ORIGINAL ARTICLE
COPD

Environmental fungal sensitisation associates with poorer clinical outcomes in COPD

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Fungal sensitisation associates with frequent exacerbations in COPD, and represents a treatable trait. Outdoor and indoor environments represent a key source of fungal allergen exposure, amenable to intervention, in "sensitised" COPD patients. https://bit.ly/2Vw3kHi

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ABSTRACT

Introduction: Allergic sensitisation to fungi such as *Aspergillus* are associated to poor clinical outcomes in asthma, bronchiectasis and cystic fibrosis; however, clinical relevance in COPD remains unclear.

Methods: Patients with stable COPD (n=446) and nondiseased controls (n=51) were prospectively recruited across three countries (Singapore, Malaysia and Hong Kong) and screened against a comprehensive allergen panel including house dust mites, pollens, cockroach and fungi. For the first time, using a metagenomics approach, we assessed outdoor and indoor environmental allergen exposure in COPD. We identified key fungi in outdoor air and developed specific-IgE assays against the top culturable fungi, linking sensitisation responses to COPD outcomes. Indoor air and surface allergens were prospectively evaluated by metagenomics in the homes of 11 COPD patients and linked to clinical outcome.

Results: High frequencies of sensitisation to a broad range of allergens occur in COPD. Fungal sensitisation associates with frequent exacerbations, and unsupervised clustering reveals a "highly sensitised fungal predominant" subgroup demonstrating significant symptomatology, frequent exacerbations and poor lung function. Outdoor and indoor environments serve as important reservoirs of fungal allergen exposure in COPD and promote a sensitisation response to outdoor air fungi. Indoor (home) environments with high fungal allergens associate with greater COPD symptoms and poorer lung function, illustrating the importance of environmental exposures on clinical outcomes in COPD.

Conclusion: Fungal sensitisation is prevalent in COPD and associates with frequent exacerbations representing a potential treatable trait. Outdoor and indoor (home) environments represent a key source of fungal allergen exposure, amenable to intervention, in "sensitised" COPD.

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Introduction

A high prevalence of allergic sensitisation is reported in chronic respiratory diseases including asthma, COPD and bronchiectasis [1–5]. Sensitisation, particularly to fungi such as *Aspergillus*, is associated with poor clinical outcomes in asthma, bronchiectasis and cystic fibrosis; however, its clinical relevance in COPD remains unclear [5–8]. Prior studies evaluating sensitisation in COPD report conflicting results: some have associated sensitisation with greater symptoms, more frequent exacerbations and poorer lung function, while others have shown no association [1, 2, 4, 9].

Rapid global urbanisation, climate change and poor air quality, particularly in Asia, has led to increasing rates of allergy and an increased burden of lung disease; however, the influence of environmental exposures on patients with COPD remains unaddressed [10, 11]. Geographical variation is important, with high prevalence of sensitisation to house dust mite (HDM) observed in atopic Asian individuals when compared to other allergens, such as pollens and animal dander, which predominate in western cohorts [12, 13]. Warm and humid climates in the Asian subcontinent further predispose to high allergen exposures and mould sensitisation, which in turn are associated with poorer outcomes in patients with chronic respiratory disease [5, 6, 14]. The natural and built environment, including climatic factors, further influences the form, variety, type and burden of allergens to which patients are exposed, and no prior studies have evaluated sensitisation in relation to environmental allergen exposure in COPD [14–17].

Current studies that assess sensitisation in COPD report inconsistent association with clinical outcomes; however, the studied populations were heterogeneous and geographically diverse, and some included patients with asthma–COPD overlap syndrome (ACOS). Differing methods of assessing sensitisation coupled to the varied allergen panels used have all influenced the indeterminate outcomes. In addition, few studies focus on Asian populations, and none have directly explored the influence of outdoor or indoor environmental allergen exposure.

Here, using a comprehensive allergen panel, we assess the role of sensitisation on clinical outcomes in COPD using a large Asian cohort recruited across three countries, and, for the first time, assess the influence of environmental allergen exposure using a metagenomics sequencing approach.

Methods

Patient recruitment

COPD

Patients aged ⩾40 years with stable COPD attending respiratory outpatient clinics for routine follow-up were recruited over a 4-year period between 2014 and 2018 at five hospitals across three Asian countries as follows: Singapore General Hospital, Changi General Hospital and Tan Tock Seng Hospital (Singapore), Prince of Wales Hospital (Hong Kong) and University Malaya Medical Centre (Kuala Lumpur, Malaysia). COPD was defined according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria [18]. Patients with any prior history of asthma (defined by variable symptoms and expiratory airflow limitation according to Global Initiative for Asthma guidelines) and those on long-term oral steroids or any immunosuppressive agents were excluded [19]. Stable COPD was defined as the absence of an exacerbation 6 weeks prior to recruitment. A frequent COPD exacerbator was defined as having more than two exacerbations in the year preceding study recruitment, and a prior history of recurrent exacerbations despite receiving COPD therapy based on GOLD guidelines [18, 20]. All recruited patients were receiving COPD therapy (including smoking cessation counselling, inhaler assessment, COPD action plans, inhalers as long-acting β-agonists, long-acting muscarinic antagonists, inhaled corticosteroids and/or short-acting bronchodilators in addition to vaccination as appropriate) based on GOLD guidelines [18, 20]. All patients underwent a blood draw and full blood count (including eosinophil count).

Non-COPD (healthy) controls

Subjects with normal spirometry and no prior history of COPD or any other respiratory disease were recruited from Nanyang Technological University (Singapore) and the University Tunku Abdul Rahman

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TABLE 1 Demographic table for the nondiseased and COPD cohorts

	Nondiseased control aged ⩽40 years	Nondiseased control aged >40 years	COPD
Subjects	26	25	446
Age years	22 (20–23)	63 (61–64)	75 (68–80)
Male	10 (38.5)	12 (48.0)	426 (96.2)
BMI kg·m^{-2}	22.0 (21.0–24.0)	24.0 (20.6–26.0)	21.5 (18.7–23.8)
Smoking status			
Current smoker	0 (0.0)	4 (16.0)	303 (67.9)
Ex-smoker	1 (3.8)	3 (12.0)	139 (31.2)
Never-smoker	25 (96.2)	18 (72.0)	4 (0.9)
Smoking history pack-years	0 (0–0)	44 (33–53)	50 (38–65)
FEV_1 % pred	96.5 (85.3–107.8)	93.5 (84.0–97.8)	45.0 (33.6–59.0)
FEV_1/FVC % pred	86.6 (82.3–93.2)	78.6 (75.0–84.7)	50.4 (41.2–59.5)
Number of exacerbations in the year preceding study entry			
0–1	NA	NA	293 (65.7)
>1	NA	NA	153 (34.3)
Hospitalisation in the year preceding study entry			
Yes			208 (46.6)
No			238 (53.4)
COPD assessment test	NA	NA	14 (9–19)
Blood eosinophil count ×10^9 cells·L^{-1}	NA	NA	0.12 (0.00–0.29)
Total IgE IU·mL^{-1}	NA	NA	24.5 (5.0–119.3)
Treatment			
SAMA/SABA			125 (28.0)
LAMA			29 (6.5)
LAMA/LABA	NA	NA	43 (9.7)
LABA/ICS			115 (25.8)
LAMA/ICS			18 (4.0)
LAMA/LABA/ICS			116 (26.0)

Data are presented as n, median (interquartile range) or n (%). BMI: body mass index; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; SAMA: short-acting muscarinic antagonist; SABA: short-acting β-agonist; LAMA: long-acting muscarinic antagonist; LABA: long-acting β-agonist; ICS: inhaled corticosteroid; NA: not applicable.

(Kampar, Malaysia) as controls and divided into subjects aged ⩽40 or >40 years for analysis. Patient demographics and clinical characteristics of all recruited cohorts are summarised in table 1.

Allergen panel

The allergen panel selected for study comprised common allergens implicated in airways disease in Asia and include *Dermatophagoides farinae*, *D. pteronyssinus*, *Blomia tropicalis*, *Elaeis guineensis*, panicoids (Johnson grass (*Sorghum halepense*)), pooids (Timothy grass (*Phleum pratense*), meadow fescue (*Festuca pratensis*), perennial ryegrass (*Lolium perenne*)), chloroids (Bermuda grass (*Cynodon dactylon*)), weeds (*Brassica* spp., *Ambrosia artemisiifolia*, *Helianthus annuus*), *Blattella germanica*, *Periplaneta americana*, *Curvularia* (*C. lunata*, *C. spicifera*, *C. inaequalis*), *Penicillium* (*P. citrinum*, *P. chrysogenum*, *P. notatum*, *P. digitatum*) and *Aspergillus fumigatus*. In addition, the top eight culturable fungi isolated from outdoor air in Singapore based on published metagenomic data were included for study as described in the supplementary material (figure E1) [13, 14, 21].

Outdoor air sampling

Outdoor air samples were collected on the rooftop of an academic building at Nanyang Technological University (Singapore), 20 m above the ground, for five consecutive days using methods described previously [14]. Briefly, air was collected using filter-based air samplers SASS3100 (Research International, Monroe, WA, USA) mounted with SASS bioaerosol electret filters (Research International). Air sampling was performed at 300 L·min^{-1} airflow rate at a height of 1.5 m above the floor at the rooftop balcony. The SASS filter was transferred to the lab for immediate processing or stored at −20°C prior to processing. For processing, the SASS filter was transferred to a sterile tube and washed with PBS-Triton X-100 in triplicate. Resultant washed solutions were filtered through 0.02 µm Anodisc filters (Whatman) and DNA extracted using the DNeasy PowerWater Kit (Qiagen, Hilden, Germany) as per manufacturer's

instructions. The extracted DNA including an extraction blank and sterile filter were sequenced using the HiSeq 2500 Illumina platform (Illumina, San Diego, CA, USA). All sequence data from the outdoor air study has been uploaded to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under project accession PRJNA436039. Details on the isolation and confirmatory identification of outdoor air fungi can be found in the supplementary material.

Indoor air and surface sampling for metagenomic sequencing

A subset of COPD patients (n=11) were recruited for home environmental sampling, which included 1) indoor (bedroom) air sampling, 2) outdoor (balcony) air sampling and 3) surface swab obtained from either an air-conditioning filter or fan from the patient's bedroom. Air samples were obtained concurrently from the patient's bedroom and balcony using filter-based air samplers SASS3100 (Research International) with a flow rate of 100 $L \cdot min^{-1}$ for eight consecutive hours. In addition, a surface (either air-conditioner or fan where applicable) were swabbed and subjected to metagenomic sequencing. The surfaces (air-conditioner or fan) were swabbed using 4N6Floq (Copan, Murrieta, CA, USA) swabs pre-moistened with 0.1% PBS-Triton-X100. DNA was then extracted from the swabs using the DNeasy PowerWater kit as per manufacturer's instructions. Extraction blanks and sterile swabs were processed in parallel as controls. Sequencing was performed using the Illumina HiSeq 2500 platform (Illumina) and library preparation including sequencing methodologies were performed using published protocols [14]. All sequence data from the indoor air study have been uploaded to the NCBI SRA under project accession PRJNA608611.

Metagenomic sequencing and data processing

Briefly, the raw metagenomic sequences with Phred scores >20 were subjected to adaptor removal and quality trimmed with Cutadapt (version 1.8.1) [22]. The metagenomic reads were then aligned to the NCBI nonredundant protein database using RAPSearch version 2.15 and imported into MEGAN for assignment of taxon ID based on NCBI taxonomy with lowest common ancestry algorithm with a minimum score of 100 and support of $\geqslant$25 [23].

Ethics approval

This study was approved by the institutional review boards of all participating hospitals and institutions. Written informed consent was obtained from all participants. Additional details can be found in the supplementary material.

Statistical analysis and data visualisation

Statistical analysis was performed using R (version 3.6.1; R Foundation for Statistical Computing, Vienna, Austria). Normality was assessed with the Shapiro–Wilk test. Continuous data are presented as median with interquartile range (IQR) (for non-normally distributed datasets) or mean±SD (for normally distributed data). For non-normal data, Kruskal–Wallis or Mann–Whitney U-tests were employed for group comparisons. Categorical data were compared by Chi-squared or Fisher exact testing as appropriate. Multiple comparisons were corrected for false discovery rate using Benjamini–Hochberg corrections. Significance was defined as $p \leqslant 0.05$. Logistic regression corrected for age, sex, body mass index (BMI) and smoking pack-years was performed using "glm" function in R. Incidence rate ratio (IRR) was performed using the "glm.nb" function of the "MASS" package. Indoor allergen abundance was visualised using "pheatmap" package with row z-score and regression plots plotted with *ggscatter* function using R "ggpubr" package with Pearson correlation. Bubble charts were plotted using MEGAN [23]. Sensitisation was defined as a specific (s)IgE binding intensity above the 95th percentile of that measured from the nondiseased control cohort. Air-fungi sensitised was defined as an individual illustrating sensitisation to one or more of the eight air fungi assessed.

Cluster analysis

Patient demographics, sIgE and treatment were employed for unsupervised clustering. Both continuous and categorical variables were converted to Gower dissimilarity matrices using the *daisy* function from the R "cluster" package. Nonmetric multidimensional scaling was then implemented on the Gower dissimilarity matrix using R "MASS" package. Unsupervised clustering with Ward's minimum variance was performed with the *hclust* function of the R "cluster" package. The optimal number of clusters was determined using the R "NbClust" package and Jaccard similarities index computed to assess cluster stability with bootstrapping >100 iterations. The mean Jaccard value for each detected cluster was 0.998, 1.0 and 0.999 respectively, suggesting that the identified clusters were highly stable.

Full details on specimen collection and processing, allergen preparation, sIgE assays, fungal identification and indoor allergen mapping are provided in the supplementary material.

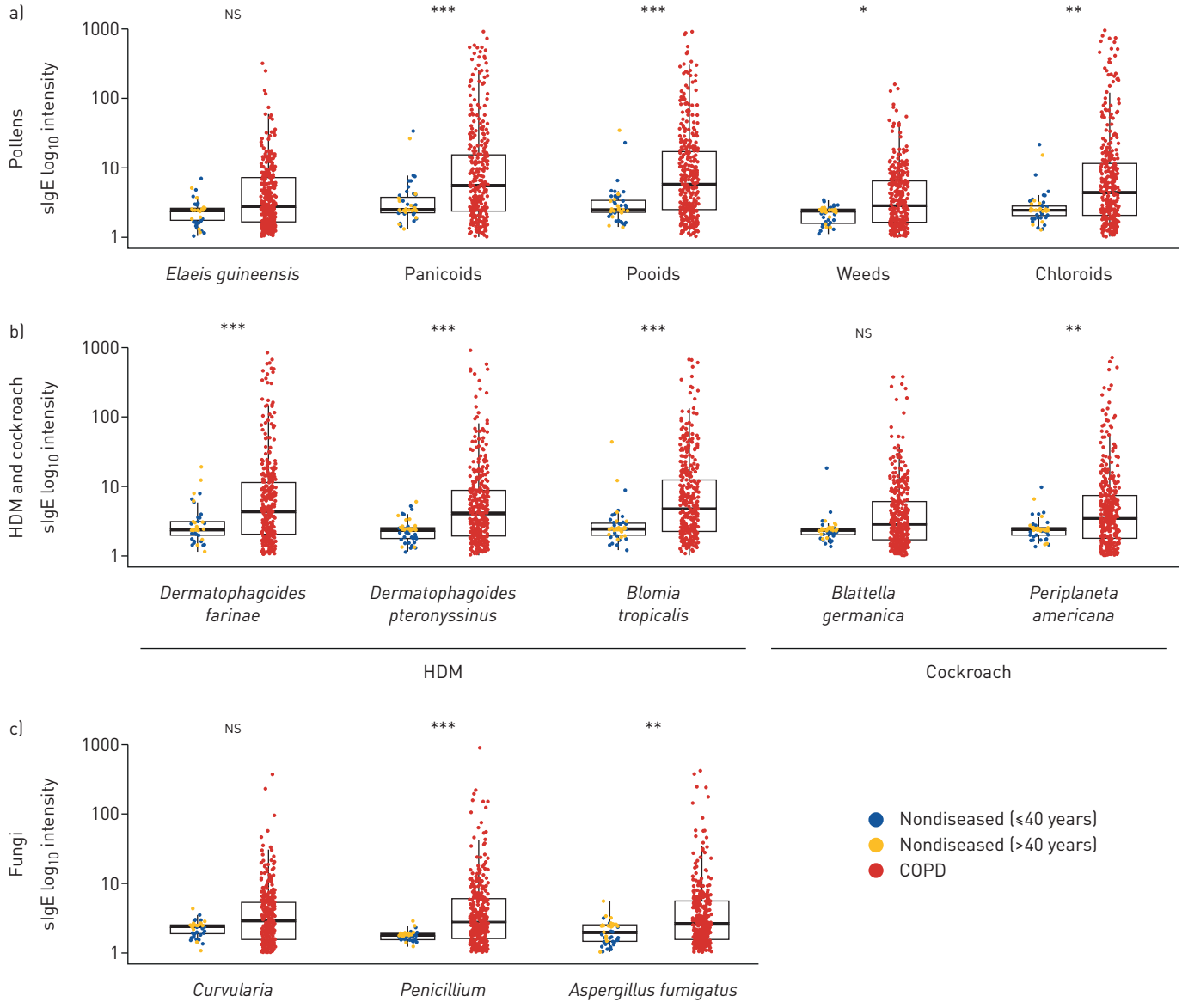

FIGURE 1 Patients with COPD exhibit increased sensitisation to a range of a) pollens, b) house dust mite (HDM) and cockroach and c) fungal allergens, compared to nondiseased (healthy) controls. Systemic specific (s)IgE binding is expressed as $\log_{10}$ OD intensity. Benjamini–Hochberg adjusted p-values are shown. NS: nonsignificant. *: $p \leqslant 0.05$, **: $p \leqslant 0.01$, ***: $p \leqslant 0.001$.

Results

Patients with COPD exhibit a high frequency of sensitisation across a broad range of allergens

sIgE levels across a broad range of allergens (pollens, HDM, cockroach and fungi) were increased in COPD (n=446) compared to nondiseased (healthy) controls (n=51) with HDM (*B. tropicalis*, *D. pteronyssinus* and *D. farinae*) and grass pollens (pooids and panicoids) illustrating the highest median sIgE-binding intensities (figure 1). Where sensitisation is classified as positive toward a particular allergen (*i.e.* sIgE-binding intensity >95th percentile of nondiseased controls), a significant number of COPD patients demonstrate sensitisation to fungi (n=249, 55.8%) and HDM (n=229, 51.3%).

Fungal sensitisation is associated with frequent exacerbations

Having identified high sensitisation rates, particularly to fungal allergens, we next evaluated association with clinical outcomes. On univariate analysis, frequent exacerbators illustrate significantly increased sIgE-binding to crude fungal allergens (*Curvularia*, *Penicillium* and *A. fumigatus*) (figure 2a) and the cockroach allergen *Bl. germanica* (supplementary figure E2), but no significant differences to pollens,

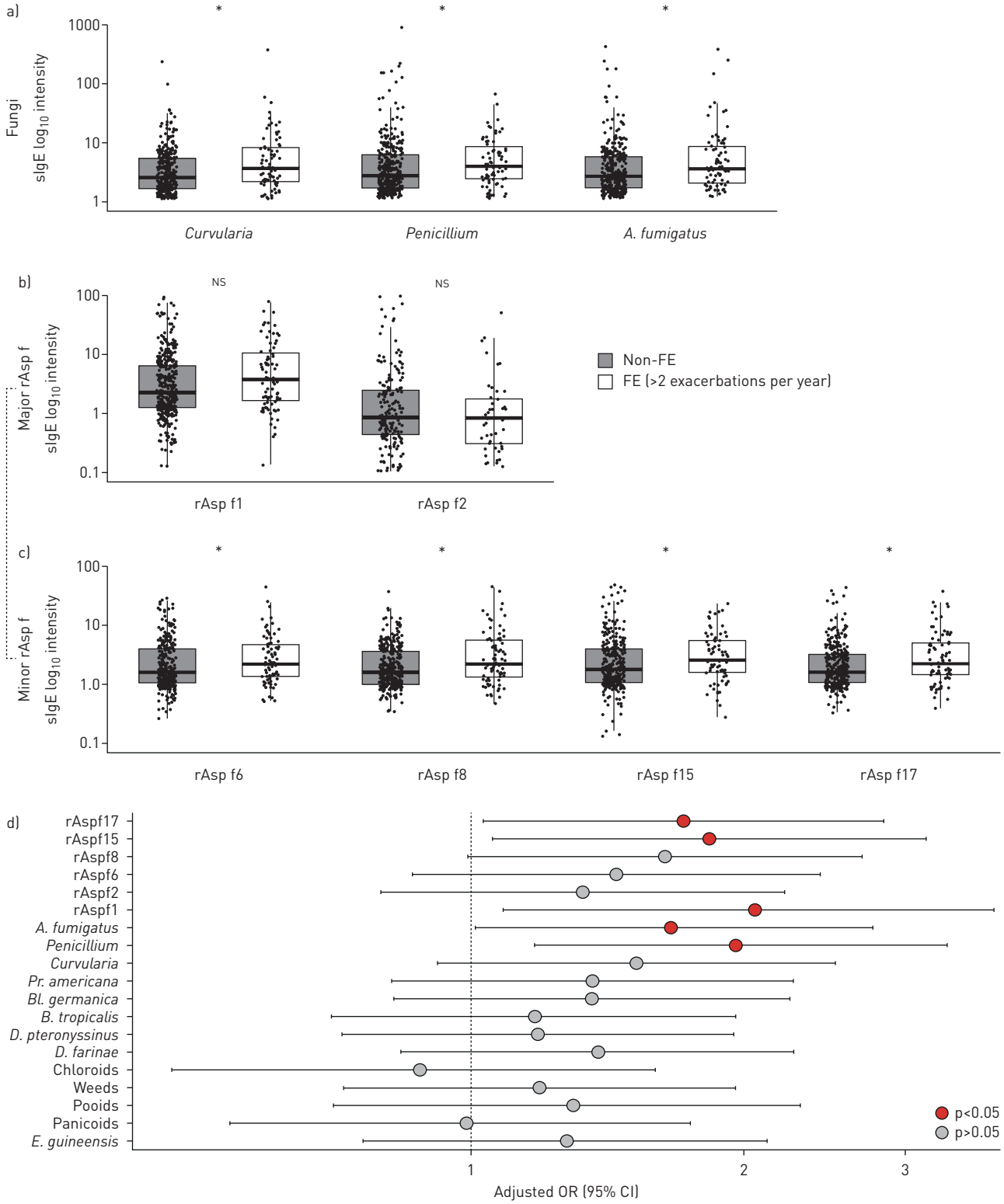

FIGURE 2 Frequent COPD exacerbators illustrate high sensitisation rates to fungal (crude) and recombinant *Aspergillus* (rAsp) allergens. Scattered boxplots illustrate systemic specific (s)IgE binding between non-frequent (non-FE) and frequent (FE) COPD exacerbators (more than two exacerbations per year) against a) *Curvularia*, *Penicillium* and *Aspergillus* (crude) allergens, b) major recombinant *Aspergillus fumigatus* allergens (rAsp f) 1, 2 and c) minor recombinant *A. fumigatus* allergens (rAsp f) 6, 8, 15 and 17. d) Forest plot illustrating multivariate logistic regression analysis for frequent COPD exacerbators after adjustment for age, sex, body mass index and smoking pack-years. Systemic sIgE binding is expressed as $\log_{10}$ OD intensity. Benjamini–Hochberg adjusted p-values are shown. *Pr. americana*: *Periplaneta americana*; *Bl. germanica*: *Blattella germanica*; *B. tropicalis*: *Blomia tropicalis*; *D. pteronyssinus*: *Dermatophagoides pteronyssinus*; *D. farinae*: *Dermatophagoides farinae*; *E. guineensis*: *Elaeis guineensis*; NS: nonsignificant. *: $p \leqslant 0.05$.

HDM or the cockroach allergen, *Pr. americana* (supplementary figure E2). In view of the importance of fungal sensitisation on exacerbation status and the relevance of *Aspergillus*-associated respiratory disease, we next evaluated the association of exacerbations with specific recombinant *A. fumigatus* allergens [5, 6, 24, 25]. Interestingly, while significant associations were seen against minor recombinant *A. fumigatus* allergens on univariate assessment (figure 2b and c), multivariate analysis (adjusted for age, sex, BMI and smoking pack-years) demonstrate that crude fungi (*Penicillium* and *A. fumigatus*) and both major (rAsp f 1) and minor recombinant *Aspergillus* allergens (rAsp f 15 and 17) remain significantly associated with frequent COPD exacerbators (figure 2d). Importantly, no association between sensitisation status and COPD GOLD stage (lung function) or GOLD group (ABCD) was detected against any of the assessed allergens (supplementary figures E3 and E4).

Unsupervised clustering reveals three COPD "sensitisation" clusters demonstrating variable clinical outcome

To assess for groups of "sensitised" COPD patients and examine associated clinical risk, we performed unsupervised clustering analyses using patient demographics (including treatment) and the measured sIgE-response against all tested allergens. We detected three highly stable clusters (by Jaccard similarities index) differentiated by their degree of sensitisation and sIgE-binding pattern (supplementary table E2) as follows: high-sensitisation fungal predominant (n=115), low-sensitisation (n=114) and moderate-sensitisation *Blomia* predominant (n=217) (figure 3a and b). The high-sensitisation fungal predominant cluster demonstrates the worst clinical outcome between clusters with greatest symptoms (median COPD assessment test (CAT) score 16, IQR 10–22; p<0.01), poorest lung function (median

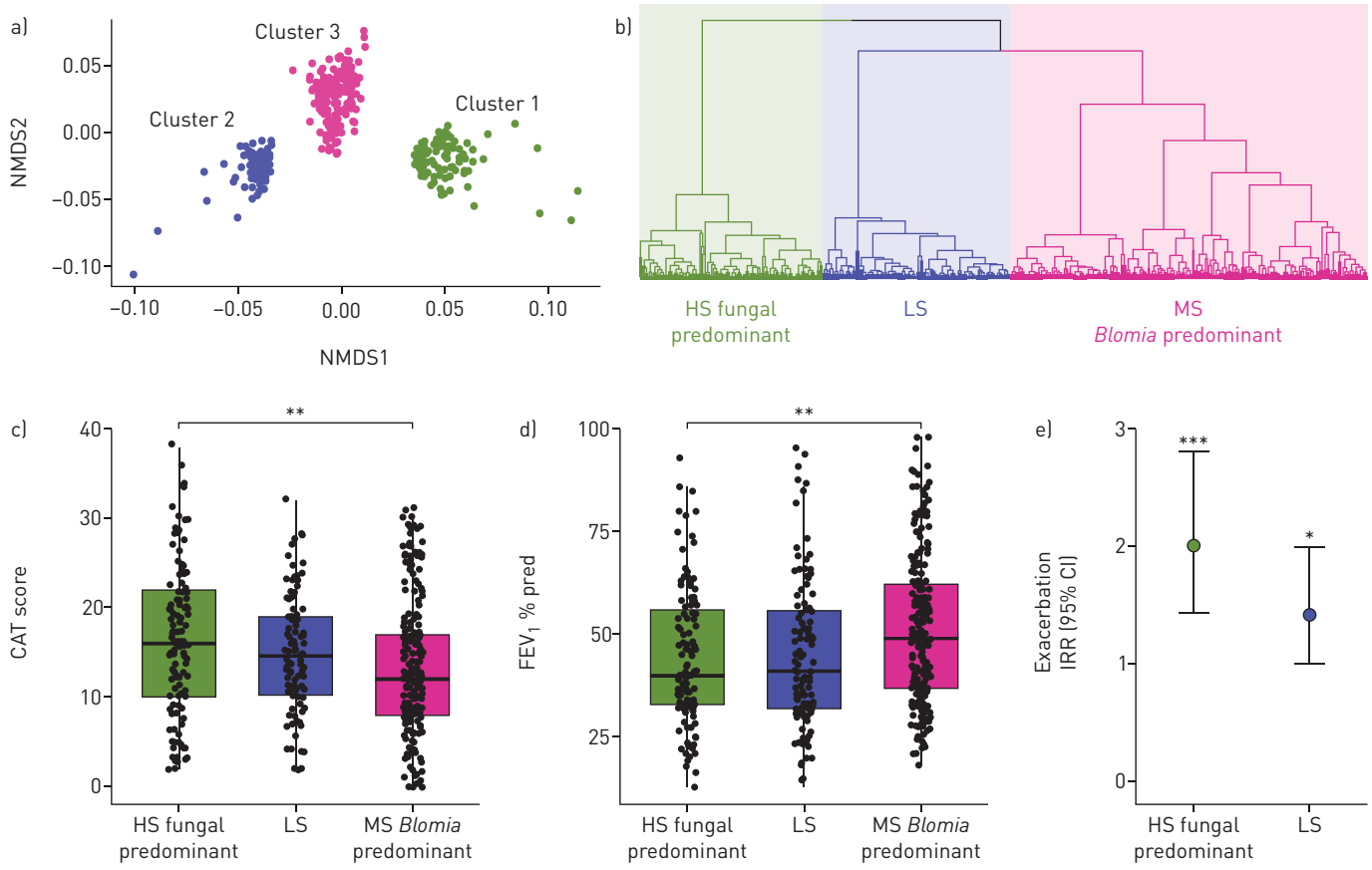

FIGURE 3 Unsupervised clustering (based on patient demographics and specific (s)IgE profiles against all examined allergens) reveals three clear patient clusters with worst clinical outcome in the "high-sensitisation (HS) fungal predominant" cluster. a) Nonmetric multidimensional plot based on Gower dissimilarity matrices and b) dendrogram illustrating the three identified patient clusters are illustrated. The three clusters were then assessed in relation to their c) symptoms (COPD assessment test (CAT) score), d) lung function (forced expiratory volume in 1 s (FEV_1) % predicted) and e) exacerbation frequency (in the year preceding recruitment) as a forest plot illustrating the incidence rate ratio (IRR) for exacerbations using the "moderate-sensitised (MS) *Blomia* predominant" cluster as reference. Cluster 1: HS fungal predominant; cluster 2: "low-sensitised" (LS); cluster 3: MS *Blomia* predominant. Error bars indicate the 95% confidence interval and dots represent each patient or the IRR for exacerbation. NS: nonsignificant. *: p≤0.05, **: p≤0.01, ***: p≤0.001.

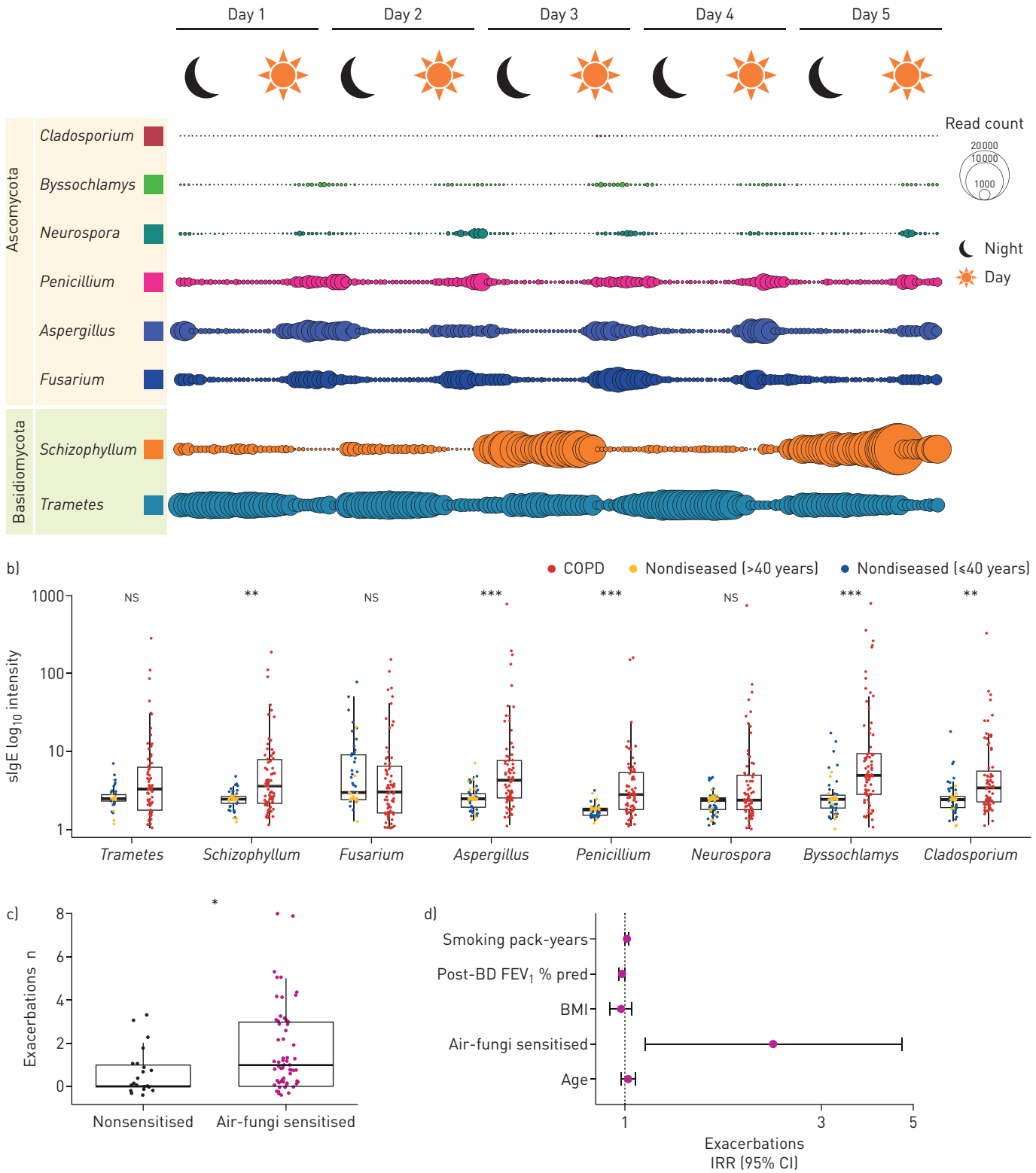

FIGURE 4 Sensitisation to outdoor air fungi in COPD associates with exacerbations. a) Bubble charts illustrating the metagenomic read abundance of the top eight culturable fungi from the outdoor air in Singapore measured over five consecutive days. Bubble size corresponds to metagenomic read; b) scattered box plots illustrating specific (s)IgE binding (expressed as $\log_{10}$ OD intensity) to the top eight outdoor air fungi in Singapore between nondiseased and Singaporean COPD patients; c) scattered box plot assessing exacerbation differences between Singaporean COPD patients with and without detectable sensitisation to outdoor air fungi; d) forest plot for exacerbation risk based on the presence of sensitisation to outdoor air fungi. Benjamini–Hochberg adjusted p-values are shown and dot colouration indicates cohort: nondiseased aged <40 years (blue), nondiseased aged >40 years (orange) and COPD (red), nonsensitised (black), air-fungi sensitised (dark pink). BMI: body mass index; post-BD FEV_1: post-bronchodilator forced expiratory volume in 1 s; NS: nonsignificant; IRR: incidence rate ratio. *: p≤0.05, **: p≤0.01, ***: p≤0.001.

forced expiratory volume in 1 s (FEV_1) 41.1% predicted, IQR 32.5–57.0% pred; p<0.01) and increased exacerbation rate (IRR 2.01, 95% CI 1.44–2.81; p<0.001), the latter using moderate-sensitisation *Blomia* predominant as reference (figure 3c–e). However, importantly, no differences in blood eosinophil counts were detected between clusters, although some patients across all clusters demonstrate elevated total eosinophil counts (supplementary figure E5).

Sensitisation to outdoor air fungi is associated with COPD exacerbations

As COPD exacerbations and poorer clinical outcome is associated with fungal sensitisation, we next assessed if the outdoor environment represents a key source of fungal exposure and the related sensitisation response. Employing the top eight culturable fungi detected by deep metagenomic sequencing of outdoor air in Singapore over five consecutive days, demonstrating a "diel cycle" (figure 4a), we next developed tailored dot-blot sIgE assays to assess the sensitisation response to these particular fungi in participants recruited from Singapore (COPD n=82 and nondisease controls n=51) (figure 4b) [14]. Interestingly, we found a significantly increased systemic sIgE response to a number of outdoor air fungi including *Schizophyllum* (p<0.01), *Aspergillus* (p<0.001), *Penicillium* (p<0.001), *Byssochlamys* (p<0.001) and *Cladosporium* (p<0.01) in COPD compared to nondiseased controls (figure 4b). To better understand the clinical implications of an outdoor air fungal response, we compared COPD patients demonstrating a measurable immune response to one or more outdoor air fungi (air-fungi sensitised, n=61) to those nonsensitised (n=21). While no relationship was observed for COPD symptoms (CAT score), lung function (FEV_1 % pred), eosinophil count and total IgE (supplementary figure E6), a significantly increased number of exacerbations was detected in the air-fungi sensitised COPD group (figure 4c), which, importantly, remains significant after adjustment for age, sex, BMI, lung function and smoking history (IRR 2.29, 95% CI 1.12–4.68; p<0.01) (figure 4d).

Indoor air and surfaces are a potential source of fungal allergen exposure and sensitisation in COPD

Having detected significant associations between sensitisation to outdoor air fungi and COPD exacerbations, we next evaluated the role of indoor (home) air and surface allergens and COPD outcomes. Prospective and consecutive home visits were conducted in n=11 stable COPD patients and air samples obtained from indoor (bedroom) and outdoor (balcony) sources in addition to a swab from an air-conditioner or fan surface in the bedroom. All samples were subjected to metagenomic sequencing and allergens identified by aligning the metagenomic reads against World Health Organization/International Union of Immunological Societies allergen nomenclature [26]. A total of 43 allergens from 11 homes were mapped and included fungi, HDM and plant allergens (figure 5a). Most allergens were fungal (n=34, 79%) with particularly high abundances detected on surfaces, and in some homes in the bedroom and balcony air (figure 5a). Abundance of indoor air and surface allergens positively correlated with COPD symptoms (r=0.75, p<0.01) (figure 5b and d) and negatively with lung function (r=−0.61, p<0.01) (figure 5c and e), suggesting that indoor air and surfaces represent a potential source of fungal allergen exposure.

Discussion

Here, we report findings from a large COPD cohort, recruited across three Asian countries and screened against the most comprehensive allergen panel reported in the COPD literature to date. For the first time, using a state-of-art metagenomics approach, we further assess the influence of outdoor and indoor environmental allergen exposure and link this to COPD outcomes. A high frequency of sensitisation to a broad range of allergens occurs in COPD. Fungal sensitisation, in particular associates with frequent exacerbations, and unsupervised clustering reveals a "highly sensitised fungal predominant" patient subgroup demonstrating poorest clinical outcome. Importantly, we observe that the outdoor and indoor (home) environment serves as an important reservoir of fungal allergen exposure translating to sensitisation responses to outdoor air fungi in a subgroup of COPD patients. Indoor (home) environments demonstrating a higher fungal allergen burden associate with greater COPD symptoms and poorer lung function illustrating the importance of environmental exposures on COPD outcomes.

Prior studies assessing the impact of sensitisation in COPD report conflicting outcomes. Studies evaluating Japanese and Brazilian cohorts report that the presence of asthma-like features associate with better COPD outcomes, and that less severe COPD associates with atopic tendencies [4, 27]. However, our work critically excluded all patients with coexisting asthma or ACOS and found no relationship between COPD sensitisation and GOLD group or stage. In fact, larger multicentre studies from Europe and the United States have illustrated that sensitisation in COPD associates with more significant symptoms and higher exacerbation rates [1, 2]. Sensitisation responses and their respective allergen profiles exhibit geographical variation, largely determined by climate, environment, genetics, cultural and social practices and account, at least in part, for the variable reports in the COPD literature [5, 16]. Furthermore, the available

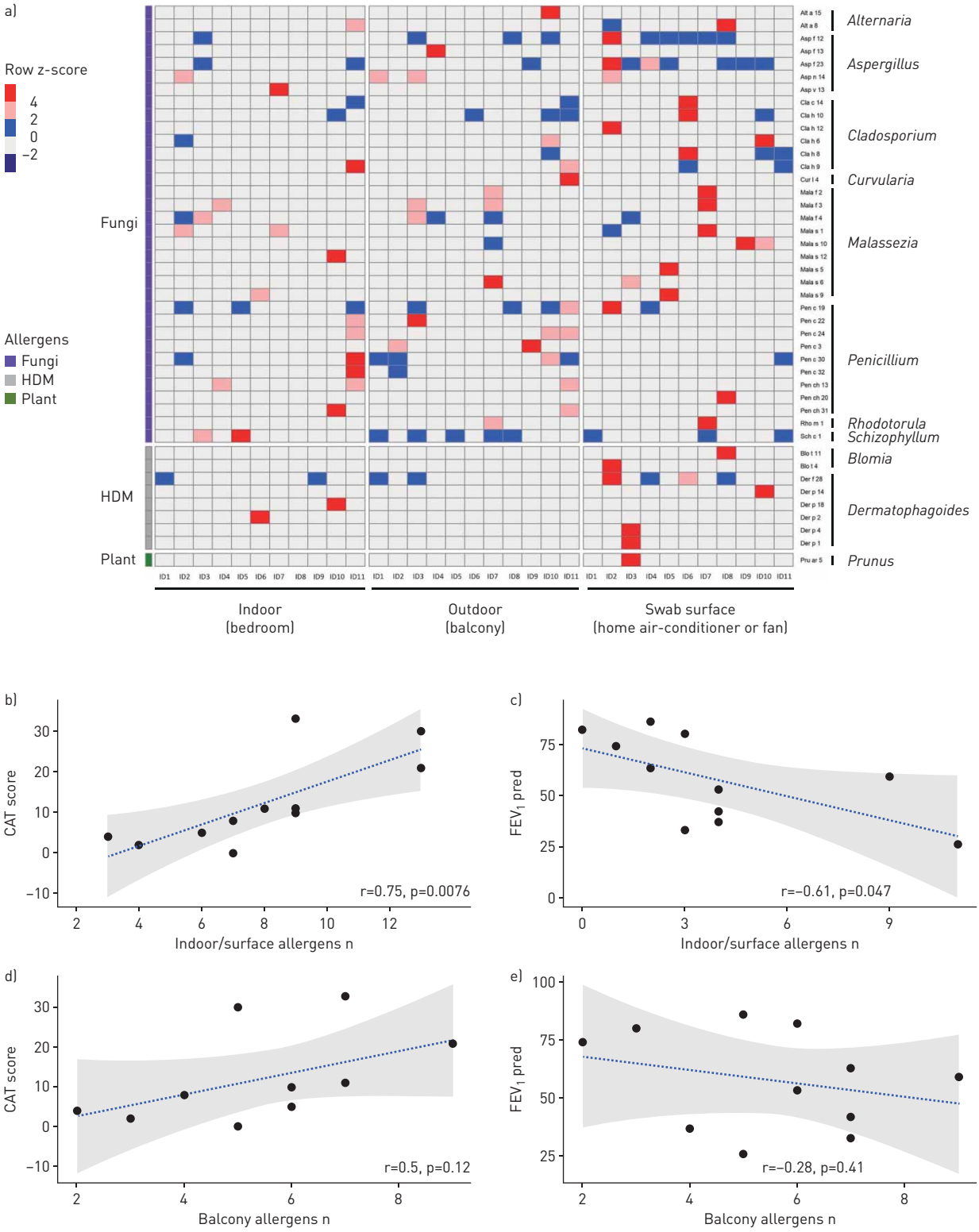

FIGURE 5 High numbers of allergens are detected in the (indoor) home environment of COPD patients and associate with greater symptoms and poorer lung function. a) Heatmap illustrating allergen abundance in n=11 homes (ID1 to ID11; x-axis) of COPD patients using shotgun metagenomic sequencing of indoor (bedroom) air, outdoor (balcony) air and indoor surface swabs (of an air-conditioner or fan) with read alignment to allergens described by World Health Organization/International Union of Immunological Societies allergen nomenclature. Scatterplots illustrating correlations between b) and c) the number of detected indoor/surface allergens (bedroom air and air conditioner/fan surface swabs) and d) and e) number of detected outdoor (balcony air) allergens with b) and d) symptoms (COPD assessment test (CAT) score) and c) and e) lung function (forced expiratory volume in the 1 s (FEV_1) % predicted). Blue dotted lines correspond to Pearson regression and the grey shaded areas represents the 95% confidence interval. HDM: house-dust mite.

outcomes in COPD assess sensitisation as a general entity, and report its presence based on collective responses to all tested allergens within a respective panel rather than provide comparisons between them in relation to clinical outcomes. Our multicentre work is the first in the Asian setting that includes three countries and evaluates COPD sensitisation based on respective individual allergens. COPD sensitisation in Asia has clinical relevance, and frequent exacerbators demonstrate a high occurrence of fungal sensitisation, a potentially treatable trait. Our unsupervised clustering approach further confirms that subgroups of "sensitised COPD" exist with the poorest clinical outcomes demonstrated in a highly-sensitised fungal predominant group.

Fungal sensitisation is an important clinical entity and is increasingly being reported in chronic airways disease [5–7, 17]. In asthma, it represents a poor prognostic indicator and relates to more severe disease and increased exacerbations [6, 28, 29]. Fungal, and in particular *Aspergillus*-associated disease is established in cystic fibrosis and our group has reported high frequencies of fungal sensitisation in bronchiectasis with poor clinical outcome [5, 8, 30, 31]. In COPD, the association of *Aspergillus* sensitisation on clinical outcomes is less clear with conflicting reports on its influence on pulmonary function; however, a clearer relationship does exist when bronchiectasis co-occurs [3, 32–34]. Prior studies in COPD differed in recruited patient groups, sample sizes, and methodologies used, and while we did not find any direct association between *A. fumigatus* sensitisation and lung function in this study, our highly sensitised fungal predominant COPD subgroup demonstrates poor clinical outcomes including lung function, symptoms and exacerbations, suggesting a meaningful clinical relevance for fungal sensitisation in COPD.

The outdoor and indoor environments represent a rich source of fungal exposure, and fungi are ubiquitously present in air [14]. Prior work reports a strong association between outdoor fungal spore concentrations and adverse asthma outcomes including exacerbations, symptoms, inhaler use and poor peak flow readings, and a heightened fungal exposure during *Alternaria* season associates with life-threatening asthma in sensitised individuals [35–37]. However, no study to date assesses the association between outdoor air fungi and sensitisation responses in COPD. Using a novel metagenomics approach and state-of-the-art air sampling techniques, we evaluated a fungal allergen panel incorporating confirmed fungi from outdoor air metagenomics and assessed sensitisation responses to these fungi from COPD patients living in the same region [14]. Like asthma, we found direct associations between sensitisation to air fungi and the occurrence of COPD exacerbations, illustrating for the first time a direct link between outdoor air environmental exposures and clinical outcomes in COPD. Therefore, fungi in the outdoor air potentially have a role in precipitating exacerbations, particularly in sensitised COPD patients. The indoor (home) environment lacks study in COPD, and, importantly, contributes to fungal exposure in an Asian setting due to climate, humidity, use of air-conditioning and air-exchange with outdoor environments [17]. Increased indoor fungal exposures in asthma, including *Aspergillus*, *Alternaria*, *Cladosporium* and *Penicillium* associate with significant symptoms and exacerbations [38]. Interestingly, our study, through metagenomics, detected comparable allergens in the home environment of COPD patients and higher allergen burdens correlate with more symptoms and poorer lung function. Allergens identified outdoors and indoors were noticeably analogous, despite sampling in separate independent experiments, further supporting the concept of micro-organism interchange between the two environments. Critically, fungal composition differed between individual homes, probably explained by dissimilar built and surrounding environments, individual resident behaviour and living activity. Nevertheless, the indoor (home) environment represents an important source of allergen exposure, and, while demonstrating individual variation, does represent a modifiable risk factor in sensitised COPD patients. Air quality and air pollution is a critical global issue of relevance in South-East Asia. Its potential role in sensitisation responses has been previously investigated in asthma; however, its association with fungal sensitisation and more specifically COPD remains unclear and should be subject of future studies.

Our study demonstrates clear strengths and novelty: it evaluates COPD sensitisation in a large multicentre Asian population using a comprehensive range of allergens including assessment of outdoor and indoor environments using metagenomics. However, it does have limitations. As assessments were cross-sectional, the stability of the COPD sensitisation response over time and measurement of longitudinal outcomes was not assessed. These are important in the context of climate change; however, seasonal variation is limited in South East Asia. Importantly, it remains unclear from our work whether the increased exacerbations associated with sensitisation is a consequence of the inflammatory milieu observed following recurrent exacerbations themselves requiring further study. While all patients underwent chest radiography, chest computed tomography was not available in all patients, and therefore a full assessment for bronchiectasis (even minor) could not be confirmed. While we made significant efforts to rule out coexisting asthma, the lack of consensus on the definition of ACOS means that some patients with asthma-like features may have been included inadvertently. Metagenomics analysis of the outdoor air was only performed in Singapore,

and therefore no definitive assessment is available for patients outside Singapore. Our preliminary work in Malaysia does suggest comparable microbial air patterns. The increased sIgE detected to outdoor air fungi does not account for duration of fungal exposure, time of day, nor fungal quantity, as these are dynamic measures out of the scope of this study. Our sIgE assays, performed by immunodot-blot, while validated against ImmunoCAP for available allergens (data not shown) could not be validated for our more customised panels, for example, against outdoor fungal air allergens (for which ImmunoCAP assays are not available). Our indoor (home) environment sampling was performed in only a small number of homes, but with significant metagenomic analyses (at least three metagenomes per home: balcony air, bedroom air and a surface swab). Further validation of our findings in non-Asian populations would provide broader clinical relevance, and future work should include longitudinal assessments that incorporate air fungi from regions outside Singapore. In addition, characteristic of cohorts in the Asian setting, the majority of subjects in our studied cohort were male (>90%); however, it remains unclear whether this is due to lower rates of female smokers in the region or differing susceptibility to the development of COPD in Asian females [39].

In summary, we illustrate that sensitisation, particularly fungal, is prevalent in COPD, associates with frequent exacerbations, and represents a potential treatable trait in Asian patients, addressing a key knowledge gap. Outdoor and indoor environments represent a key source of allergen exposure in COPD, which is amenable to precision intervention approaches to prevent adverse clinical outcomes in "sensitised" COPD.

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