and/or sABPA in bronchiectasis with high false negative rates in both groups (Figure 5C). Additionally, false positives were also observed in a small number of ND patients (Figure 5C). Patients with sABPA exhibited significantly more exacerbations, poorer pulmonary function and the severest disease compared to other groups (Figure 5D-F). AS was associated with greater exacerbations while AC portended toward more exacerbations with preserved pulmonary function and less severe disease (Figure 5D-F). AC, AS and sABPA therefore appear to exist along an increasing

continuum of disease severity. Only a single patient met criteria for sCPA precluding further analysis of this group. *Aspergillus*-associated disease in bronchiectasis is therefore frequent, variable and clinically relevant including in patients appearing clinically stable; a characteristic inclusion criteria for our study.

***Aspergillus*-associated disease in bronchiectasis has distinct mycobiome profiles:** As high frequencies of *Aspergillus*-associated disease were observed in bronchiectasis based on our modified classification system, we next assessed for the presence of distinct taxonomic profiles that may associate with four of the five clinical states: ND, AC, AS and sABPA, excluding sCPA because this group contained only a single patient. Although unsupervised beta-diversity analysis revealed three distinct mycobiome groups (Figure E3) their association with clinical phenotype was not evident. We therefore adopted a supervised strategy comparing mycobiome profiles between our identified immunological classes. Each clinical state revealed mycobiome profiles characterised by a combination of varying fungal genera (Figure 6). The ND group exhibited apparent high abundance of *Phellinus*, *Magnusiomyces* and *Phlebia* with lower relative abundance of *Saccharomyces*, *Aspergillus* and *Penicillium* (Figure 6A). These latter three genera however increased in their relative abundance across a spectrum from ND to AC, AS and sABPA (Figure 6, Table E5). The genera *Cryptococcus* and *Clavispora* followed similar and comparable spectra with increasing relative abundance from AC, AS to sABPA. *Mycosphaerella*, and *Botrytis* were present only in diseased states without specific pattern (Figure 6B-D). Other characteristic fungal genera included *Trichosporon* and *Cladosporium* in the AC state and; *Wickerhamomyces* and *Alternaria* in AS and sABPA (Figure 6B). Importantly, the relative abundance of *Aspergillus* detected from mycobiome profiles were in agreement with our qPCR-based quantification of conidial burden for *A. fumigatus* and *A. terreus* across all

disease categories with greater exacerbations noted in AS and sABPA (Figure 6). Mycobiome profiles unique to each *Aspergillus*-associated disease state are therefore potentially useful for future bronchiectasis studies that focus on the diagnosis and endo-phenotyping of fungal disease.

DISCUSSION

We describe the first, culture-independent analysis of the pulmonary mycobiome performed to date in bronchiectasis and, to our knowledge, the first such respiratory study to meticulously match geographically distinct patient cohorts allowing us to assess potential regional variation in the mycobiome. By applying high-throughput 18S-28S ITS sequencing and other molecular techniques, we delineated the mycobiome constituents and their associated clinically relevant states. Our analysis identified *Aspergillus* as a major fungal genus in bronchiectasis, similar to that described in other chronic respiratory diseases [6, 12]. An interesting observation was the prevalence of specific *Aspergillus* species characterising our Singapore/Kuala Lumpur and Dundee cohorts with *A. fumigatus* and *A. terreus* most frequently detected within each respective group, the latter associated to exacerbations. Further patient stratification into groups including AC, AS and sABPA revealed high occurrences of clinically significant disease, even in 'stable' patients and, the existence of distinct mycobiome profiles of discriminant taxa for each group.

Changes to regional lung growth conditions creates a conducive environment for colonisation and infection by pathogenic microbes [2]. Given the gross anatomical distortion characteristic of bronchiectasis, it is therefore unsurprising that microbial differences are observed when compared to non-diseased states. The chronic bronchial insult coupled to anatomic distortion, compromised mucociliary clearance, sustained neutrophilic response and

recurrent infection all favour the growth of pathogenic fungi and their ensuing clinical consequences in bronchiectasis [6, 24].

As observed in this and other studies, the airway mycobiome is dominated by Ascomycota [25]. Ascomycota-dominant profiles are characteristic of bronchiectasis although an important minority have high airway Basidiomycota. Basidiospores are environmentally more abundant and smaller than ascospores, explaining their airway presence [26]. Compared to Ascomycota, which includes the established respiratory pathogens *Aspergillus* and *Candida*, the specific role of Basidiomycota in bronchiectasis is unclear. Notably pathogenic Basidiomycota including *Cryptococcus, Schizophyllum, Phellinus, Ceriporia* and *Trichsporon* were all detected in our work [27]. *Cryptococcus* is significantly elevated in bronchiectasis; an observation previously documented, which now warrants further investigation [28]. The existence of pulmonary Basidiomycota might reflect dynamic trafficking of 'rare biosphere' fungi ('immigration and elimination'), suggestive of a healthier respiratory state rather than the sustained fungal outgrowth and unfavourable environment associated with Ascomycota [2, 5]. The cross-sectional design of our study however precluded longitudinal assessment of the stability and transiency of the mycobiome over time, a key consideration for future work.

Bronchiectasis-associated fungal genera include *Aspergillus, Issatchenkia, Wickerhamomyces* and *Simplicillium*. Where dedicated species-specific qPCR for *Aspergillus* was performed, only *A. fumigatus* and *A. terreus* were identifiable in bronchiectasis. Interestingly, *A. fumigatus* had preponderance within the Asian cohorts from Singapore and Kuala Lumpur whilst *A. terreus* exhibited higher prevalence and burden in patients from Dundee. Even in patients with both species, geographic origin dictated which proportionally dominated. Our patients were stringently matched for age, sex and disease severity to

confront the heterogeneity that plagues bronchiectasis research and allowed us to better assess potential geographic variation. Studies of additional cohorts from Europe and Asia will be required to determine if these are truly geographical differences or reflect differences in patient selection or referral patterns at the participating sites. These geographic phenotypes were accompanied by differences in lung mycobiome profiles: *Penicillium*, *Clavispora* and *Cryptococcus* had markedly higher frequency in patients from Dundee while Basiodiomycota of the genera *Phlebia*, *Trichosporon*, *Ceriporia*, *Phellinus*, *Schizophyllum*, *Psathyrella* and *Peniophora* were exclusive to patients from Singapore/Kuala Lumpur who exhibit higher overall Basidiomycota. Our identified differences may reflect a myriad of regionally variable factors such as contrasting atmospheric conditions between temperate and tropical climates including temperature and humidity, each documented to affect fungi [29, 30]. Interestingly, our identified geographic variation of *A. terreus* predominance in Dundee occurred despite low reported UK prevalence rates of this fungus suggestive of enrichment in the bronchiectasis population [31]. CF-related bronchiectasis also associates with *A. terreus* and an unidentified 'environmental exposure' of likely relevance in our Dundee cohort is ascribed to this species [32, 33]. Other key geographic factors to consider include dietary preferences, genetics, air quality and/or lifestyle, all influences on acquisition and persistence of microbes including fungi [34]. Interestingly, the marked BMI difference between our 'matched' cohorts raises questions about potential differences in gut microbiomes that in turn may associate with the airway mycobiome given the emerging lung-gut axis relationship; an area of increasing relevance to respiratory health [35]. Other confounders to consider include regional differences in pharmacological prescribing and their consequent implications for the mycobiome; important considerations given differences between our Dundee and Singapore/Kuala Lumpur cohorts in terms of inhaled corticosteroids, long-term prophylactic antibiotics (greater in Dundee) and mucolytic use (greater in Singapore/Kuala Lumpur)

(Table 1) – all features of potential influence on mycobiome composition which warrant further study. While our findings could be peculiar to Dundee, Singapore and Malaysia; generalizability is supported by data showing that Dundee patients are similar and data generated in Scotland has been generalizable across more than 10 European cohorts in recent analyses [36, 37].

Our group and others have previously investigated the role of *Aspergillus*-associated disease in CF-related bronchiectasis; however focused work in non-CF bronchiectasis is lacking [7, 34, 38-40]. Importantly however, Everaerts *et al* assessed occurrence of *A. fumigatus* sensitisation in COPD and, when detectable, associated it with a high risk for bronchiectasis [21]. These data support the role for *Aspergillus* as a bronchiectasis pathogen either as a cause or consequence. Our data further corroborates such associations with high observable sIgE levels to *A. fumigatus* and *A. terreus*. Using a modified immunologic classification, adapted from CF, we describe high rates of AS and sABPA in the CAMEB cohort outlining its clinical relevance even in patients appearing clinically stable [14]. TARC, a proposed marker of CF-ABPA was found to have poor sensitivity and specificity when applied to the CAMEB cohort illustrating difficulties in translating findings from CF to non-CF bronchiectasis [23]. Each of our classified clinical states is accompanied by a distinct mycobiome pattern suggestive of mechanistic links necessitating future studies.

A potential role for *A. terreus* in bronchiectasis is a novel finding of this work. When compared to *A. fumigatus*, *A. terreus* is lesser-studied. It is however noted for colonising the CF lung, is capable of causing ABPA, and associates with opportunistic infections[41-43]. Its resistance to polyene antifungals confers survival advantages in the host compared to *A. fumigatus* [44-45]. For instance, *A. terreus* conidia are rapidly phagocytosed through dectin-1

and mannose receptors, however, exhibit an increased macrophage survival compared to *A. fumigatus* [45]. Rapid germination of *A. fumigatus* within the macrophage comes at the cost of its increased vulnerability to host immunity. In contrast, *A. terreus* germinates at lower rates favouring its persistence. Our work highlights high proportions of bronchiectasis patients with both *A. fumigatus* and *A. terreus*. Moreover, such patients demonstrate greater exacerbations, suggestive that, in combination these fungi have important clinical effects on distinct host pathways that worsen disease. As a consequence, assessment of fungal airway conidial burden is important in bronchiectasis.

While our work is novel, it does have limitations including its cross sectional design. This is a consequence of our attempt to robustly match Asian and European cohorts within the study to address disease heterogeneity, a key issue in bronchiectasis research. In addition, we must consider the 'generalizability' of our three participating countries in comparison to the wider Asian and European sub-continents including the potential variation in aetiology for exacerbations, their management including bronchiectasis treatments and geographic differences in climate, temperature and air quality. Epidemiologic fungal studies have in fact illustrated species differences between countries even within the same sub-continent [31]. Furthermore, we included only a small group of non-diseased (healthy) individuals (all from Singapore) which precluded us from defining a 'healthy' mycobiome clearly which was not the primary aim of this work. Future studies however should focus on assessing the non-diseased lung mycobiome and include individuals from more than a single region to assess potential geographic variation. In addition, targeted amplicon sequencing – even through our parallel shotgun sequencing of ITS1 and ITS2 - has lower resolution compared to the more costly and analytically challenging whole genome shotgun metagenomics that provides superior speciation and functional annotation. We focused on *Aspergillus* as the predominant

bronchiectasis-associated fungal pathogen however our mycobiome analysis suggests that other fungal genera may also have roles. Finally, when the CAMEB cohort was conceived, a decision was made to match across Singapore/Kuala Lumpur and Dundee based on age, sex and disease severity. This matching on total BSI rather than its specific components including exacerbations, radiology or microbiology limits our ability to perform more specific analyses. Our cohort enrolled predominantly severe patients with bronchiectasis with a relatively low number of patients with mild disease (BSI 0-4).

We have performed the first fungal profiling study in bronchiectasis to date using two 'matched' cohorts across three countries. We illustrate high levels of airway fungi and key differences between cohorts. These data suggest that the role for fungi and specifically *Aspergillus* in bronchiectasis is significant and routine screening for *Aspergillus*-associated diseases should be considered for inclusion in future bronchiectasis guidelines. Screening may include skin testing, *Aspergillus*–associated sIgE and *Aspergillus*-specific IgG with potential follow up immunological monitoring for those clinically affected. Our identified mycobiome profiles that relate to clinical disease should also be explicated in future work.

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595 **Table 1.** Demographics of the study population including non-diseased (healthy) controls and
596 stable bronchiectasis patients comprising Asian and European matched cohorts.

Characteristic	Non-diseased controls (n=10)	Bronchiectasis patients (n=238)	Bronchiectasis patients SG-KL (n=138)	Matched cohorts	
				SG-KL (n=100)	DD (n=100)
Age : median (IQR)	37 (30-49)	68 (64-71)	65 (58-73)	65 (58-74)	69 (64-76)
Gender : n (%)					
Female	4 (40%)	130 (55%)	77 (55%)	59 (59%)	53 (53%)
Male	6 (60%)	108 (45%)	61 (45%)	41 (41%)	47 (47%)
Aetiology n (%)					
Idiopathic	-	145 (61%)	85 (62%)	63 (63%)	60 (60%)
Post-infection	-	56 (23.5%)	43 (31%)	27 (27%)	27 (27%)
Other	-	37 (15.5%)	10 (7%)	10 (10%)	13 (13%)
Smoking status n (%)					
Never	-	170 (70%)	108 (78%)	80 (80%)	62 (62%)
Current	-	11 (5%)	7 (5%)	4 (4%)	4 (4%)
Past	-	57 (25%)	23 (17%)	16 (16%)	34 (34%)
BSI status : n (%)					
Severe	-	147 (62%)	84 (61%)	63 (63%)	63 (63%)
Moderate	-	71 (30%)	45 (33%)	26 (26%)	26 (26%)
Mild	-	20 (8%)	9 (6%)	11 (11%)	11 (11%)
BSI score : median (IQR)	-	9 (6-13)	10 (7-14)	10 (7-14)	9 (6-12)
BMI (kg/m2) : median (IQR)	24(22-24)	21 (18-27)	19 (17-22)	19 (17-22)	27 (22-31)
MRC dyspnea score : n (%)					
1-3	-	200 (84%)	121 (88%)	90 (90%)	79 (79%)
4	-	26 (11%)	10 (7%)	6 (6%)	16 (16%)
5	-	12 (5%)	7 (5%)	4 (4%)	5 (5%)
FEV_1 % predicted: : median (IQR)	-	74 (54-87)	69 (51-84)	69 (52-84)	76 (57-96)
Radiological severity : n (%)					
1-2 lobes involved	-	106 (45%)	62 (45%)	43 (43%)	44 (44%)
3 or more lobes involved	-	132 (55%)	76 (55%)	57 (57%)	56 (56%)
No. of exacerbations in previous year : n (%)					
0	-	84 (35%)	69 (50%)	44 (44%)	15 (15%)
1-2	-	82 (35%)	51 (37%)	41 (41%)	31 (31%)
3 or more	-	72 (30%)	18 (13%)	15 (15%)	54 (54%)
Hospital admissions before study : n (%)					
Yes	-	88 (37%)	63 (46%)	43 (43%)	25 (25%)
No	-	150 (63%)	75 (54%)	57 (57%)	75 (75%)
Colonization with other organisms : n (%)					
Yes	-	127 (53%)	60 (43%)	44 (44%)	67 (67%)
No	-	111 (47%)	78 (57%)	56 (56%)	33 (33%)
Pseudomonas colonisation : n (%)					
Yes	-	23 (10%)	18 (13%)	15 (15%)	5 (5%)
No	-	215 (90%)	120 (87%)	85 (85%)	95 (95%)
Bronchodialator use					
Yes	-	107 (45%)	58 (42%)	39 (39%)	49 (49%)
No	-	131 (55%)	80 (58%)	61 (61%)	51 (51%)
Inhaled corticosteroids					
Yes	-	80 (34%)	21 (15%)	14 (14%)	59 (59%)
No	-	158 (66%)	117 (85%)	86 (86%)	41 (41%)
Mucolytics					
Yes	-	118 (50%)	60 (44%)	45 (45%)	13 (13%)
No	-	120 (50%)	78 (56%)	55 (55%)	87 (87%)
Long-term antibiotics					
Yes	-	48 (20%)	22 (16%)	14 (14%)	26 (26%)
No	-	190 (80%)	116 (84%)	86 (86%)	74 (74%)

597

598 Data are presented as median (interquartile range; IQR) or n (percentage; %). Patients in the
599 matched cohorts were matched on age, gender and disease severity according to the
600 Bronchiectasis Severity Index (BSI). The variables defining composite BSI score including
601 Body Mass Index (BMI), shortness of breath (MRC) dyspnoea score, Forced expiratory
602 volume in the 1st second (FEV_1) % predicted values, Radiological severity, number of
603 exacerbations in the preceding year defined by established consensus [46], hospitalisations in
604 the preceding year, microbial colonisation with other organisms and colonisation by *P.*
605 *aeruginosa* are also reported.
606

FIGURE LEGENDS

Figure 1. The pulmonary mycobiome in stable bronchiectasis (a) Mirrored Sankey plots illustrating the relative abundance of read classification by taxonomic rank from phylum (centre; blue) to genus level (margins; red). Non-diseased (healthy) controls (n=10) are compared to patients with stable bronchiectasis (n=238). Central coloured bars demonstrate phylum-level abundance of Ascomycota (yellow) versus Basidiomycota (blue) between the cohorts. Cohort-specific fungal genera are indicated in bold. (b) Individual patient phylum-level classification of the pulmonary mycobiome in stable bronchiectasis (n=238) illustrating the relative abundance of Ascomycota (yellow) versus Basidiomycota (blue) for individual patients (c) Percent prevalence of fungal genera (present at >1% relative abundance) in non-diseased (healthy) controls (n=10) and patients with stable bronchiectasis (n=238). The prevalence of the top genera (observed at >1% relative abundance) are illustrated across non-diseased controls and patients with stable bronchiectasis. Coloured bars indicate membership to either Ascomycota (yellow) or Basidiomycota (blue) phyla. Filled dots (●) indicate genera found in bronchiectasis and open dots (○) genera in non-diseased (healthy) controls.

Figure 2. The pulmonary mycobiome differs between Singapore/Kuala Lumpur (SG-KL) and Dundee (DD) cohorts of stable bronchiectasis (a) Genus level classification of the pulmonary mycobiome in non-diseased (ND, n=10), stable bronchiectasis (BR, n=238) and matched SG-KL (n = 100) and DD (n = 100) cohorts. The relative abundance of identified taxa is colour-illustrated. (b) Percent prevalence of observed fungal genera (present at >1% relative abundance) in matched bronchiectasis cohorts from SG-KL (red, n=100) and DD (blue, n=100). Filled squares (■) indicate genera found only in the DD cohort while dots (●) indicate genera detected only in the SG-KL cohort. Significant differences in prevalence between cohorts are indicated; ** $p < 0.01$, *** $p < 0.001$.

Figure 3. Identification of specific airway *Aspergillus* species and their association with exacerbations in bronchiectasis (a) Quantitative polymerase chain reaction (qPCR)-based screening for the presence of the major specific *Aspergillus* species: *A. fumigatus*, *A. terreus*, *A. flavus* and *A. niger* in non-diseased (healthy) controls (ND, n=10) and stable bronchiectasis (BR, n = 238). Bar colouration indicates the relative proportion of positive Singapore/Kuala Lumpur (SG-KL) (red) and Dundee (DD) (blue) patients respectively according to *Aspergillus* species. (b) Exacerbations for the year preceding study recruitment in patients with qPCR detectable *A. fumigatus* (left) and *A. terreus* (right). (c) Classification of qPCR detectable *Aspergillus* species by percentage (%) of the total bronchiectasis patient cohort (n=238). Patients are classified as having no detectable species (none, n=39), *A. fumigatus* only (AF, n=26), *A. terreus* only (AT, n=78) or both *A. fumigatus* and *A. terreus* (both, n=95). Bar colouration indicates the relative proportion of positive patients from SG-KL (red) and DD (blue) respectively by classification. (d) Exacerbations for the year preceding study recruitment in patients with no detectable species (None), *A. fumigatus* only (AF), *A. terreus* only (AT) or both *A. fumigatus* and *A. terreus* (Both). Dot colouration indicates patient origin: SG-KL (red) and DD (blue). Median number of exacerbations per group is indicated. ns – non-significant , * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 4. Quantification of airway *Aspergillus* conidial burden and its association with exacerbations in bronchiectasis (n =238). (a) Conidial burden per gram (g) of sputum was quantified for *A. fumigatus* and *A. terreus* respectively and classified according to load as Low (<500 conidia / g sputum), Intermediate (500-2000 conidia / g sputum) or High (>2000 conidia / g sputum). Conidial load categories are illustrated according to fungal airway status as *A. fumigatus* only (AF), *A. terreus* only (AT) or the presence of both species (Both). Dot colouration indicates patient origin: Singapore/Kuala Lumpur (SG-KL) (red) and Dundee (DD) (blue). (b) Scatter plot of *A. fumigatus* (x-axis) and *A. terreus* (y-axis) conidial load in stable bronchiectasis. Patients with single (-●-) and both (●) species are indicated. Patients with both species are classified by their relative *A. fumigatus* and *A. terreus* burdens respectively into low burden of both (LL), low burden of *A. fumigatus* and high burden of *A. terreus* (LH), high burden of *A. fumigatus* and low burden of *A. terreus* (HL) and high burden of both (HH). Dotted lines indicate cut-off levels for a high conidial burden (>2000 conidia / g sputum). Dot colouration indicates patient origin: SG-KL (red) and DD (blue). (c) Exacerbations for the year preceding study recruitment in bronchiectasis patients with detectable conidial burdens of both *A. fumigatus* and *A. terreus* classified as LL, LH, HL and HH respectively. Dot colouration indicates patient origin: SG-KL (red) and DD (blue). Median number of exacerbations per group is indicated and Benjamini–Hochberg-adjusted p-values for all groups compared to 'None' are shown and significance indicated as *p=0.01 and **p=0.006 respectively. .

Figure 5. Immunologic classification reveals high frequencies of Aspergillosis in stable bronchiectasis and an association of serological allergic bronchopulmonary aspergillosis (sABPA) with greater exacerbations, poorer pulmonary function and more severe disease. Measured levels of specific IgE (sIgE) responses to (a) *A. fumigatus* and (b) *A. terreus* respectively where Aspergillosis is classified immunologically as non-diseased (ND, n = 4); *Aspergillus*-colonized (AC, n=8); *Aspergillus*-sensitized (AS, n = 182); serological allergic bronchopulmonary aspergillosis (sABPA, n = 43) and suspected chronic pulmonary aspergillosis (sCPA, n =1) (Supplementary table 4). Thymus and activation regulated chemokine (TARC); a proposed ABPA-marker in cystic fibrosis [23] was assessed according to the same classification. Broken lines indicate respective cut-offs indicating a positive test for each marker (Specific IgE ; 0.35 kU/L, TARC ; 386 pg/mL) (d), Exacerbations for the year preceding study recruitment (e), Pulmonary function (as percent predicted FEV_1) and (f) disease severity (as BSI) was assessed according to immunologic aspergillosis class. Dot colouration indicates patient origin: SG-KL (red) and DD (blue). Mean values are indicated except for exacerbations and BSI where median values are shown. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. FEV_1: Forced Expiratory Volume in the first second; BSI: Bronchiectasis Severity Index.

Figure 6. Pulmonary Mycobiome profiles illustrate specific taxa-associated patterns according to immunological classification of Aspergillosis in stable bronchiectasis. The Mycobiome profiles of (a) Non-diseased (ND, n = 4); (b) *Aspergillus*-colonized (AC, n=8); (c) *Aspergillus*-sensitized (AS, n = 182) and (d) serological allergic bronchopulmonary aspergillosis (sABPA, n = 43) illustrate the different composition by relative abundance of reads classified to genus level, with the (b) AC, (c) AS and (d) sABPA states exhibiting increased exacerbations and higher conidial load. Only a single patient had suspected chronic pulmonary aspergillosis and therefore data is not shown. Mycobiome profiles are represented by pie-charts with colour coding according to the taxonomic legend. Adjacent colour illustrated log-scaled bar charts detail the observed taxa patterns in each immunological patient class (Formal statistical assessment is provided in Table E5). Exacerbations in the preceding year (x-axis) are plotted against *Aspergillus* conidial load (y-axis) and colour coded according to qPCR detection status of *Aspergillus* species into *A. fumigatus* alone (green), *A. terreus* alone (orange), presence of Both (purple) or None (grey). . Patients are further classified according to their individual conidial load as Negative ('Neg' ; no detected conidia / g sputum), Low (<500 conidia / g sputum), Intermediate ('Int' ; 500-2000 conidia / g sputum) or High (>2000 conidia / g sputum).