A high-risk airway mycobiome is associated with frequent exacerbation and mortality in COPD

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ABSTRACT

Introduction: The chronic obstructive pulmonary disease (COPD) bacteriome associates with disease severity, exacerbations and mortality. While COPD patients are susceptible to fungal sensitisation, the role of the fungal mycobiome remains uncertain.

Methods: We report the largest multicentre evaluation of the COPD airway mycobiome to date, including participants from Asia (Singapore and Malaysia) and the UK (Scotland) when stable (n=337) and during exacerbations (n=66) as well as nondiseased (healthy) controls (n=47). Longitudinal mycobiome analysis was performed during and following COPD exacerbations (n=34), and examined in terms of exacerbation frequency, 2-year mortality and occurrence of serum specific IgE (sIgE) against selected fungi.

Results: A distinct mycobiome profile is observed in COPD compared with controls as evidenced by increased α-diversity (Shannon index; $p<0.001$). Significant airway mycobiome differences, including greater interfungal interaction (by co-occurrence), characterise very frequent COPD exacerbators (three or more exacerbations per year) (permutational multivariate ANOVA; adjusted $p<0.001$). Longitudinal analyses during exacerbations and following treatment with antibiotics and corticosteroids did not reveal any significant change in airway mycobiome profile. Unsupervised clustering resulted in two clinically distinct COPD groups: one with increased symptoms (COPD Assessment Test score) and *Saccharomyces* dominance, and another with very frequent exacerbations and higher mortality characterised by *Aspergillus*, *Curvularia* and *Penicillium* with a concomitant increase in serum sIgE levels against the same fungi. During acute exacerbations of COPD, lower fungal diversity associates with higher 2-year mortality.

Conclusion: The airway mycobiome in COPD is characterised by specific fungal genera associated with exacerbations and increased mortality.

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Introduction

The role of fungi, in particular *Aspergillus* species, in chronic respiratory disease states including asthma, bronchiectasis and cystic fibrosis (CF) is increasingly being recognised and researched [1–4]. A major challenge in clinical settings is the varied presentation of fungal-associated disease that ranges from simple nodules to allergic sensitisation, and includes chronic nonspecific illness and life-threatening invasive disease [5, 6]. Patients with asthma, bronchiectasis, CF and, more recently, chronic obstructive pulmonary disease (COPD) have been shown to be susceptible to fungal sensitisation and allergic bronchopulmonary mycoses (ABPM) with poorer clinical outcomes [2, 3, 5, 7].

Despite prior work illustrating the importance of fungal sensitisation, the true significance of fungal infection remains poorly defined in COPD [4, 7, 8]. Prior work suggests associations with disease severity and worse clinical outcomes [4, 9, 10]. For example *Aspergillus* sensitisation in COPD is associated with increased symptoms, lung function decline, exacerbations and presence of bronchiectasis [4, 7, 8, 11]. Long-term inhaled corticosteroid use and frequent oral steroid bursts during exacerbations impair host immunity and may predispose patients to fungal-associated disease [12, 13]. While isolation of fungi from the airway can be associated with clinical disease, the significance of detecting fungi without overt invasive fungal disease in stable COPD remains unknown [10]. Positive fungal culture is reported in up to 43% of COPD patients, and has been associated with inhaled corticosteroid dose, activation of the host response and airway neutrophilia [4].

Prior studies on the COPD bacterial microbiome have shown associations with disease severity, exacerbations and mortality; however, the role of the fungal mycobiome has lacked dedicated study [14–19]. Although present in lower absolute abundance compared with bacteria, a healthy airway mycobiome does exist, demonstrating a predominance of *Candida*, *Saccharomyces* and *Grammothele*, and fungal dysbiosis is linked to disease severity in asthma, bronchiectasis and CF [2, 20, 21]. In COPD, however, only a single study has assessed the role of the mycobiome in a specific patient subgroup with HIV and found that *Pneumocystis jirovecii* was the predominant airway taxa [22]. Therefore, no study to date has evaluated the role of the airway mycobiome in COPD and related it to treatment and clinical outcomes.

Here, we describe a large multicentre study characterising the airway mycobiome in COPD participants from Singapore, Malaysia and the UK. In addition, we provide a longitudinal analysis of the mycobiome during and following COPD exacerbations, and relate it to clinical outcomes and the systemic immune response in COPD.

Methods

Study recruitment

The nondiseased (healthy) cohort (n=47) comprised non-COPD subjects recruited from community volunteers at Nanyang Technological University, Singapore (forced expiratory volume in 1 s (FEV_1)/forced vital capacity (FVC) >0.7 with normal FEV_1 (⩾80% predicted)). The COPD cohorts comprised individuals with COPD aged ⩾40 years prospectively recruited in three countries (Singapore, Malaysia and the UK) across five hospital sites, divided into three separate independent cohorts: a stable COPD cohort (n=337), an acute exacerbation of COPD (AECOPD) cohort (n=66) and a longitudinal COPD cohort (n=34). COPD was diagnosed based on Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria [23]. Participants with any prior history of asthma (defined by variable symptoms and expiratory airflow limitation according to Global Initiative for Asthma guidelines [24]), those receiving long-term oral steroids (>5 $mg \cdot day^{-1}$) and/or other immunosuppressive therapy, malignancy on active therapy and/or active mycobacterial disease were excluded.

Hospital sites included Singapore General Hospital, Changi General Hospital and Tan Tock Seng Hospital (all Singapore), University of Malaya Medical Centre (Kuala Lumpur, Malaysia), and Ninewells Hospital (Dundee, Scotland, UK). Recruitment for the stable and longitudinal COPD cohorts occurred between January 2014 and June 2019; the AECOPD cohort was recruited between January 2014 and January 2018.

For COPD participants recruited into the stable or AECOPD arms, stable COPD was defined as the absence of an exacerbation in the 4-week period immediately preceding study recruitment and an acute exacerbation was defined as an acute worsening of respiratory symptoms (increase dyspnoea, cough, sputum purulence and volume or wheeze) necessitating treatment with antibiotics and/or corticosteroids based on GOLD guidelines [23]. Participants in the AECOPD cohort were followed prospectively over a 2-year period following sampling during an acute exacerbation and their mortality outcome recorded (as documented on the death certification). Only respiratory causes of death were considered, defined as death secondary to pneumonia, COPD and/or respiratory failure.

A separate cohort of COPD participants was recruited into the longitudinal study arm with airway specimen samples collected at baseline (during stability), within 24 h of a documented exacerbation, and

then at 2 weeks post-exacerbation and upon completion of treatment. All participants in the longitudinal arm received standard treatment with an oral antibiotic (200 mg doxycycline on day 1 followed by 100 mg for 6 days or 625 mg twice daily co-amoxiclav for 1 week) and a 5-day course of prednisolone at 30 or 40 mg·day^{-1} based on the patient's weight.

At recruitment, a representative airway (sputum) sample and blood draw (for serum) was performed for each patient, and complete demographic and clinical data (including GOLD group, stage and treatment received) for each respective cohort were collected (table 1). For COPD, we define frequent exacerbators as those with two or more exacerbations per year, very frequent exacerbators as those with three or more exacerbations per year and nonfrequent exacerbators as those with less than two exacerbations per year, based on the year preceding recruitment [25]. Use of inhaled corticosteroids was defined as daily maintenance doses of at least 100 µg fluticasone propionate or an equivalent inhaled corticosteroid.

This study was approved by the institutional review boards of all participating hospitals and institutions.

Ethical approval, methods and statistical analysis

Full details of ethical approvals, specimen collection and processing, DNA extraction and mycobiome sequencing, specific IgE (sIgE) assays, and statistical analysis are provided in the supplementary material.

TABLE 1 Demographics of the nondiseased (healthy) and chronic obstructive pulmonary disease (COPD) cohorts

	Nondiseased (healthy)	Stable COPD			AECOPD			Longitudinal[#]
		Overall	SG/KL	DD	Overall	SG/KL	DD	
Subjects	47	337	175	162	66	29	37	34
Age years	39 (28–63)	72 (67–77)	72 (67–77)	72 (66–77)	69 (64–76)	68 (63–72)	70 (65–77)	70 (65–77)
BMI kg·m^{-2}	22.5 (19.6–25.7)	24.7 (21.9–29.0)	22.5 (20.2–26.3)	27.0 (24.0–31.0)	25.0 (21.0–30.6)	21.7 (17.5–27.9)	28.0 (24.3–31.8)	25.0 (23.0–29.9)
Male	17 (36.2)	273 (81.0)	167 (95.4)	106 (65.4)	53 (80.3)	29 (100)	24 (64.9)	26 (76.5)
Current smoker	0 (0.0)	186 (55.2)	61 (34.9)	125 (77.2)	44 (66.7)	11 (38.0)	33 (89.2)	27 (79.4)
Ex-smoker	0 (0.0)	151 (44.8)	114 (65.1)	37 (22.8)	22 (33.3)	18 (62.0)	4 (10.8)	7 (20.6)
Smoking pack-years	NA	50.0 (33.1–64.5)	50.0 (40.0–80.0)	40.0 (30.0–54.5)	41.5 (37.8–67.3)	49.0 (40.0–75.0)	40.0 (30.0–60.0)	40.0 (26.3–69.5)
CAT score	NA	20.0 (12.0–25.0)	20.0 (11.3–25.0)	19.0 (13.0–25.0)	23.0 (18.0–27.0)	22.0 (17.5–27.0)	23.0 (18.8–27.3)	23.0 (17.0–27.3)
FEV_1 % pred	96.5 (85.8–107.3)	59.0 (42.0–74.5)	52.0 (37.0–66.0)	67.5 (51.8–79.3)	51.7 (41.0–73.0)	49.0 (41.0–59.0)	61.8 (43.4–79.9)	61.8 (50.7–76.9)
FEV_1/FVC % pred	86.6 (81.5–93.1)	53.0 (44.0–63.0)	55.0 (44.0–65.0)	52.0 (43.1–60.9)	50.7 (42.3–59.8)	49.0 (43.0–59.0)	51.0 (42.0–60.0)	49.0 (41.0–56.0)
Exacerbations per year	NA	1 (0–3)	0 (0–2)	2 (1–4)	3 (2–4)	2 (1–3)	3 (2–4)	3 (2–4)
GOLD group								
A	NA	81 (24.0)	45 (25.7)	36 (22.2)	3 (4.5)	0 (0.0)	3 (8.1)	1 (2.9)
B	NA	104 (30.9)	76 (43.4)	28 (17.3)	1 (1.5)	7 (24.1)	1 (2.7)	5 (14.7)
C	NA	41 (12.2)	8 (4.6)	33 (20.4)	10 (15.2)	0 (0.0)	10 (27.0)	0 (0.0)
D	NA	111 (32.9)	46 (26.3)	65 (40.1)	52 (78.8)	22 (75.9)	23 (62.2)	28 (82.4)
GOLD grade								
1	NA	57 (16.9)	20 (11.4)	37 (22.8)	12 (18.2)	3 (10.4)	9 (24.3)	8 (23.5)
2	NA	163 (48.4)	71 (40.6)	92 (56.8)	29 (43.9)	11 (37.9)	18 (48.7)	19 (55.9)
3	NA	92 (27.3)	66 (37.7)	26 (16.1)	20 (30.3)	13 (44.8)	7 (18.9)	4 (11.8)
4	NA	25 (7.4)	18 (10.3)	7 (4.3)	5 (7.6)	2 (6.9)	3 (8.1)	3 (8.8)
LABA monotherapy	NA	8 (2.4)	3 (1.7)	5 (3.1)	1 (1.5)	0 (0.0)	1 (2.7)	1 (2.9)
LAMA monotherapy	NA	34 (10.1)	20 (11.4)	14 (8.6)	5 (7.6)	4 (13.8)	1 (2.7)	2 (5.9)
LABA/LAMA	NA	112 (33.2)	70 (40.0)	42 (25.9)	13 (19.7)	6 (20.7)	7 (18.9)	6 (17.6)
LABA/ICS	NA	41 (12.1)	14 (8.0)	27 (16.7)	5 (7.6)	1 (3.4)	4 (10.8)	4 (11.8)
LABA/LAMA/ICS	NA	130 (38.6)	63 (36.0)	67 (41.4)	38 (57.5)	17 (58.7)	21 (56.8)	19 (55.9)
SAMA/SABA	NA	12 (3.6)	5 (2.9)	7 (4.3)	4 (6.1)	1 (3.4)	3 (8.1)	2 (5.9)
Macrolide	NA	34 (10.1)	10 (5.7)	24 (14.8)	8 (12.1)	1 (3.4)	7 (18.9)	5 (14.7)

Data are presented as n, n (%) or median (interquartile range). SG/KL: Singapore/Kuala Lumpur; DD: Dundee; AECOPD: acute exacerbation of COPD; BMI: body mass index; CAT: COPD Assessment Test; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; NA: not applicable; GOLD: Global Initiative for Chronic Obstructive Lung Disease; LABA: long-acting β-agonist; LAMA: long-acting muscarinic antagonist; ICS: inhaled corticosteroid; SAMA: short-acting muscarinic antagonist; SABA: short-acting β-agonist. [#]: pre–during–post COPD exacerbation.

Results

The airway mycobiome in stable COPD is diverse and illustrates geographic variation

The airway mycobiome exhibited significantly greater diversity in stable COPD (n=337) compared with nondiseased (healthy) individuals (n=47) (p=0.0006) (figure 1a). Characterised by distinct fungal genera, including *Alternaria*, *Aspergillus*, *Cladosporium*, *Cryptococcus*, *Mycosphaerella*, *Penicillium*, *Trametes* and *Wickerhamomyces*, the COPD mycobiome also contained other fungi shared with a healthy mycobiome but at a higher proportion (figure 1b and c). Geographic variation between mycobiome profiles was observed from participants recruited in Singapore/Kuala Lumpur (n=175) compared with Dundee (n=162), where *Saccharomyces*, *Curvularia*, *Aspergillus*, *Schizophyllum*, *Penicillium* and *Grammothele* predominated in the Singapore/Kuala Lumpur cohorts, and *Cladosporium*, *Debaryomyces*, *Hanseniaspora*, *Trametes* and *Wickerhamomyces* predominated in the Dundee cohorts, by linear discriminant analysis (LDA) (LDA score >3.5; p<0.05) (figure 1d and e, and supplementary figure E6a). Importantly, mycobiome composition remained unaltered by GOLD ABCD group or GOLD lung function grade in stable COPD (supplementary figures E1 and E2).

Frequent COPD exacerbators illustrate altered airway mycobiomes that demonstrate increased fungal interaction and are unaffected by acute exacerbation, antibiotics and/or corticosteroids

Very frequent COPD exacerbators (n=92) demonstrated airway mycobiomes discriminated by *Wickerhamomyces* (LDA score >3.5) (figure 2a and supplementary figure E6b). These mycobiomes also illustrated contrasting β-diversity compared with nonfrequent exacerbators (n=245) (median (interquartile range (IQR)) distance to centroid 0.61 (0.49–0.67) *versus* 0.53 (0.39–0.69); p<0.05) (figure 2b and c). Differences in mycobiome profiles between very frequent exacerbators and nonfrequent exacerbators remained significant following permutational multivariate ANOVA (PERMANOVA) adjusted for age, sex, smoking pack-year history, body mass index (BMI), geographic origin and inhaled corticosteroid use (p=0.0022). When fungal burden was assessed semiquantitatively by PCR, derived amplicon concentrations between the nonfrequent exacerbator and very frequent exacerbator COPD groups were comparable (supplementary figure E7). Having detected significant differences in the airway mycobiome of very frequent exacerbators, we next evaluated fungal interactions within the mycobiome using network analyses. Employing the top 15 fungal genera (all with >1% relative abundance and present in >5% of the participants), we identified an increased number of fungal interactions in the very frequent exacerbator group compared with the nonfrequent exacerbator group (figure 2d and supplementary table E2). In very frequent exacerbators, the key taxa responsible for maintaining network integrity included *Alternaria*, *Aspergillus*, *Cryptococcus*, *Curvularia*, *Lodderomyces*, *Malassezia*, *Penicillium* and *Saccharomyces*, which demonstrated the highest number of interactions (edge counts) and their critical (indicated by stress centrality) and influential (measured by betweenness centrality) roles in maintaining mycobiome network integrity (supplementary table E2). Prospective and longitudinal analysis of the mycobiome in 34 participants with COPD was then assessed at baseline (pre-exacerbation), within 24 h of an acute exacerbation and again 2 weeks post-exacerbation following treatment with 1 week of oral antibiotics (either doxycycline or co-amoxiclav) and 5 days of oral corticosteroids (prednisolone). No significant changes were observed in airway mycobiome profiles or their α-diversity (Shannon index; p=0.9393 and Simpson index; p=0.9144) or β-diversity (PERMANOVA; p=0.6963) over the course and treatment of an acute exacerbation (figure 2e–h and supplementary figure E6c). In addition, when mycobiome profiles were compared between stable COPD participants receiving long-term treatment with inhaled corticosteroids, no differences were detectable, suggesting that the altered mycobiome observed in very frequent exacerbators is not strongly influenced by exacerbations, antibiotics and/or oral/inhaled corticosteroids (supplementary figure E3).

Unsupervised hierarchical clustering of the COPD mycobiome reveals two distinct patient clusters with variable clinical outcomes

We next sought to determine if specific mycobiome signatures or individual fungal genera relate to COPD outcomes. Unsupervised hierarchical clustering (n=337) (excluding *Candida* as this taxa represents >50% relative abundance) revealed two distinct COPD patient clusters (figure 3a). Cluster 1 (n=178) was characterised by *Saccharomyces* (figure 3b), while Cluster 2 (n=159) associated with significant levels of *Aspergillus*, *Curvularia* and *Penicillium* (figure 3c–e). Multivariate logistic regression with adjustment for age, sex, BMI, smoking pack-year history and lung function (FEV_1 % pred), and where Cluster 2 was the dependent variable, demonstrated the clinical relevance of the two identified clusters in that Cluster 1 demonstrated significant symptomatology (COPD Assessment Test (CAT) score >10) (OR 0.45, 95% CI 0.24–0.85; p=0.0135), and Cluster 2 demonstrated a higher number of exacerbations (OR 1.12, 95% CI 1.01–1.24; p=0.0393) and higher mortality (OR 2.43, 95% CI 1.04–5.69; p=0.0408: 31 deaths (9.2%) from 337 participants) (figure 3f). As additional confirmation of the increased symptoms (CAT score >10) in Cluster 1, logistic regression was repeated with CAT score >10 as the "dependent variable" and adjusted for

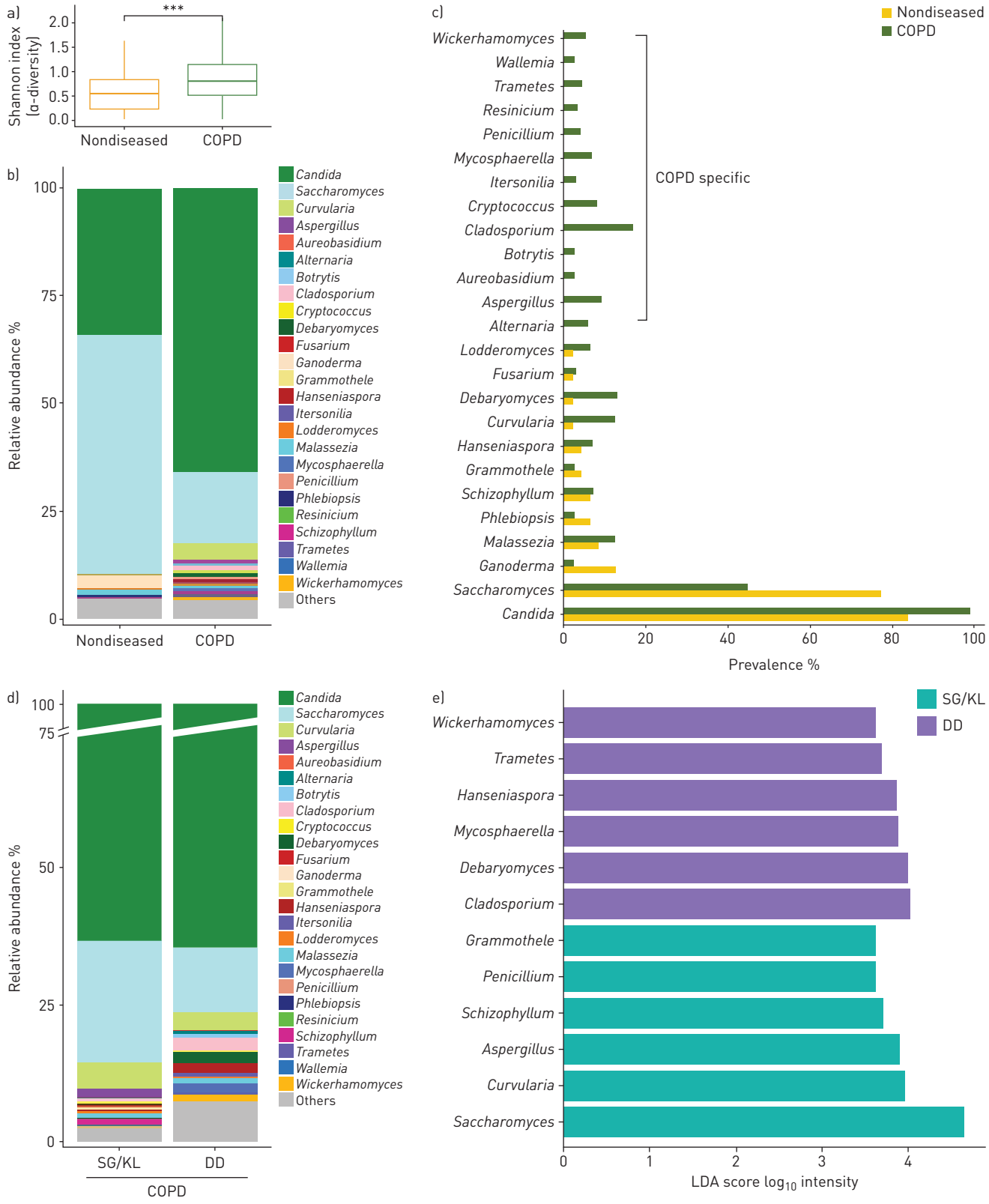

FIGURE 1 The airway mycobiome in chronic obstructive pulmonary disease (COPD) is diverse and demonstrates geographic variation. a) Shannon index (α-diversity) between nondiseased (healthy) (n=47) and participants with stable COPD (n=337). b) Relative abundance and c) prevalence (present at >1% relative abundance) of various fungal genera between nondiseased (healthy) individuals (n=47) and participants with COPD (n=337). d) Airway mycobiome composition between participants with COPD from Singapore/Kuala Lumpur (SG/KL) and Dundee (DD), with the top 25 fungal genera. e) Linear discriminant analysis (LDA) effect size illustrating the most discriminant fungal genera between respective groups. Box plot shows median, interquartile range and minimum–maximum range. ***: p<0.001.

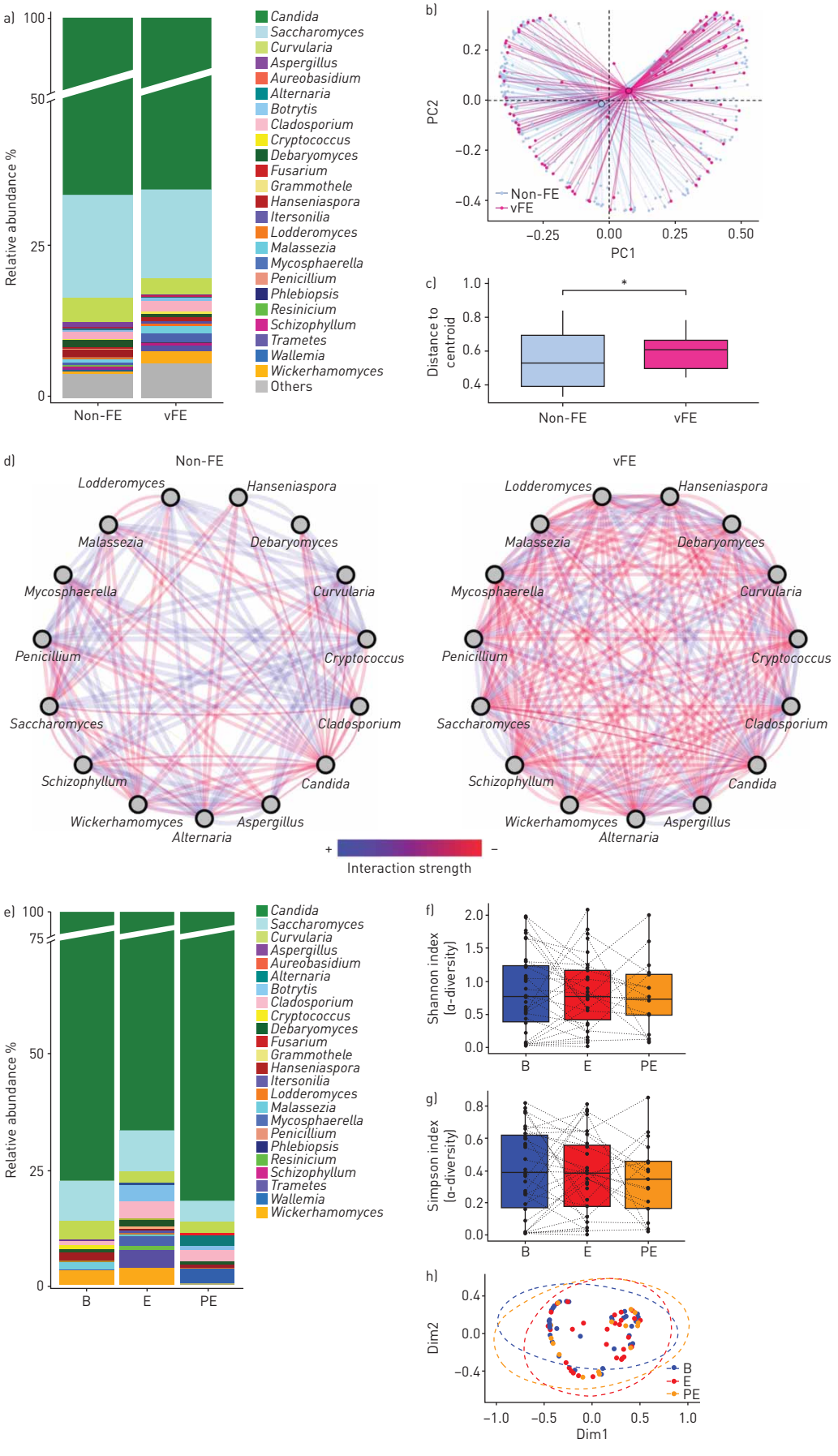
a)
Relative abundance %
Non-FE
vFE
Candida
Saccharomyces
Curvularia
Aspergillus
Aureobasidium
Alternaria
Botrytis
Cladosporium
Cryptococcus
Debaryomyces
Fusarium
Grammothele
Hanseniaspora
Itersonilia
Lodderomyces
Malassezia
Mycosphaerella
Penicillium
Phlebiopsis
Resinicium
Schizophyllum
Trametes
Wallemia
Wickerhamomyces
Others
b)
PC2
PC1
Non-FE
vFE
c)
Distance to centroid
d)
Non-FE
vFE
Lodderomyces
Hanseniaspora
Malassezia
Debaryomyces
Mycosphaerella
Curvularia
Penicillium
Cryptococcus
Saccharomyces
Cladosporium
Schizophyllum
Candida
Wickerhamomyces
Aspergillus
Alternaria
Interaction strength
e)
Relative abundance %
B
E
PE
f)
Shannon index (α-diversity)
g)
Simpson index (α-diversity)
h)
Dim2
Dim1

FIGURE 2 Very frequent chronic obstructive pulmonary disease (COPD) exacerbators (vFE) illustrate an altered airway mycobiome characterised by increased fungal interactions which is unaffected by exacerbation, antibiotics and/or corticosteroids. non-FE: nonfrequent exacerbators. a) Relative abundance of the top 25 fungal genera between the non-FE (n=245) and vFE (n=92) groups. b) Principle coordinate analysis illustrating β-diversity between the non-FE and vFE groups using the Bray–Curtis dissimilarity index. c) Average distance to centroid between groups illustrating significant differences based on the preceding principle coordinate analysis. d) Co-occurrence analysis between the non-FE and vFE groups revealing a more complex and increased fungal interaction present within the airway mycobiome in the vFE group compared with the non-FE group. Fungal genera demonstrating >1% relative abundance in >5% of the study population are illustrated. The colour scale denotes if an interaction is positive (co-occurrence) or negative (co-exclusion). e) Relative abundance of the top 25 fungal genera in the airway mycobiome following longitudinal analysis in n=34 participants with COPD at baseline (B; pre-exacerbation), during exacerbation (E) and following treatment (PE; post-exacerbation), which illustrate no significant change by f) Shannon index, g) Simpson index and h) principle coordinate analysis plot. Box plots show median, interquartile range and minimum–maximum range; dotted lines connect each individual patient at the respective time-points in f) and g). *: p<0.05.

age, sex, BMI, lung function, mortality and exacerbations. The OR for CAT score >10 in Cluster 1 was 2.41 (95% CI 1.25–4.66; p=0.01), confirming the increased symptomatology in this cluster. As the mycobiome in very frequent exacerbators was altered (figure 2a–d), we next assessed the occurrence of very frequent exacerbators between our two identified clusters and found no significant difference in the number of very frequent exacerbators between clusters (41 (23.0%) in Cluster 1 *versus* 48 (30.2%) in Cluster 2; p=0.173). Overall, Cluster 2 was characterised by *Aspergillus*, *Curvularia* and *Penicillium*, and demonstrates the poorest clinical outcome with increased exacerbations (median (IQR) 2 (0–3) *versus* Cluster 1 with 1 (0–2); p=0.0016) and higher mortality (21 (13.2%) *versus* Cluster 1 with 10 (5.6%); p=0.0266) (figure 3f).

A "high-risk" COPD mycobiome characterised by Aspergillus, Curvularia *and* Penicillium *associates with systemic immune responses to these fungi*

We next evaluated if a systemic and specific immune response was detectable to these fungi in participants belonging to this "high-risk" cluster employing a subset of stable COPD participants recruited from Singapore/Kuala Lumpur into our original cohort (n=42). Systemic sIgE binding using immuno-dot blot assays was screened against a panel of fungi that included *Aspergillus*, *Curvularia* and *Penicillium* (all characteristic of Cluster 2) and *Cladosporium*, *Fusarium*, *Schizophyllum* and *Trametes* (as controls, no specific association with either cluster). We detected a significantly elevated sIgE response to *Aspergillus*, *Curvularia* and *Penicillium* (all p<0.05) in participants from Cluster 2, corresponding with the increased relative abundance of the same respective taxa from their airways (figure 4a–c). Importantly, no significant differences in systemic sIgE responses were observed between the clusters using the control fungi that illustrated no cluster association (figure E4).

COPD mycobiomes illustrating lower fungal diversity during acute exacerbations associate with worse 2-year survival

While no significant change was observed to airway mycobiomes, including α- or β-diversity, over longitudinal assessment during AECOPD, we next assessed if mycobiome profiles examined during an AECOPD in an independent subset of 66 participants (recruited from Singapore and Dundee) and followed over 2 years related to survival outcomes (supplementary table E3). Mycobiome composition differed between COPD survivors (alive at 2 years; n=51) and nonsurvivors (death from a respiratory cause at 2 years; n=15), and in the latter illustrated a significantly lower α-diversity (p<0.01) with an inverse hazard ratio (HR) (lower α-diversity associated with high mortality) after adjustment for age, sex, BMI, smoking pack-year history, lung function (FEV_1) and comorbidities, including cardiovascular disease, diabetes mellitus, osteoporosis, malignancy and anxiety disorder (Shannon index HR 0.25, 95% CI 0.07–0.085; p=0.03 and Simpson index HR 0.07, 95% CI 0.005–0.88; p=0.04) (figure 5a–d). Of the fungal genera characterising the "high-risk" mycobiome Cluster 2, only *Penicillium* was exclusively detected in nonsurvivors, while *Aspergillus* and *Curvularia* were identified in varying proportions between the survival groups (figure 5a). Interestingly, in addition to *Penicillium*, *Cladosporium*, *Trametes* and *Lodderomyces* were identified at higher proportions in (but not exclusive to) nonsurvivors (figure 5a). Nonsurvivors further illustrated contrasting β-diversity compared with survivors, with no differences observed between their country of origin (adjusted PERMANOVA; p<0.05) (figure 5e). Collectively these data suggest that targeted analyses of mycobiome profiles during AECOPD may have prognostic implications and identify patients who are at higher risk of death.

Discussion

Here, we report the largest multicentre evaluation of the airway mycobiome in COPD, including participants recruited in Asia (Singapore and Malaysia) and the UK (Dundee), and additionally evaluate longitudinally mycobiome profiles during and following exacerbations.

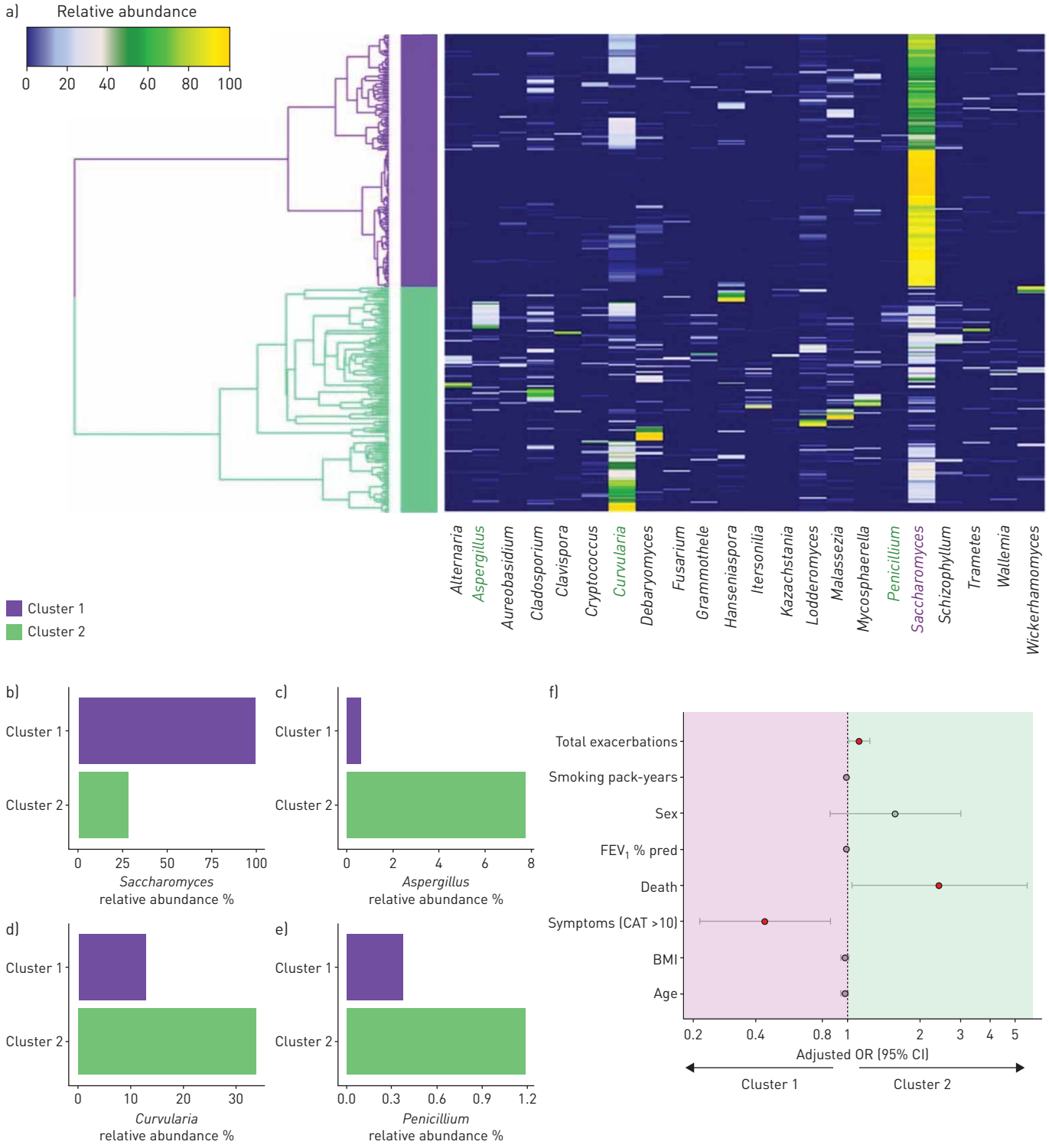

FIGURE 3 Unsupervised hierarchical clustering of fungal genera from the airway mycobiome in chronic obstructive pulmonary disease (COPD) reveals two distinct clusters with variable clinical outcomes. a) Heatmap illustrating the relative abundance of the various fungal genera within the mycobiome in their two distinct clusters differentiated based on abundance of b) *Saccharomyces*, c) *Aspergillus*, d) *Curvularia* and e) *Penicillium*. b–e) $p<0.05$. f) Forest plot with adjusted odds ratios and 95% confidence intervals illustrating clinical differences between the two clusters. Cluster 1 has significantly greater symptoms (CAT score >10) ($p<0.05$), whereas Cluster 2 demonstrates significantly more exacerbations ($p<0.05$) and higher mortality ($p<0.05$). Circle colour indicates significance level: $p<0.05$ (red) and not significant (grey). FEV_1: forced expiratory volume in 1 s; CAT: COPD Assessment Test; BMI: body mass index.

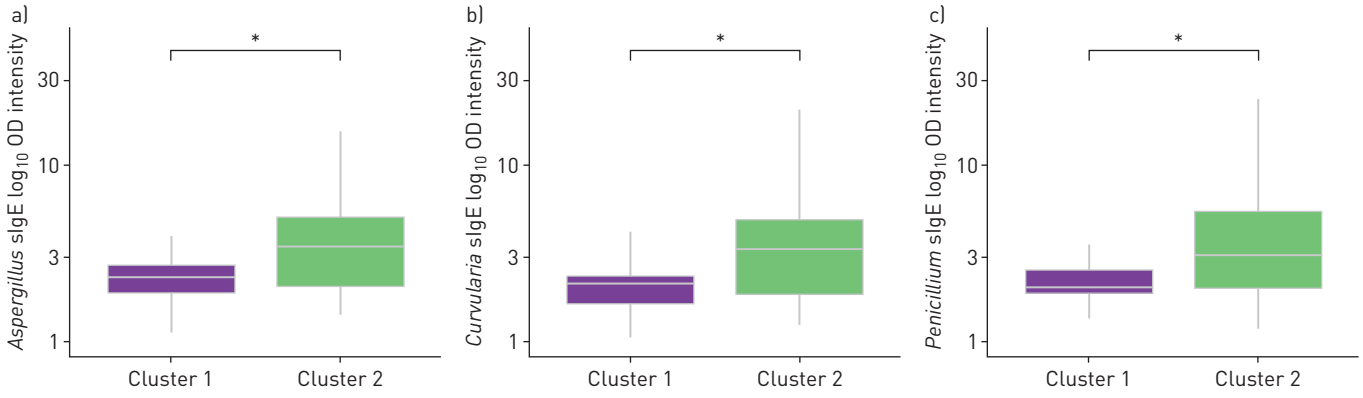

FIGURE 4 A "high-risk" chronic obstructive pulmonary disease (COPD) mycobiome (Cluster 2) characterised by *Aspergillus*, *Curvularia* and *Penicillium* demonstrates a measurable systemic immune response to these fungi. Systemic sIgE binding to a) *Aspergillus*, b) *Curvularia* and c) *Penicillium* was measured in a subset of stable COPD participants from Singapore/Kuala Lumpur (n=42) and illustrated as box plots for comparison between clusters. Box plots show median, interquartile range and minimum–maximum range. *: p<0.05. sIgE: specific IgE; OD: optical density.

The COPD mycobiome is diverse and demonstrates geographic variation. Very frequent COPD exacerbators are characterised by altered mycobiomes and increased interfungal interaction; however, mycobiome composition and diversity remain unaffected by the exacerbation event itself, antibiotics and/or corticosteroid use, suggesting that perturbation of the COPD mycobiome results from factors other than treatment. Two important patient clusters were identified based on mycobiome signatures: Cluster 1 characterised by significant symptoms and *Saccharomyces*, and Cluster 2 characterised by exacerbations,

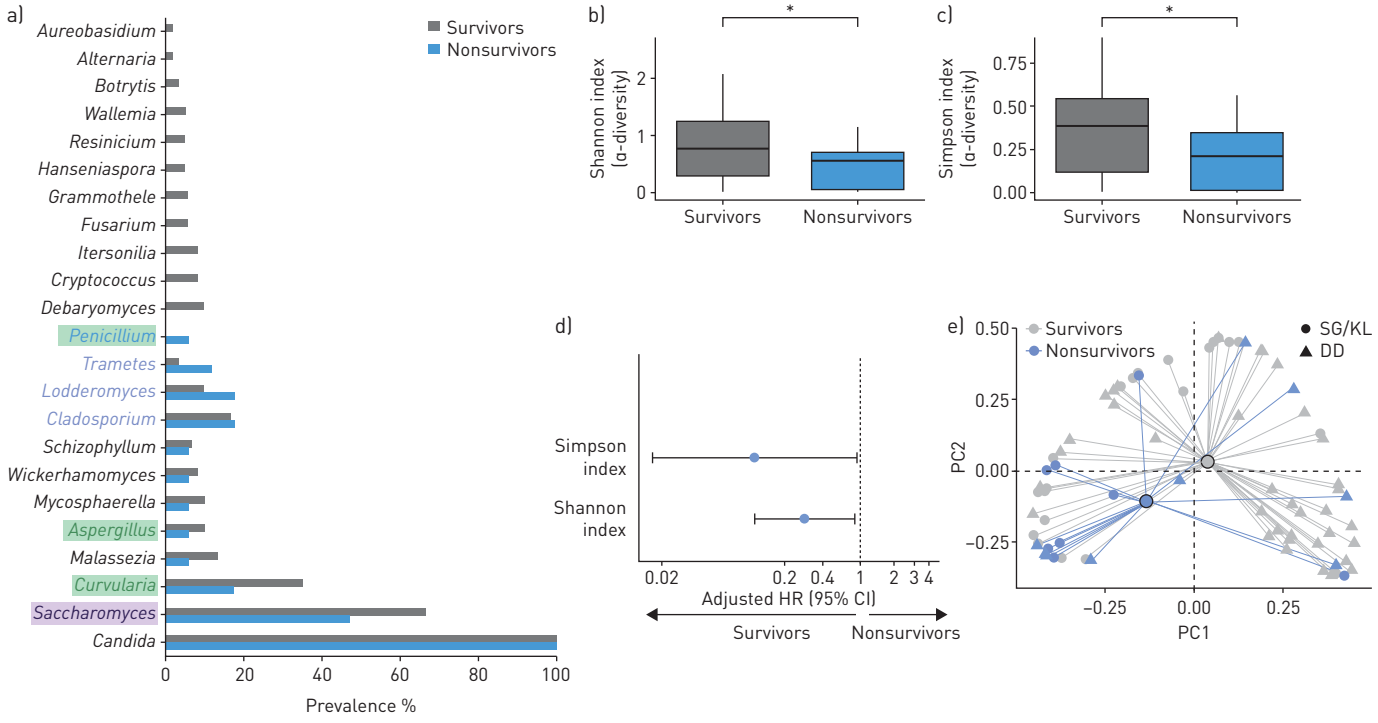

FIGURE 5 Chronic obstructive pulmonary disease (COPD) mycobiomes with lower fungal diversity at exacerbation associate with higher 2-year mortality. a) Prevalence rates of various fungal genera (with relative abundance >1%) from COPD mycobiomes obtained at acute exacerbation (n=66) between survivors (n=51) and nonsurvivors (n=15) at 2-year follow-up. The text and bar colours represent the predominant taxa between the nonsurvivors and survivors. Previously identified cluster-associated taxa are highlighted: Cluster 1 (purple) and Cluster 2 (green). b, c) A significantly decreased α-diversity of the mycobiome is observed in COPD nonsurvivors compared with survivors measured by b) Shannon index and c) Simpson index between groups. d) Forest plot with adjusted hazard ratios (p<0.05) and 95% confidence intervals illustrating inverse association with increase in α-diversity indexes in survivors at 2-year follow-up. Hazard ratios are adjusted for age, sex, smoking pack-year history and body mass index. e) Principle coordinate analysis illustrating β-diversity between survivors and nonsurvivors at 2-year follow-up using the Bray–Curtis dissimilarity index. Box plots show median, interquartile range and minimum–maximum range. *: p<0.05. SG/KL: Singapore/Kuala Lumpur; DD: Dundee.

higher mortality, and *Aspergillus*, *Curvularia* and *Penicillium*. The latter group demonstrates systemic immune responses to these fungi. Lower mycobiome diversity during exacerbations represents an important risk for 2-year mortality.

In line with work in bronchiectasis, we observe differing mycobiome profiles between participants recruited in different countries [2]. The detected geographic variability may be attributed to contrasting climates, humidity and air quality, all important for fungal growth and survival. Host factors, including genetics, lifestyle and dietary differences, may contribute further, but remain beyond the scope of this work. Importantly, the respiratory tract represents an organ system in continuous communication with the external environment, *i.e.* a rich fungal source, allowing temporary passage and potentially retention of environmental fungi under appropriate conditions. The importance of geographic location and the specific environmental influence on host mycobiome profiles in relation to COPD has also been the subject of prior work [7]. Using metagenomic sequencing of outdoor and indoor air, a measurable sensitisation response to environmental fungi relates to COPD outcomes. Taken together with the findings presented in this study, geographic location and local environment remain important determinants of the host mycobiome.

Prior studies in COPD demonstrate alterations in the bacterial microbiome, particularly in frequent exacerbators [26, 27]. This current study identifies significant changes to the airway mycobiome in very frequent COPD exacerbators (three or more exacerbations per year) who interestingly illustrate a more complex mycobiome characterised by increased antagonistic interfungal interaction. COPD exacerbations may promote initial bacterial dysbiosis and disruption to fungal communities may only occur as exacerbation frequency increases. In addition to an alteration in fungal community membership with increased exacerbation frequency, dynamic variation also occurs. The increased negative (co-exclusive) interactions observed in very frequent COPD exacerbators may result in fungal overgrowth, particularly of specific fungal taxa such as *Wickerhamomyces*, which in turn drives the observed clinical phenotype. These fungi are widespread in natural habitats, including soil, trees and plants; however, there has been a reported case of clinical fungaemia due to *Wickerhamomyces* [28]. Although very frequent COPD exacerbators generally receive significantly more treatment, including antibiotics and/or systemic steroids, compared with nonexacerbators, longitudinal mycobiome analysis reveals its relative stability despite such treatment and even during exacerbations, consistent with work performed in CF, suggesting alternate mechanisms to explain the observed fungal dysbiosis in very frequent exacerbators [29, 30]. As bacteria and viruses (rather than fungi) are commonly identified triggers of COPD exacerbations, it is not unexpected that the airway mycobiome remains stable during AECOPD in our longitudinal analysis. However, with increased exacerbation frequency and its associated immunoinflammatory change, a "threshold" in very frequent COPD exacerbators may be reached where alterations to the airway mycobiome begin to appear. Such a change, occurring over time, likely involves a complex interaction between microbial kingdoms, the surrounding environment and the underlying host immunoinflammatory response rather than an acute alteration during a single COPD exacerbation where bacterial and/or viral change may be more apparent [16]. Therefore, the airway mycobiome in COPD is likely most useful in identifying patients at greatest risk of longer-term adverse outcomes, including a risk of frequent exacerbations and mortality [31, 32]. Interestingly, inhaled corticosteroid use demonstrates no impact on mycobiome profiles. This contrasts with bacteriome studies in COPD, where microbiome change is observed over the course and treatment of exacerbations, which are influenced further by COPD therapy [16]. Differences in study outcomes between bacterial and fungal microbiomes suggest that bacteria and fungi play different roles during exacerbations and are differently impacted by COPD treatments.

While our current understanding of the mycobiome's contribution to respiratory disease pathogenesis remains indeterminate, available data does suggest that specific mycobiome profiles do exist in chronic respiratory disease states, with an increased abundance of *Candida* and *Aspergillus* in CF, an increased abundance of *Psathyrella*, *Malassezia*, *Termitomyces* and *Grifola* in asthma, and an increased abundance of *Aspergillus*, *Cryptococcus* and *Clavispora* in bronchiectasis [2, 20, 33–35]. In the context of COPD, prior mycobiome studies have focused purely on the HIV-infected COPD patient group where the key change to airway mycobiomes observed was an increased abundance of *P. jirovecii* [22]. Importantly, our work did not detect any *Pneumocystis* fungi in our COPD cohorts; however, we did identify specific fungal taxa associated to COPD, including *Alternaria*, *Aspergillus*, *Cladosporium*, *Cryptococcus*, *Mycosphaerella*, *Wickerhamomyces*, *Trametes* and *Penicillium*. Differences in the chosen study populations and methodology used likely accounted for at least some of these differences.

Unsupervised clustering analysis reveals two clinically important COPD patient groups: one (Cluster 1) with increased symptoms (CAT score) and *Saccharomyces* dominance, and another (Cluster 2) with increased exacerbations and higher mortality characterised by *Aspergillus*, *Curvularia* and *Penicillium*. In the latter group, systemic sIgE responses to *Aspergillus*, *Curvularia* and *Penicillium* were detectable. The

first group, *i.e.* characterised by increased COPD symptoms and *Saccharomyces*, is particularly interesting because existing data in COPD and, more recently, bronchiectasis suggest that the presence of symptoms can indicate a risk of future exacerbations and a response to treatment [36–40]. Whether these concepts hold true regarding airway fungi in COPD remains to be established. The second group, *i.e.* characterised by *Aspergillus*, *Curvularia* and *Penicillium*, should be considered "high risk", and warrants early identification and close clinical follow-up. *Aspergillus*, *Curvularia* and *Penicillium* are thermophilic fungi with survival capabilities in the human airway and pathogenic potential. Importantly, these fungi commonly associate with ABPM, and airway isolation of *Aspergillus* and *Penicillium* has been associated with sensitisation and reduced lung function in asthma [41]. The importance of fungal sensitisation in COPD has recently been described by our group and contributes to COPD exacerbations in line with earlier studies in asthma, where fungal sensitisation demonstrates key roles in disease severity and exacerbations [3, 7]. Therefore, it is plausible that increases in specific airway fungi in COPD activate host immunity, with resulting sensitisation and poorer clinical outcomes [7]. Importantly, despite striking geographical, environmental and ethnic differences, both clusters were consistently identified in participants from Asia and the UK.

Evaluating the COPD mycobiome during acute exacerbations reveals that a loss of diversity is a signal for increased 2-year mortality. This is consistent with prior bacteriome studies in COPD where survival, assessed at 1 year following exacerbation, demonstrates a similar lack of diversity [17]. Fungal genera characterising poorer survival include *Penicillium*, *Cladosporium*, *Trametes* and *Lodderomyces*. Of interest, *Penicillium* also characterised the "high-risk" cluster in the stable COPD state, suggesting that its specific role in COPD pathogenesis warrants further study. The fungi identified in the nonsurvivors have been associated with invasive fungal infection and/or sensitisation in humans; however, their specific roles in COPD remain to be fully expounded. The COPD mycobiome clearly holds important information with potential prognostic implications and may be used to identify "high-risk" patients with worse 2-year survival following an acute exacerbation.

Our study is the largest and, to the best of our knowledge, the first multicentre COPD study to evaluate the airway mycobiome including longitudinal sampling. Despite its clear strengths, our work is limited by a small healthy cohort for comparison, all recruited from a single site (Singapore), and therefore may not be generalisable to the wider healthy population. In addition, our AECOPD cohort was relatively small (n=66) and therefore the survival analysis is limited; however, it does represent the largest study of the airway mycobiome in COPD to date, complete with 2-year survival data. Further investigations building on this work in larger cohorts will help to explicate the clinical significance and survival-related aspects of our findings. While we report unique mycobiome signatures in COPD, we did not specifically exclude patients with bronchiectasis–COPD overlap (24% (81 out of 337)), although importantly, no differences between mycobiome profiles were detectable between patients with COPD (n=256) and patients with bronchiectasis–COPD overlap (n=81). In addition, we did not assess the COPD mycobiome's potential interaction with other microbiomes (bacterial and viral). Future studies integrating the various microbiomes accounting for the host response will be important in understanding the specific impact of the mycobiome in COPD pathogenesis. Despite optimising our internal transcribed spacer protocol as previously published, inherent limitations persist in the field, including weak fungal reference databases that preclude confident resolution to the species level [42]. In addition, targeted amplicon sequencing lacks functional annotation when compared with metagenomic sequencing approaches, and therefore our findings presented here can only describe associations and cannot imply causation or demonstrate a pathogenic role for any of the detected fungal taxa. Future work focused on mechanistic confirmation should be pursued. Unlike bacterial microbiome studies in COPD [19, 43], we did not detect associations between disease severity and the COPD mycobiome; however, it does appear that the mycobiome composition is influenced beyond a certain "threshold" of COPD exacerbation frequency, exemplified by our "very frequent exacerbator" group. The COPD mycobiome may therefore represent a "marker" for specific disease traits in COPD that requires further study. Finally, our systemic sIgE assays were performed only in a subset of participants and with selected fungi, and this an avenue for further exploration. A validation of our study findings with a more comprehensive fungal allergen panel and inclusion of other ethnic populations should be pursued in future work.

In summary, we show that the COPD mycobiome provides important information in terms of clinical outcomes and prognosis, and should be further evaluated.

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M.S. Koh, A. Tee and J.A. Abisheganaden: patient recruitment, clinical data and specimen collection. M. Mac Aogáin: performance of experimental optimisation and work. S.L. Pang and B.Q.Y. Chua: performance of experimental work and data collection. B.E. Miller and R. Tal-Singer: patient recruitment and funding. F.T. Chew: conception of experiments and interpretation of results. J.D. Chalmers and S.H. Chotirmall: study design and conception of experiments, data collection, interpretation and analysis, obtained study funding, and writing of the final manuscript.

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