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ENVIRONMENTAL FUNGAL SENSITISATION ASSOCIATES WITH POORER CLINICAL OUTCOMES IN COPD

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Take home message: Fungal sensitization 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 'sensitized' COPD patients.

Abstract

Introduction: Allergic sensitization 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 non-diseased 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 assess outdoor and indoor environmental allergen exposure in COPD. We identify key fungi in outdoor air and develop specific-IgE assays against the top culturable fungi, linking sensitization responses to COPD outcomes. Indoor air and surface allergens were prospectively evaluated by metagenomics in the homes of n=11 COPD patients and linked to clinical outcome. **Results**: High frequencies of sensitization to a broad range of allergens occurs in COPD. Fungal sensitization associates with frequent exacerbations, and, unsupervised clustering reveals a 'highly sensitized fungal predominant' sub-group 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 sensitization 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 sensitization 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 'sensitized' COPD.

Keywords: COPD, sensitization, fungi, *Aspergillus*, environment

Introduction

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

Rapid global urbanization, climate change and 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]. Geographic variation is important with high prevalence of sensitization 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 sub-continent further predispose to high allergen exposures and mould sensitization, which, in turn is associated with poorer outcomes in patients with chronic respiratory disease [5, 6, 14]. The natural and built environments, including climatic factors, further influence the form, variety, type and burden of allergens patients are exposed to, and, no prior studies have evaluated sensitization in relation to environmental allergen exposure in COPD [14-17].

Current studies that assess sensitization in COPD report inconsistent association with clinical outcomes, however, the studied populations were heterogenous, geographically diverse and some included ACOS. Differing methods of assessing sensitization 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 sensitization 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 old with stable COPD attending respiratory outpatient clinics for routine follow up were recruited over a four-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 GINA guidelines), those on long term oral steroids or any immunosuppressive agents were excluded [19]. Stable COPD was defined as the absence of an exacerbation six weeks prior to recruitment. A frequent COPD exacerbator was defined as having >2 exacerbations in the year preceding study recruitment, and, with 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 had a blood draw and full blood count (including eosinophil count) performed. *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 (NTU), Singapore and the University Tunku Abdul Rahman (UTAR) Kampar, Malaysia as controls and divided into subjects

≤40 or >40 years old for analysis. Patient demographics and clinical characteristics of all recruited cohorts are summarized in Table 1.

Allergen panel: The allergen panel selected for study comprised of common allergens implicated in airways disease in Asia and include *Dermatophagoides farinae, Dermatophagoides pteronyssinus, Blomia tropicalis, Elaeis guineensis,* Panicoids (Johnson grass (*Sorghum halepense)*), Pooids (Timothy grass (*Phleum pratense*), Meadow fescue (*Festuca pratesis)*, 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.inequalis), 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 also included for study as described in the online supplement [13, 14, 21] (Figure E1).

Outdoor Air sampling: Outdoor air samples were collected on the rooftop of a academic building at Nanyang Technological University, Singapore, 20m above the ground for 5 consecutive days using methods previously described [14]. Briefly, air was collected using filter-based air samplers SASS3100 (Research International, USA) mounted with SASS bioaerosol electret filters (Research International). Air sampling was performed at 300 L/min airflow rate at a height of 1.5 metres above the floor at the rooftop balcony. The SASS filter was transferred to the lab for immediate processing or stored at -20ºC 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 0.02µm Anodisc filters (Whatman) and DNA extracted using the Dneasy PowerWater Kit (Qiagen) as per manufacturer's instructions. The extracted DNA including an extraction blank and sterile filter were sequenced using the HiSeq 2500 Illumina platform (Illumina, USA). All sequence data from the outdoor air study has been uploaded to the National Center for Biotechnology Information (NCBI)

Sequence read archives (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 (a) indoor (bedroom) air sampling (b) outdoor (balcony) air sampling and (c) surface swab obtained from either an air-conditioning filter or fan from the patient's bedroom. Air samples were concurrently obtained from the patient's bedroom and balcony using filter-based air samplers SASS3100 (Research International, USA) with a flow rate of 100 L/min for 8 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, 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, USA) and library preparation including sequencing methodologies were performed using published protocols [14]. All sequence data from the indoor air study has been uploaded to the National Center for Biotechnology Information (NCBI) Sequence read archives (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 (LCA) algorithm with a minimum score of 100 and support of ≥25 [23].

Ethics approval: This study was approved by the Institutional Review Boards (IRBs) 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 visualization: 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 ±standard deviation (SD) (for normally distributed data). For non-normal data, Kruskal-Wallis or Mann-Witney U test 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 (FDR) using Benjamin Hochberg corrections. Significance is defined as $p \leq 0.05$. Logistic regression corrected for age, gender, 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 visualized 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]. Sensitization was defined as a specific-IgE (sIgE) binding intensity above the 95^{th} percentile of that measured from the non-diseased control cohort. Air-fungi sensitized was defined an individual illustrating sensitization to one or more of the 8 air fungi assessed.

Cluster analysis: Patient demographics, specific-IgE (sIgE) and treatment was employed for unsupervised clustering. Both continuous and categorical variables were converted to Gower dissimilarity matrices using the *daisy* function from the R "cluster" package. Non-metric 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 over 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, specific-IgE assays, fungal identification and indoor allergen mapping are provided in the supplementary material.

Results

Patients with COPD exhibit a high frequency of sensitization across a broad range of allergens: Specific-IgE (sIgE) levels across a broad range of allergens (pollens, house-dust mite, cockroach and fungi) were increased in COPD (n=446) compared to non-diseased (healthy) controls (n=51) with house dust mites (HDM) (Blo t, Der p and Der f) and grass pollens (Pooids and Panicoids) illustrating the highest median sIgE-binding intensities (Figure 1). Where sensitization is classified as positive toward a particular allergen (i.e. sIgE-binding intensity>95th percentile of non-diseased controls), a significant number of COPD patients demonstrate sensitization to fungi (n=249, 55.8%) and HDM (n=229, 51.3%).

Fungal sensitization is associated with frequent exacerbations: Having identified high sensitization 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, *Blattella germanica* (Figure E2) but no significant differences to pollens, HDM or the cockroach allergen, *Periplaneta americana* (Figure E2). In view of the importance of fungal sensitization 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 2c), multivariate analysis (adjusted for age, gender, body mass index 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,

however, no association between sensitization status and COPD GOLD stage (lung function) or GOLD group (ABCD) was detected against any of the assessed allergens (Figure E3 and E4).

Unsupervised clustering reveals three COPD 'sensitization' clusters demonstrating variable clinical outcome: To assess for groups of 'sensitized' 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 detect three highly stable clusters (by Jaccard similarities index) differentiated by their degree of sensitization and sIgE-binding pattern (Table E2) as follows: high-sensitization (HS) fungal predominant (n=115), low-sensitization (LS) (n=114) and moderate-sensitization (MS) *Blomia* predominant (n=217) (Figure 3a and 3b). The HS fungal predominant cluster demonstrates the worst clinical outcome between clusters with greatest symptoms (median CAT score 16, IQR 10-22; $p<0.01$), poorest lung function (median FEV_1% predicted: 41.1, IQR 32.5-57.0; $p<0.01$) and increased exacerbation rate (IRR: 2.01, 95% CI: 1.44-2.81, $p<0.001$), the latter using MS *Blomia* predominant as reference (Figure 3c-e). Importantly, however, no differences in blood eosinophil counts were detected between clusters, although some patients across all clusters demonstrate elevated total eosinophil counts (Figure E5).

Sensitization to outdoor air fungi is associated with COPD exacerbations: As COPD exacerbations and poorer clinical outcome is associated with fungal sensitization, we next assessed if the outdoor environment represents a key source of fungal exposure and the related sensitization 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 sensitization response to these particular fungi in participants recruited from Singapore (COPD: n=82 and non-disease 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 non-diseased 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 sensitized, n=61) to those non-sensitized (n=21). While no relationship was observed for COPD symptoms (CAT score), lung function (FEV_1% predicted), eosinophil count and total IgE (Figure E6), a significantly increased number of exacerbations was detected in the air-fungi sensitized COPD group (Figure 4c) which importantly remains significant after adjustment for age, gender, 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 sensitization in COPD: Having detected significant associations between sensitization 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 WHO/IUIS allergen nomenclature [26]. A total of 43 allergens from 11 homes were mapped and included fungi, house-dust mites and plant allergens (Figure 5a). Most allergens were fungal (n=34; 79%) with particularly high abundances detected on surfaces, and, in some homes 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 5d) and negatively with lung function ($r=-0.61$, $p<0.01$) (Figure 5c and 5e) 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 sensitization to a broad range of allergens occurs in COPD. Fungal sensitization, in particular; associates with frequent exacerbations, and, unsupervised clustering reveals a 'highly sensitized fungal predominant' patient sub-group 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 sensitization responses to outdoor air fungi in a sub-group 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 sensitization 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]. Our work, however, critically excluded all patients with co-existing asthma or ACOS and found no relationship between COPD sensitization and GOLD group or stage. In fact, larger multicentre studies from Europe and the US have illustrated that sensitization in COPD associates with more significant symptoms and higher exacerbation rates [1, 2]. Sensitization responses and their respective allergen profiles exhibit geographic variation, largely determined by climate, environment, genetics, cultural and social practices, and, at least in part account for the variable reports in the COPD literature [5, 16]. Furthermore, the available outcomes in COPD assess sensitization 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 sensitization based on respective individual allergens. COPD sensitization in Asia has clinical relevance, and, frequent exacerbators demonstrate a high occurrence of fungal sensitization, a potentially treatable trait. Our unsupervised clustering approach further confirms that sub-groups of 'sensitized COPD' exist with the poorest clinical outcomes demonstrated in a 'highly-sensitized fungal predominant' group.

Fungal sensitization 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 CF and recently, our group has reported high frequencies of fungal sensitization in bronchiectasis with poor clinical outcome [5, 30-32]. In COPD, however, the association of *Aspergillus*-sensitization on clinical outcomes are less clear with conflicting reports on its influence on pulmonary function, however, a clearer relationship does exist when bronchiectasis co-occurs [3, 33-35]. 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* sensitization and lung function in this study, our 'highly-sensitized fungal predominant' COPD sub-group demonstrates poor clinical outcomes including lung function, symptoms and exacerbations suggesting a meaningful clinical relevance for fungal sensitization 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 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 sensitized individuals [36-38]. No study to date, however, assesses the association between outdoor air-fungi and sensitization 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 sensitization responses to these fungi from COPD patients living in the same region [14]. Like asthma, we found direct associations between sensitization 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. Fungi in the outdoor air therefore potentially have a role in precipitating exacerbations particularly in 'sensitized 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 [39]. 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, although sampled in separate independent experiments were noticeably analogous, further supporting the concept of microorganism interchange between the two environments. Critically, however, fungal composition differed between individual homes, likely 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 sensitized COPD patients. Air quality and air pollution is a critical global issue of relevance in South-East Asia. Its potential role in sensitization responses has been previously investigated in asthma, however its association with fungal sensitization and more specifically COPD remains unclear and should be subject of future studies.

Our study demonstrates clear strengths and novelty: it evaluates COPD sensitization in a large multicentre Asian population using a comprehensive range of allergens including assessment of outdoor and indoor environments using metagenomics, however, does have limitations. As assessments were cross-sectional, the stability of the COPD sensitization response over time, and, measurement of longitudinal outcomes was not assessed, and, 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 sensitization is a consequence of the inflammatory milieu observed following recurrent exacerbations themselves requiring further study. While all patients had chest radiography (CXR) performed, chest computed tomography (CT) was not available in all patients and therefore a full assessment for bronchiectasis (even minor) could not be confirmed. While we made significant effort to rule out co-existing asthma, the lack of consensus on the definition of ACOS means that some patients with asthma-like features may have

inadvertently been included. Metagenomics analysis of the outdoor air was only performed in Singapore, and, therefore no definitive assessment available for patients outside Singapore, however, 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 this study's scope. Our sIgE assays, performed by immuno-dot 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 unavailable). Our indoor (home) environment sampling was performed in only a small number of homes but with significant metagenomic analyses (at least 3 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 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 [40].

In summary, we illustrate that sensitization, 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 'sensitized' COPD.

Author contributions: PYT: Study design, patient recruitment and performance of experimental work, data collection, interpretation and analysis including writing of the final manuscript. FXI, MMA: data interpretation, bioinformatics and statistical analysis. FWSK, MEP, HX, MK, DSCH, JAA, AT: patient recruitment, clinical data and specimen collection. SLP, SAM, SYY, JXKL, TKJ, AU, NEG, DDM: experimental work and data collection. SCS, FTC: Study design and conception of experiments, obtained study funding, interpretation of results and data analysis. SHC: Study design and conception of experiments, obtained study funding, interpretation of results, data analysis and writing of the final manuscript.

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Table 1

Demographic	Non-diseased control (<=40 Years old)	Non-diseased control (>40 Years old)	COPD
n (%)	26	25	446
Age (years), Median (IQR)	22 (20-23)	63 (61-64)	75 (68-80)
Sex (Male), n (%)	10 (38.5)	12 (48.0)	426 (96.2)
BMI (kg/m^2), Median (IQR)	22.0 (21.0-24.0)	24.0 (20.6-26.0)	21.5 (18.7-23.8)
Smoking status, n (%)			
Current	0 (0.0)	4 (16.0)	303 (67.9)
Ex-smoker	1 (3.8)	3 (12.0)	139 (31.2)
Never	25 (96.2)	18 (72.0)	4 (0.9)
Smoking pack years, Median (IQR)	0 (0-0)	44 (33-53)	50 (38-65)
FEV1 % predicted, Median (IQR)	96.5 (85.3-107.8)	93.5 (84.0-97.8)	45.0 (33.6-59.0)
FEV1/FVC (% predicted), Median (IQR)	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, n (%)			
0-1	NA	NA	293 (65.7)
>1	NA	NA	153 (34.3)
Hospitalisation in the year preceding study entry, n (%)			
Yes			208 (46.6)
No			238 (53.4)
COPD assessment test (CAT), Median (IQR)	NA	NA	14 (9-19)
Blood eosinophil count ($x10^9/L$)	NA	NA	0.12 (0.00-0.29)
Total IgE (IU/ml)	NA	NA	24.5 (5.0-119.3)
Treatment, n (%)			
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)

Table 1: Demographic table for the non-diseased and COPD cohorts. Data is presented as the number of patients (n) (with percentage; %) or median (and interquartile range; IQR) as appropriate. COPD: Chronic Obstructive Pulmonary Disease, BMI: body mass index, FEV_1: forced expiratory volume in the 1st second, FVC: forced vital capacity, IQR: interquartile range, NA: not applicable. SAMA: short acting muscarinic antagonist, SABA: short-acting beta agonist, LAMA: long-acting muscarinic antagonist, LABA: long-acting beta agonist, ICS: inhaled corticosteroid.

Figure 1: Patients with chronic obstructive pulmonary disease (COPD) exhibit increased sensitization to a range of (a) pollens, (b) house dust mite, cockroaches and (c) fungal allergens compared to non-diseased (healthy) controls. Systemic specific IgE (sIgE) binding is expressed as $\log_{10}$ OD intensity. Benjamin-Hochberg adjusted p-values are shown and dot coloration indicates the respective patient cohort: non-diseased ≤40 years (blue), non-diseased >40 years (orange) and COPD (red). COPD: chronic obstructive pulmonary disease, HDM: House Dust Mite, ns: not-significant, *p≤0.05, **p≤0.01, ***p≤0.001

Figure 2: Frequent COPD exacerbators illustrate high sensitization rates to fungal (crude) and recombinant *Aspergillus* (rAsp) allergens. Scattered boxplots illustrate systemic specific-IgE binding between non-frequent (Non-FE) and frequent COPD exacerbators (>2 exacerbations/year) against (a) *Curvularia*, *Penicillium* and *Aspergillus* (crude) allergens, (b) major recombinant A*spergillus fumigatus* allergens (rAsp) f 1, f 2 and (c) minor recombinant A*spergillus 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, gender, body mass index and smoking pack years. Systemic specific IgE (sIgE) binding is expressed as $\log_{10}$ OD intensity. Benjamin-Hochberg adjusted p-values are shown and boxplot colouration corresponds to exacerbator status: non-frequent exacerbator (grey), frequent exacerbator (pink). Error bars indicate the 95% confidence interval (CI) and dots represent the odds ratios (ORs) with colour indicating significance level: red ($p<0.05$), grey ($p>0.05$). FE:

frequent exacerbator, rAsp f: recombinant *Aspergillus fumigatus*, Der f: *Dermatophagoides farinae*, Der p: *Dermatophagoides pteronyssinus*, Blo t: *Blomia tropicalis*, Bla g: *Blattella germanica*, CI: confidence interval, ns: non-significant. *p≤0.05.

Figure 3: Unsupervised clustering (based on patient demographics and sIgE profiles against all examined allergens) reveals three clear patient clusters with worst clinical outcome in the "High sensitization (HS) fungal predominant" cluster. (a) Non-metric multi-dimensional 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 (CAT score), (d) lung function (FEV_1% predicted) and (e) exacerbation frequency (in the year preceding recruitment) as a forest plot illustrating the incidence rate ratio for exacerbations using the "moderate sensitized (MS) *Blomia* predominant" cluster as reference. Cluster colouration denotes patient membership: (cluster 1) High sensitized (HS) fungal predominant (turquoise); (cluster 2) Low sensitized (LS) (purple) and (cluster 3) Moderately-sensitized *Blomia* predominant (violet-red). Error bar indicates the 95% confidence interval (CI) and dots each respective patient or the incidence rate ratio for exacerbation respectively. HS: High-sensitization, MS: Moderate-sensitization, LS: Low-sensitization, CI: confidence interval, ns: non-significant. *p≤0.05, **p≤0.01, ***p≤0.001.

Figure 4: Sensitization 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 count with day and night sampling indicated by sun and moon symbols respectively. Bubble colour corresponds to respective fungi: *Trametes* (bluish-green), *Schizophyllum* (orange), *Fusarium* (navy blue), *Aspergillus* (blue), *Penicillium* (dark pink), *Neurospora* (bluish-green), *Byssochlamys* (green) and *Cladosporium* (red) (b) Scattered box plots illustrating specific IgE (sIgE) binding (expressed as $\log_{10}$ OD intensity) to the top eight outdoor air fungi in Singapore between non-diseased and

Singaporean COPD patients. (c) Scattered box plot assessing exacerbation differences between Singaporean COPD patients with and without detectable sensitization to outdoor air fungi and (d) forest plot (indicating incidence rate ratio with 95% CI) for exacerbation risk based on the presence of sensitization to outdoor air fungi. Benjamin-Hochberg adjusted p-values are shown and dot colouration indicates cohort: non-diseased <40 years old (blue), non-diseased >40 years old (orange) and COPD (red), non-sensitized (black), air-fungi sensitized (dark pink). BMI: body mass index, Post BD FEV_1: post bronchodilator forced expiratory volume in the 1st second, CI: confidence interval, ns: non-significant. *p≤0.05, **p≤0.01, ***p≤0.001.

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 WHO/IUIS allergen nomenclature. Highest abundances are indicated in red and side colours bars denote allergen source: fungi (purple), house dust mite (grey) and plants (green) (y-axis). (b) and (c) Scatterplots illustrating correlations between 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 (CAT score) and (c and e) lung function (FEV_1% predicted). Blue dotted lines correspond to Pearson regression and the grey shaded areas represent the 95% confidence interval. CAT: COPD assessment test; FEV_1: forced expiratory volume in the 1st second; HDM: house-dust mite.

Figure 1

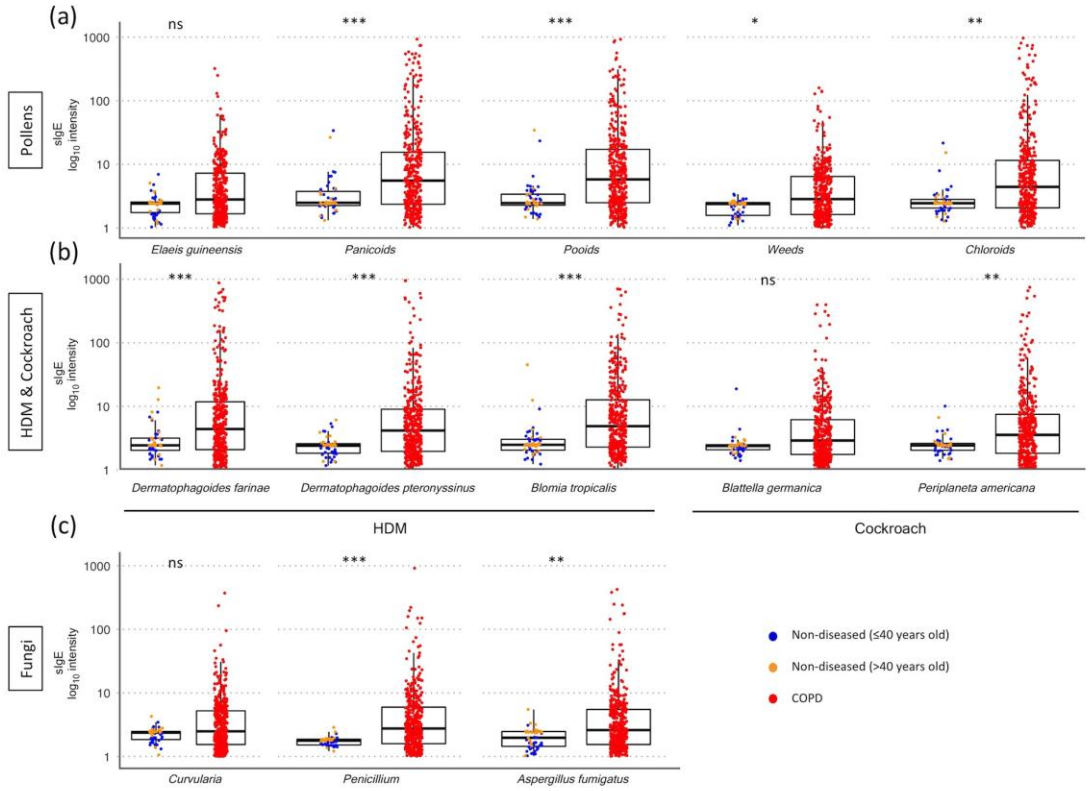

(a)
Pollens
sIgE log10 intensity
ns
***
***
*
**
Elaeis guineensis
Panicoids
Pooids
Weeds
Chloroids
(b)
HDM & Cockroach
***
***
***
ns
**
Dermatophagoides farinae
Dermatophagoides pteronyssinus
Blomia tropicalis
Blattella germanica
Periplaneta americana
HDM
Cockroach
(c)
Fungi
ns
***
**
Curvularia
Penicillium
Aspergillus fumigatus
Non-diseased (≤40 years old)
Non-diseased (>40 years old)
COPD

Figure 2

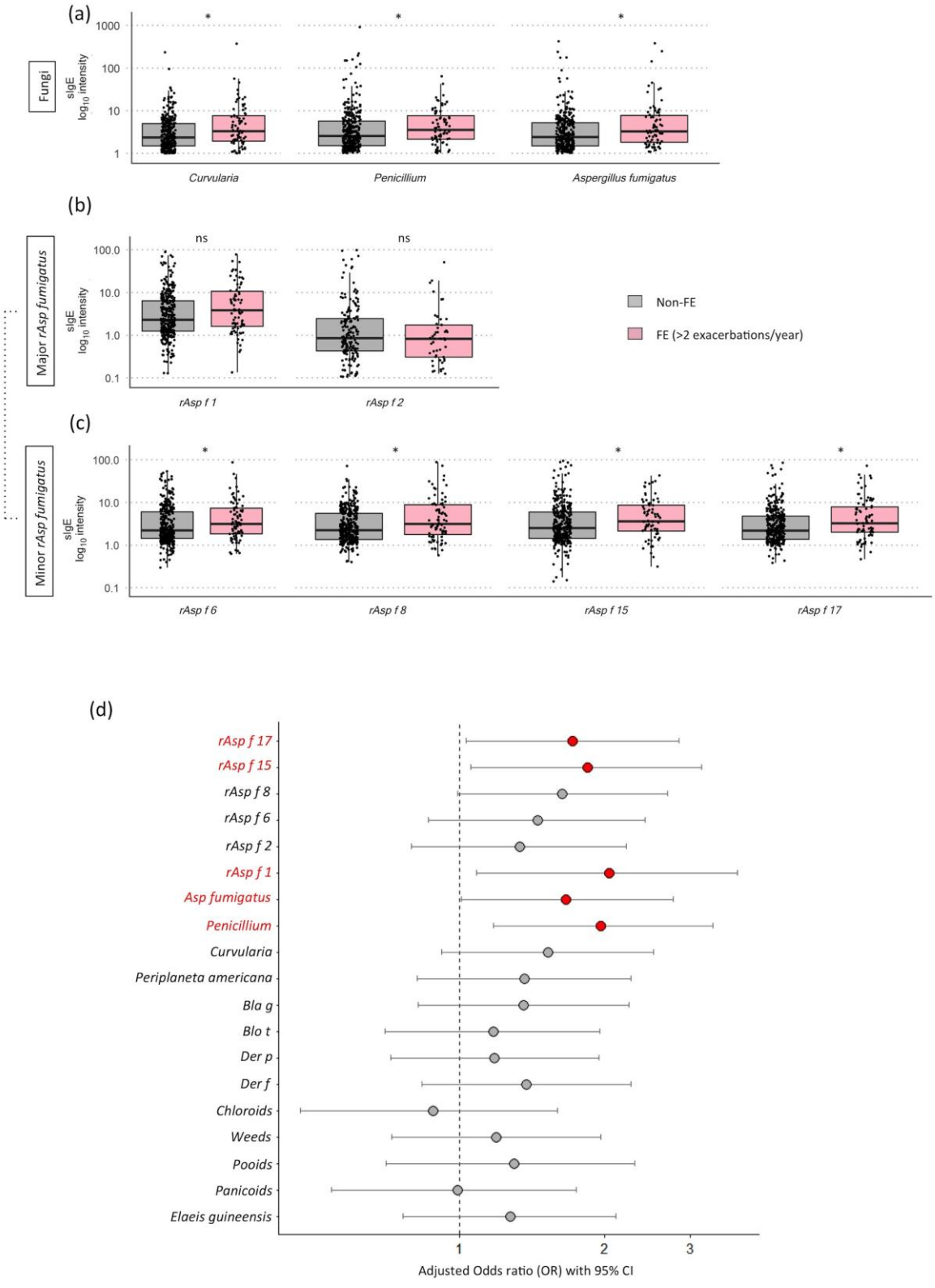

(a)
Fungi
sIgE log10 intensity
1000
100
10
1
*
*
*
Curvularia
Penicillium
Aspergillus fumigatus
(b)
Major rAsp fumigatus
sIgE log10 intensity
100.0
10.0
1.0
0.1
ns
ns
rAsp f 1
rAsp f 2
Non-FE
FE (>2 exacerbations/year)
(c)
Minor rAsp fumigatus
sIgE log10 intensity
100.0
10.0
1.0
0.1
*
*
*
*
rAsp f 6
rAsp f 8
rAsp f 15
rAsp f 17
(d)
rAsp f 17
rAsp f 15
rAsp f 8
rAsp f 6
rAsp f 2
rAsp f 1
Asp fumigatus
Penicillium
Curvularia
Periplaneta americana
Bla g
Blo t
Der p
Der f
Chloroids
Weeds
Pooids
Panicoids
Elaeis guineensis
1
2
3
Adjusted Odds ratio (OR) with 95% CI

Figure 3

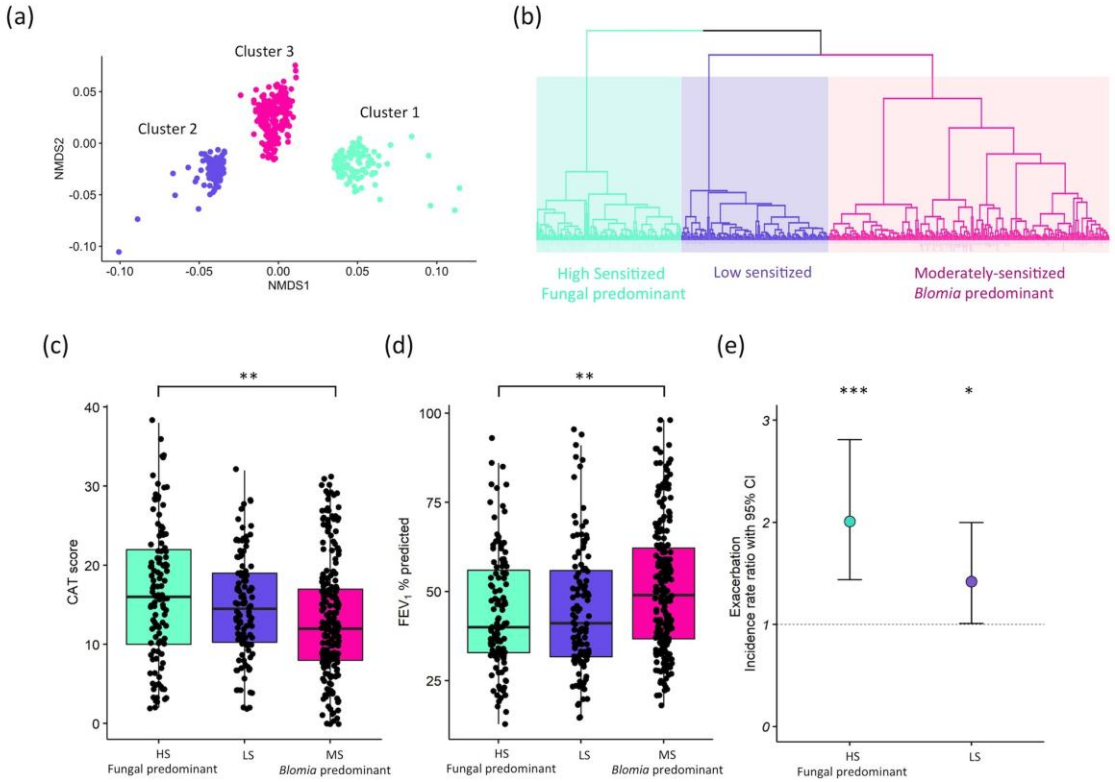

(a)
Cluster 3
Cluster 2
Cluster 1
NMDS2
NMDS1
0.05
0.00
-0.05
-0.10
-0.10
-0.05
0.00
0.05
0.10
(b)
High Sensitized
Fungal predominant
Low sensitized
Moderately-sensitized
Blomia predominant
(c)
CAT score
**
HS
Fungal predominant
LS
MS
Blomia predominant
(d)
FEV1 % predicted
**
HS
Fungal predominant
LS
MS
Blomia predominant
(e)
Exacerbation
Incidence rate ratio with 95% CI
***
*
HS
Fungal predominant
LS

Figure 4

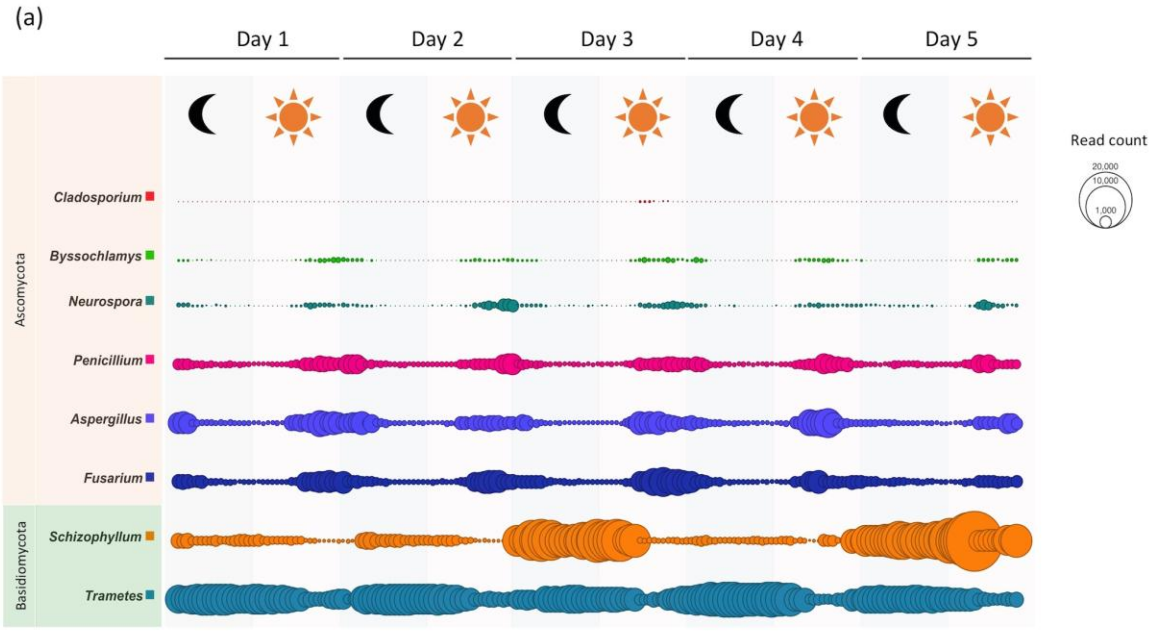


Figure 4

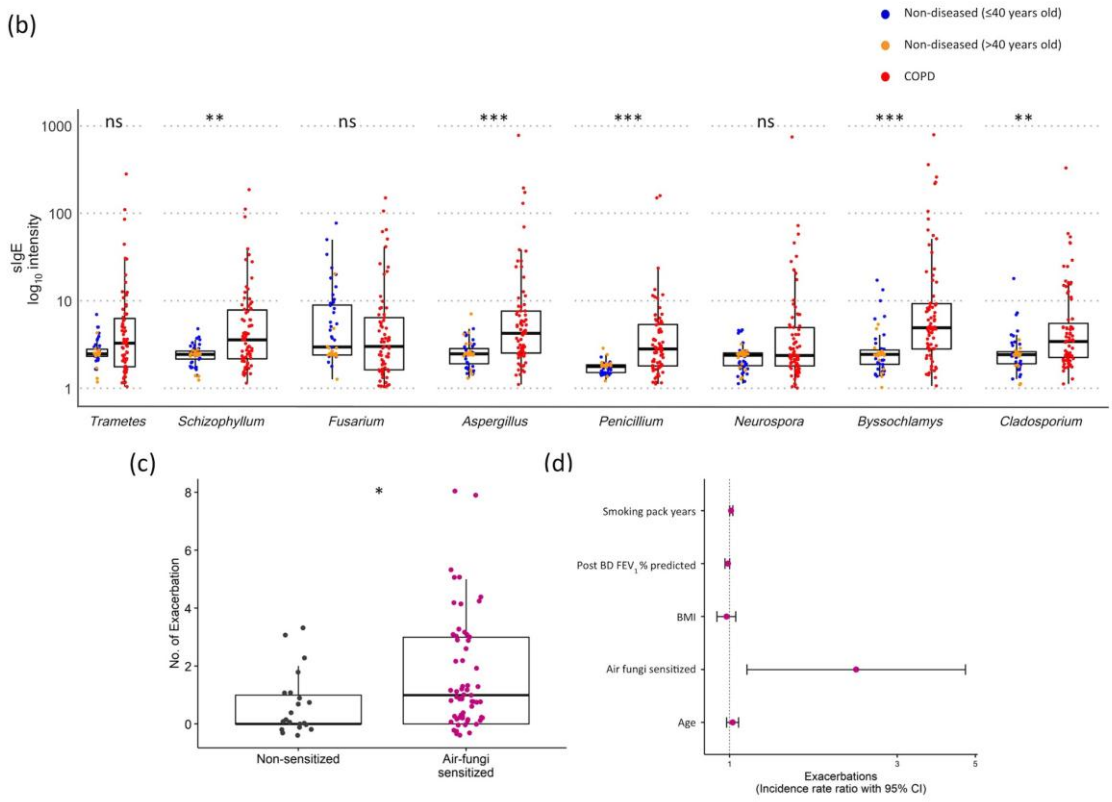
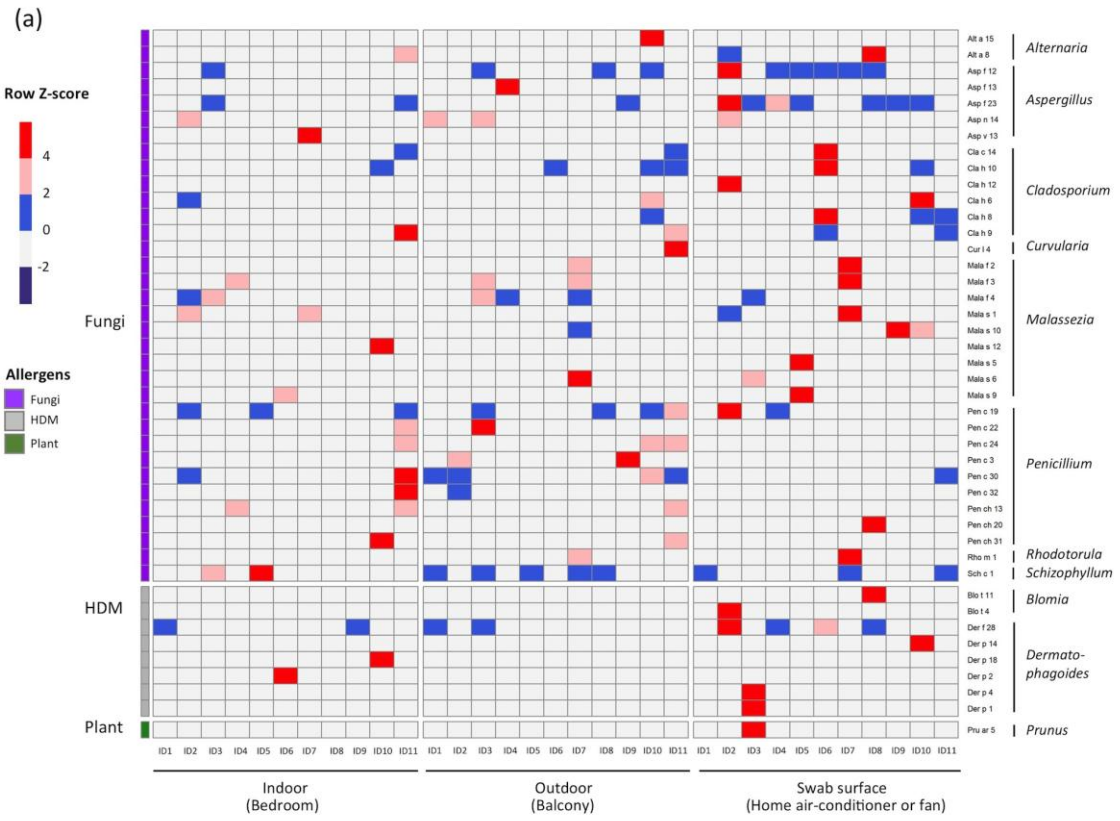
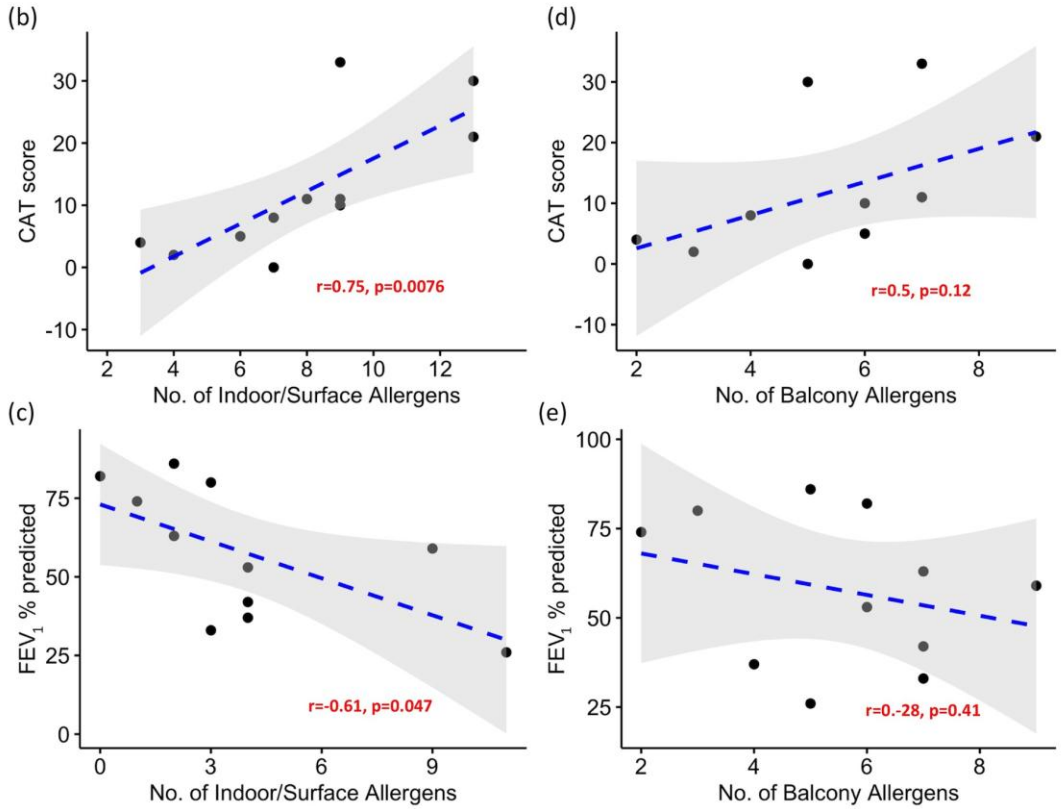


Figure 5


(a)
Row Z-score
4
2
0
-2
Allergens
Fungi
HDM
Plant
Fungi
HDM
Plant
Alt a 15
Alt a 8
Asp f 12
Asp f 13
Asp f 23
Asp n 14
Asp v 13
Cla c 14
Cla h 10
Cla h 12
Cla h 6
Cla h 8
Cla h 9
Cur l 4
Mala f 2
Mala f 3
Mala f 4
Mala s 1
Mala s 10
Mala s 12
Mala s 5
Mala s 6
Mala s 9
Pen c 19
Pen c 22
Pen c 24
Pen c 3
Pen c 30
Pen c 32
Pen ch 13
Pen ch 20
Pen ch 31
Rho m 1
Sch c 1
Blo t 11
Blo t 4
Der f 28
Der p 14
Der p 18
Der p 2
Der p 4
Der p 1
Pru ar 5
Alternaria
Aspergillus
Cladosporium
Curvularia
Malassezia
Penicillium
Rhodotorula
Schizophyllum
Blomia
Dermato-phagoides
Prunus
ID1 ID2 ID3 ID4 ID5 ID6 ID7 ID8 ID9 ID10 ID11
Indoor (Bedroom)
Outdoor (Balcony)
Swab surface (Home air-conditioner or fan)


(b)
CAT score
No. of Indoor/Surface Allergens
r=0.75, p=0.0076
(d)
CAT score
No. of Balcony Allergens
r=0.5, p=0.12
(c)
FEV1 % predicted
No. of Indoor/Surface Allergens
r=-0.61, p=0.047
(e)
FEV1 % predicted
No. of Balcony Allergens
r=0.-28, p=0.41

Online supplement

ENVIRONMENTAL FUNGAL SENSITISATION ASSOCIATES WITH POORER CLINICAL OUTCOMES IN COPD

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SUPPLEMENTARY MATERIALS AND METHODS

Ethics approval: Reference numbers pertaining to ethical approvals at each site was as follows: CIRB 2016/2549 (2013/184/C), CIRB 2016/2715, CIRB 2017/2933, CIRB 2017/2109 (all mutually recognized by DSRB, Singapore), UMMC 2018725-6524 (Malaysia), CREC 2011.146, CREC 2015.164 and CREC 2018.042 (Hong Kong). Non-diseased (healthy) control recruitment was approved by Nanyang Technological University (NTU) and UTAR Institutional Review Boards (IRBs) under IRB-2017-12-010 (Singapore), U/SERC/03/2015 and U/SERC/03/2016 (Malaysia).

Venous blood sampling and processing: Venous blood was collected at recruitment. Blood was centrifuged at 1300g for 10 minutes at 18℃, plasma was isolated and stored in aliquots at -80°C until further processing as described.

Total Immunoglobulin (Ig)-E: Total IgE was measured using the Human IgE ELISA Kit (Abcam ab108650) as per manufacturers instructions. Briefly, twenty microlitres of plasma from each patient (in duplicate) were screened against a set of standards within each microplate.

Crude fungal allergen preparation: Fungi were homogenised with phosphate-buffered saline (PBS) using mortar and pestle. The homogenised fungi were incubated at 4℃ for 4h followed by centrifugation to isolate the supernatant. Glycerol (20%) was added to supernatants and filtered. Protein concentrations were determined by Bradford assays and stored at -20℃ for immunological assessment.

Immuno-dot blot assay for specific-IgE (sIgE) measurements: Immuno-dot blot assays were used to assess for specific-IgE (sIgE) responses to the crude proteins *Dermatophagoides farinae* (Der f),

Dermatophagoides pteronyssinus (Der p), *Blomia tropicalis* (Blo t), *Elaeis guineensis*, Panicoids, Pooids, Chloroids, Weeds, *Blattella germanica* (Bla g), *Periplaneta Americana*, *Curvularia*, *Penicillium, Aspergillus fumigatus* (*A..fumigatus*) including air fungi and *Aspergillus* recombinant proteins as per published methodology by our group and others [1-4]. The recombinant proteins used in this study included: Asp f 1 (M83781), Asp f 2 (U56938), Asp f 4 (AJ001732), Asp f 6 (U53561), Asp f 8 (AJ224333), Asp f 15 (AJ002026) and Asp f 17 (AJ224865). Briefly, each allergen was blotted onto nitrocellulose membranes (in duplicate at a standardized concentration of 0.25mg/allergen) with Bovine Serum Albumin (BSA) used as protein control and PBS included as a negative control. Membranes were blocked with 1X PBS 0.1% Tween-20 for 40 minutes and incubated overnight (16 hours) with diluted plasma (1:8 in PBS) at 4℃. Washing steps were performed with 1X PBS 0.05% Tween-20 for 15 minutes followed by 7-minute washes in replicate. Subsequently, membranes were incubated with anti-human IgE antibodies (1:1000) conjugated with alkaline phosphatase (Sigma Aldrich, USA) for 2h. Nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3'-indolyphosphate (BCIP) solution (Thermo Fisher Scientific) was then added for detection of alkaline phosphatase activity for 10 minutes and data analyzed with Syngene imaging software. Inter and intra assay reproducibility was 90% and 95% respectively and specific Ig-E binding is presented as optical density (OD) intensity.

Isolation and confirmatory identification of outdoor air fungi

Top outdoor air fungi: Based on outdoor air metagenomic profiles as described by Gusareva *et al*, we cultivated the top 8 fungi for further immunological analysis[5]. *Schizophyllum commune* was collected from the National Institute of Education Carpark 3 at Nanyang Technological University, *Trametes sanguinea* from Kent Ridge Park, Singapore. Permission for fungal specimen collection was filed under NParks Permit NP/RP19-078. *Aspergillus spp.* (*A. terreus, A. sydowii*), *Cladosporium spp.* (*C. tenussimum, C. xantochromaticum*), *Fusarium spp.* (*F. solani, F. moniliforme*), *Neurospora spp., Byssochlamys spectabilis* and *Penicillium spp.* were directly isolated from air samples collected at a rooftop balcony at Nanyang Technological University, Singapore using a Spin-Air agar impactor

(IUL S.A., Barcelona, Spain) at a sampling height of 156cm. Air was impacted onto various nutrient media including Malt Extract Agar (MEA) (Sigma-Aldrich, USA), cellulose agar (Sigma-Aldrich, USA), Potato Dextrose Agar (Sigma-Aldrich, USA), Reasoner's 2A Agar (Becton-Dickinson, USA) and Tryptic Soy Agar (Becton-Dickinson, USA) in 90mm Petri dishes at an air flow rate of 100L/min for 1 min following protocols described by Sánchez-Muñoz, Muñoz-Vicente [6]. Impacted agar plates were then incubated at 25℃ for 3-5 days and single fungal colonies selected and re-streaked onto fresh MEA plates. The selection and streaking process was repeated for 3 generations until plate morphology appeared to represent a single fungal isolate and to ensure the isolate species was free from contamination. Each of the fungal species isolated was then confirmed using molecular analysis as described.

Fungal species identification: DNA was extracted from single fungal isolates using the Qiagen PowerWater DNA extraction kit (Qiagen, Germany) and subsequently amplified by polymerase chain reaction (PCR) targeting the ITS-4 and ITS-5 (internal transcribed spacer) rRNA genes. ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS-5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') primers were used and PCR conditions were as follows: 2x KAPA HiFi Hotstart Ready Mix (12.5 µl) (Kapa Biosystems, USA), primers (for both forward and reverse primers each 0.75µl of 10µM), template DNA (1µl of 5 ng/µl) and H_2O (10 µl) for each reaction (total 25 µl). PCR cycles for ITS-4 and ITS-5 were performed as follows: 95℃ for 3 minutes, 35 cycles of 95℃ for 30 seconds, 55℃ for 30 seconds, 72℃ for 30 seconds with a final elongation step at 72ºC for 5 minutes. The PCR products were sent to AITbiotech Singapore for Sanger sequencing and read quality analysed with 4Peaks (Mekentosj, Amsterdam) and Chromas (Technelysium, Australia) where sites below Phred score 20 were filtered and trimmed. Forward and reverse reads were merged using BioEdit v7.2.6.1 [7] and blastn [8] used to query the sequences to confirm species identity. Only once each species was confirmed was the fungal extract used for further immunological analyses.

Indoor Allergen mapping: Data for 977 established allergens were extracted from the Allergen Nomenclature (http://www.allergen.org/) using the R "rvest" package [9]. Nucleotide sequences of the 786 allergens whose GenBank Accession was available were retrieved using the R "ape" package [10]. The raw paired-end Illumina HiSeq sequencing reads with a minimum Phred score of 20 and >30bp length were selected and adapter-trimmed using Cutadapt (version 1.8.1) [11]. The trimmed reads were then mapped against the GRCh38 human reference genome using bowtie2 (version 2.3.3.1) [12]. The unmapped non-human reads were extracted using samtools (version 1.3.1) and bedtools2 (version 2.8.0), and, aligned to the 786 allergens nucleotide sequences using bowtie2 in its local mode [13, 14]. Samtools was next used to calculate the number of alignments with mapping quality >30 (raw hits). The number of raw hits (H) to a specific allergen's nucleotide sequence was then normalized using similar methodology to ShortBRED [15], whereby read length (R), allergen nucleotide sequence length (L), and sequencing depth were adjusted after removal of human reads (N) according to the following formula:

$$C = \frac{H}{\left(\frac{|L-R|}{10^3}\right)\left(\frac{N}{10^6}\right)} = \frac{H}{|L-R| \cdot N} \times 10^9$$

The normalized count (relative abundance) is then presented in units of reads per kilobase of reference sequence per million sample reads (RKPM).

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Table E1

Demographic	
Number of patient homes sampled	11
Age (years), Mean ±SD	69 ± 3
Sex (Male), n (%)	11 (100)
BMI (kg/m^2), Mean ±SD	22.5 ±3.7
Smoking status, n (%)	
Current	5 (45.5)
Ex-smoker	6 (54.5)
Never	0 (0.0)
Smoking pack years, Median (IQR)	50.0 (47.5-65.0)
FEV_1 % predicted, Mean ±SD	57.7±21.2
FEV_1/FVC (% predicted), Mean ±SD	56.7 ±12.5
Hospitalization in the year preceding study, n (%)	
Yes	3 (27)
No	8 (73)
Exacerbation number in the year preceding study, n (%)	
0-1	10 (91)
>1	1 (9)
COPD assessment test (CAT), Mean ±SD	12.3±11.1
Blood Eosinophil ($x10^9/L$)	0.21 (0.09-0.42)
Total IgE (IU/ml)	26.2 (5.0-251.6)
Treatment, n (%)	
LAMA	4 (36)
LAMA/LABA	4 (36)
LAMA/LABA/ICS	3 (28)

Table E2

Allergens	**Total, OD,**	**HS fungal predominant, OD**	**LS, OD**	**MS *Blomia* predominant, OD**	**p-value**
	Median (IQR)	**Median (IQR)**	**Median (IQR)**	**Median (IQR)**	
Elaeis guineensis	2.59 (1.55-6.51)	**3.31 (1.68-9.44)**	2.55 (1.55-7.5)	2.47 (1.48-5.55)	**0.01**
Panicoids	5.20 (2.01-16.92)	7.98 (2.49-22.24)	4.60 (1.86-16.57)	4.76 (1.95-15.52)	ns
Pooids	5.56 (2.35-17.94)	7.49 (3.02-23.24)	4.93 (2.16-15.88)	5.22 (2.26-15.86)	ns
Chloroids	4.09 (1.85-12.78)	5.22 (2.1-18.11)	3.40 (1.66-10.47)	3.75 (1.84-11.77)	ns
Weeds	2.51 (1.48-6.06)	**3.39 (1.77-6.49)**	1.93 (1.31-6.15)	2.35 (1.5-5.66)	**0.04**
Blomia tropicalis	4.88 (2.25-15.6)	5.34 (2.21-16.8)	3.68 (1.86-9.55)	**5.89 (2.67-17.52)**	**0.05**
Dermatophagoides farina	4.34 (1.96-12.71)	4.56 (2.27-12.9)	4.27 (1.65-11.79)	4.17 (1.89-13.32)	ns
Dermatophagoides pteronyssinus	4.20 (1.92-9.53)	4.92 (2.21-11.32)	3.43 (1.66-8.56)	4.23 (1.84-9.62)	ns
Blattella germanica	2.63 (1.57-6.06)	2.91 (1.87-6.93)	2.31 (1.4-6.06)	2.62 (1.57-5.65)	ns
Periplaneta americana	3.37 (1.65-7.56)	3.83 (2.03-8.59)	3.29 (1.48-6.18)	3.24 (1.59-8.44)	ns
Curvularia spp.	2.35 (1.43-5.13)	2.68 (1.63-5.44)	2.05 (1.26-5.77)	2.32 (1.45-4.65)	ns
Penicillium spp.	2.58 (1.48-5.98)	**3.19 (1.82-6.92)**	2.30 (1.33-6.16)	2.51 (1.45-5.53)	**0.05**
Aspergillus fumigatus	2.34 (1.44-5.26)	**3.39 (1.64-6.38)**	2.04 (1.42-5.05)	2.31 (1.44-4.75)	**0.02**
rAsp f 1	2.66 (1.26-7.51)	3.23 (1.15-8.98)	2.90 (1.47-7.81)	2.31 (1.14-6.78)	ns
rAsp f 2	0.13 (0.02-1.02)	0.19 (0.02-1.69)	0.09 (0.03-0.93)	0.12 (0.02-0.86)	ns
rAsp f 6	2.40 (1.48-6.26)	2.87 (1.54-7.33)	2.11 (1.49-6.18)	2.34 (1.4-5.81)	ns
rAsp f 8	2.41 (1.42-6.03)	**3.00 (1.58-7.3)**	2.29 (1.39-5.83)	2.25 (1.31-5.69)	**0.03**
rAsp f 15	2.83 (1.48-6.63)	3.50 (1.59-7.81)	2.56 (1.49-6.04)	2.57 (1.43-6.31)	ns
rAsp f 17	2.44 (1.41-5.71)	**3.10 (1.51-6.88)**	2.11 (1.32-4.82)	2.39 (1.43-5.08)	**0.04**

Table E1: Demographic table of the COPD patient cohort participating in the indoor home air and surface sampling (n=11). Data are presented as number of homes/patients (n) (with percentage; %) or mean ± standard deviation (SD) or median (interquartile range; IQR). BMI: body mass index; FEV_1: forced expiratory volume in the 1st second; FVC: forced vital capacity.

Table E2: Table showing systemic specific IgE (sIgE) binding (expressed as OD intensity) for each tested allergen within the three defined patient clusters. Data are presented as median (interquartile range; IQR) with p-value indicated where significant. OD: optical density.

Figure E1: Overall summary of the allergen panels used in this study. HDM: house-dust mite, Der f: *Dermatophagoides farinae*, Der p: *Dermatophagoides pteronyssinus*, Blo t: *Blomia tropicalis*, Bla g: *Blattella germanica*, and rAsp f: recombinant *Aspergillus fumigatus*.

Figure E2: Sensitization rates to (a) pollens, (b) house dust mite and cockroach allergens (except *Blattella germanica*) does not differ based on frequent exacerbator status in COPD. Systemic specific IgE (sIgE) binding is expressed as $\log_{10}$ OD intensity. Boxplot colouration corresponds to exacerbator status: non-frequent exacerbator (grey) and frequent exacerbator (pink). HDM: house-dust mite, ns: non-significant. *$p<0.05$.

Figure E3: Sensitization rates to (a) pollens, (b) house dust mite, cockroach and (c-e) fungal allergens (crude and recombinant) does not differ based on GOLD (lung function) stage. Systemic specific IgE (sIgE) binding is expressed as $\log_{10}$ OD intensity. Boxplot colouration corresponds to GOLD (lung function; FEV_1) stage: GOLD stage 1 (light grey), GOLD stage 2 (medium grey), GOLD stage 3 (grey), GOLD stage 4 (dark grey). ns: non-significant.

Figure E4: Sensitization rates to (a) pollens, (b) house dust mite, cockroach and (c-e) fungal allergens (crude and recombinant) does not differ based on GOLD group (ABCD). Systemic specific IgE (sIgE) binding is expressed as $\log_{10}$ OD intensity. Boxplot colouration corresponds to GOLD group: GOLD A (light grey), GOLD B (medium grey), GOLD C (grey), GOLD D (dark grey). ns: non-significant.

Figure E5: Scattered box plots illustrating absolute eosinophil counts (expressed as x 10^9/L) between the detected patient clusters. Colour denotes cluster membership: HS fungal predominant (turquoise), LS (blue), MS *Blomia* predominant (violet-red). HS: high-sensitization; LS: low-sensitization; MS: moderate-sensitization; ns: non-significant. Dotted lines indicates standard eosinophil count cut-off (0.45 x 10^9/L) (grey line) and COPD GOLD cut-off (0.30 x 10^9/L) (red line) to indicate elevated total eosinophil count.

Figure E6: Comparison of (a) symptoms (CAT score), (b) lung function (FEV_1 % predicted), (c) total blood eosinophil count (as 10^9/L) and (d) total serum IgE between non-sensitized and air-fungi sensitized Singaporean COPD patients. CAT: COPD assessment test, FEV_1: forced expiratory volume in the 1st second, IgE: immunoglobulin-E, ns: non-significant.

Figure E1

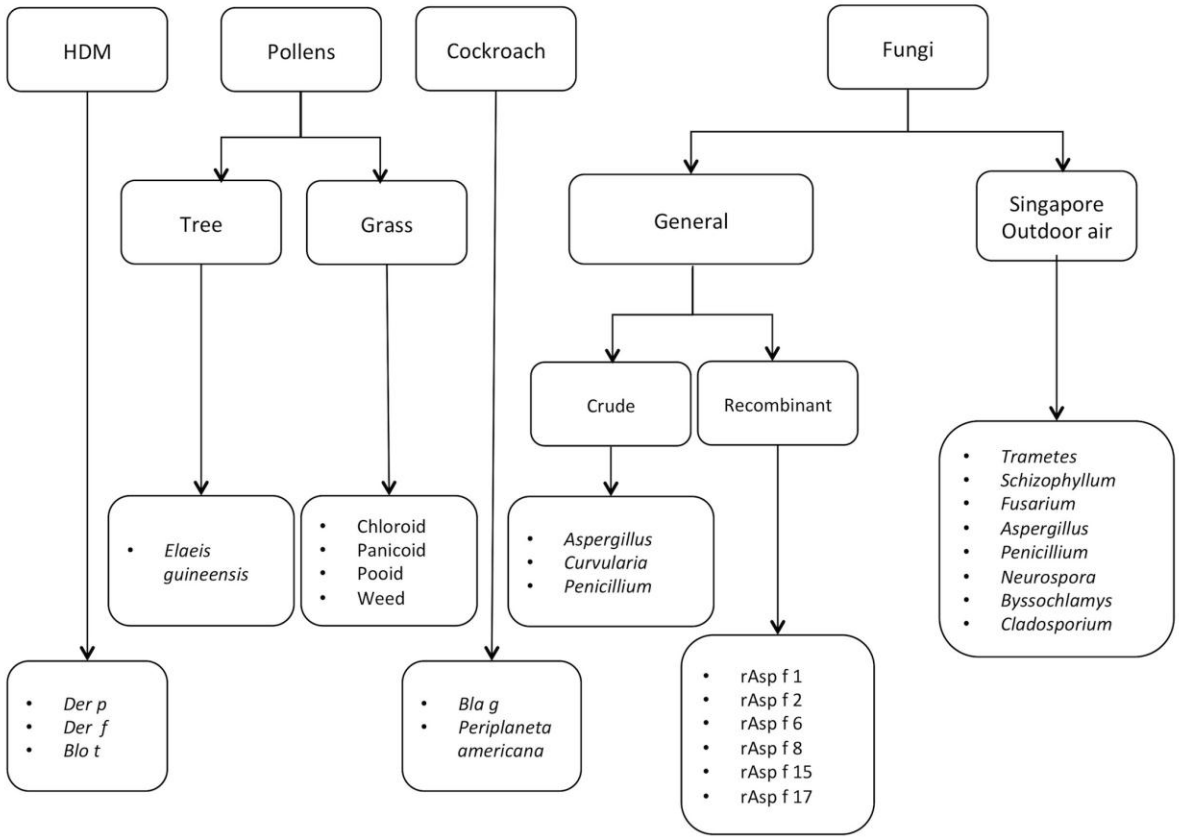

HDM
Pollens
Cockroach
Fungi
Tree
Grass
General
Singapore Outdoor air
Crude
Recombinant
• Elaeis guineensis
• Chloroid
• Panicoid
• Pooid
• Weed
• Aspergillus
• Curvularia
• Penicillium
• Trametes
• Schizophyllum
• Fusarium
• Aspergillus
• Penicillium
• Neurospora
• Byssochlamys
• Cladosporium
• Der p
• Der f
• Blo t
• Bla g
• Periplaneta americana
• rAsp f 1
• rAsp f 2
• rAsp f 6
• rAsp f 8
• rAsp f 15
• rAsp f 17

Figure E2

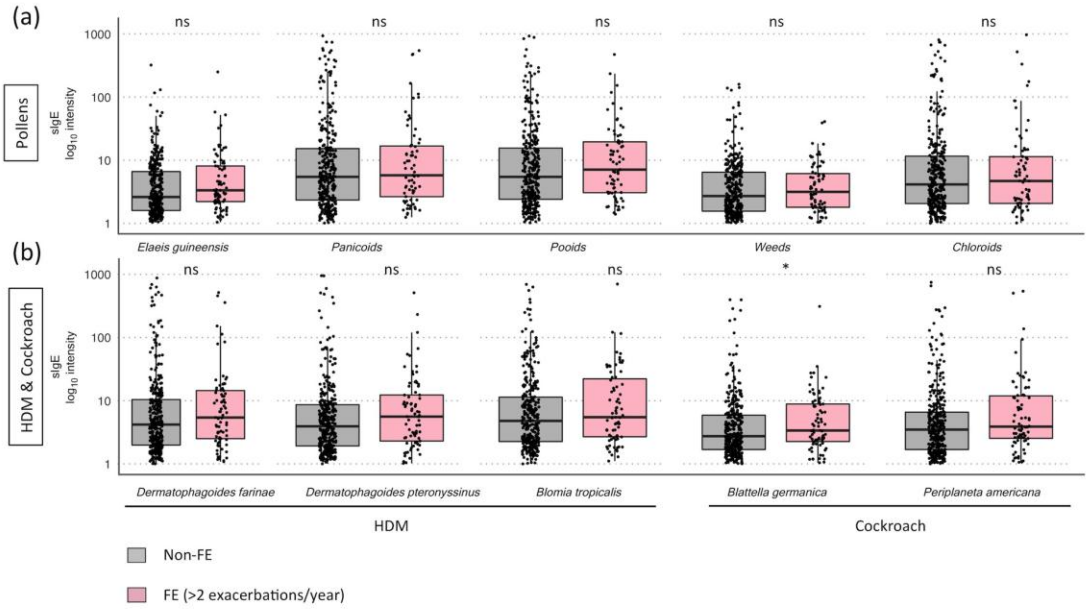
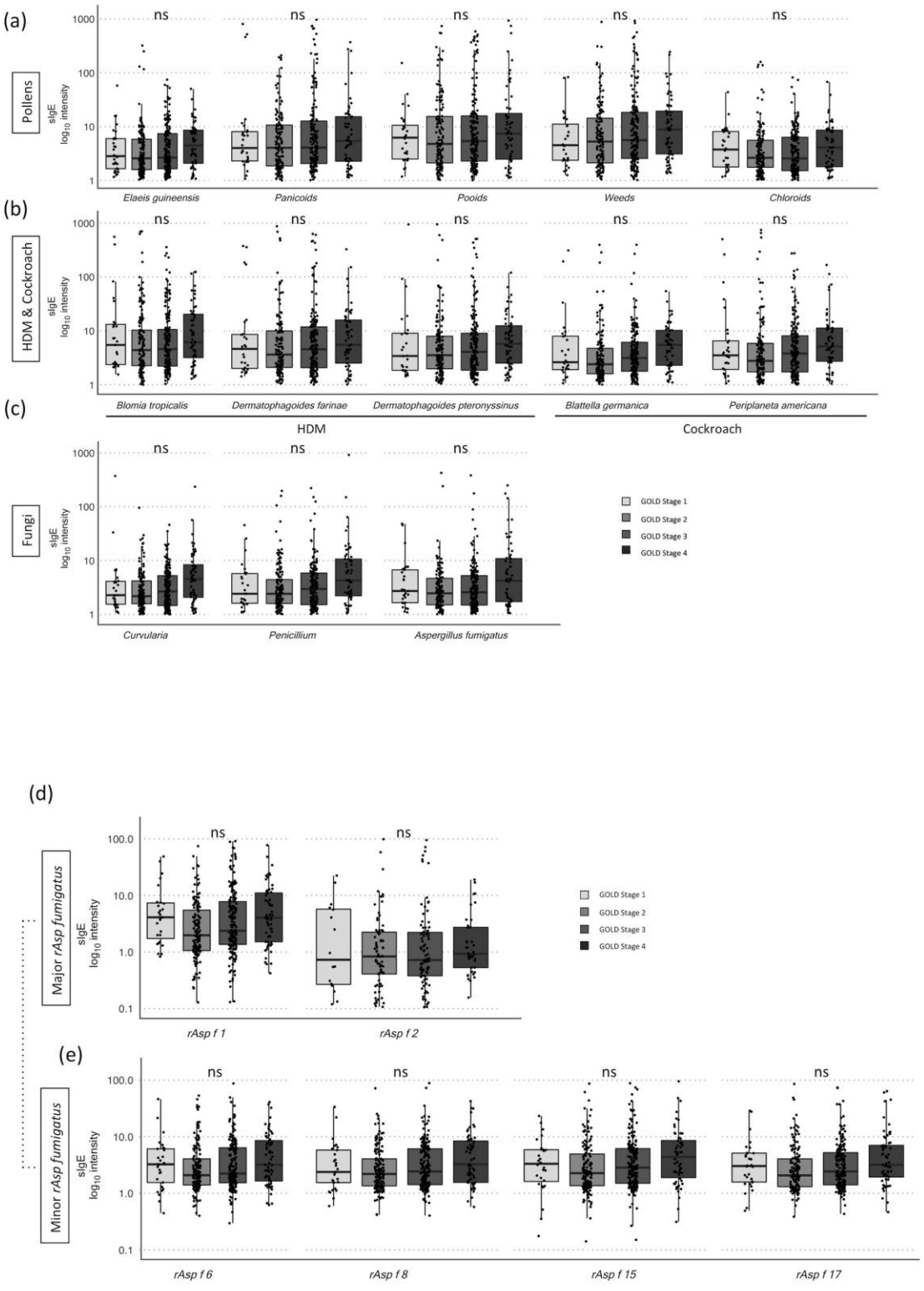

(a)
Pollens
sIgE
log10 intensity
1000
100
10
1
ns
ns
ns
ns
ns
Elaeis guineensis
Panicoids
Pooids
Weeds
Chloroids
(b)
HDM & Cockroach
sIgE
log10 intensity
1000
100
10
1
ns
ns
ns
*
ns
Dermatophagoides farinae
Dermatophagoides pteronyssinus
Blomia tropicalis
Blattella germanica
Periplaneta americana
HDM
Cockroach
Non-FE
FE (>2 exacerbations/year)

Figure E3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Pollens
sIgE log10 intensity
ns
Elaeis guineensis
Panicoids
Pooids
Weeds
Chloroids
(b)
HDM & Cockroach
Blomia tropicalis
Dermatophagoides farinae
Dermatophagoides pteronyssinus
Blattella germanica
Periplaneta americana
HDM
Cockroach
(c)
Fungi
Curvularia
Penicillium
Aspergillus fumigatus
GOLD Stage 1
GOLD Stage 2
GOLD Stage 3
GOLD Stage 4
(d)
Major rAsp fumigatus
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rAsp f 2
(e)
Minor rAsp fumigatus
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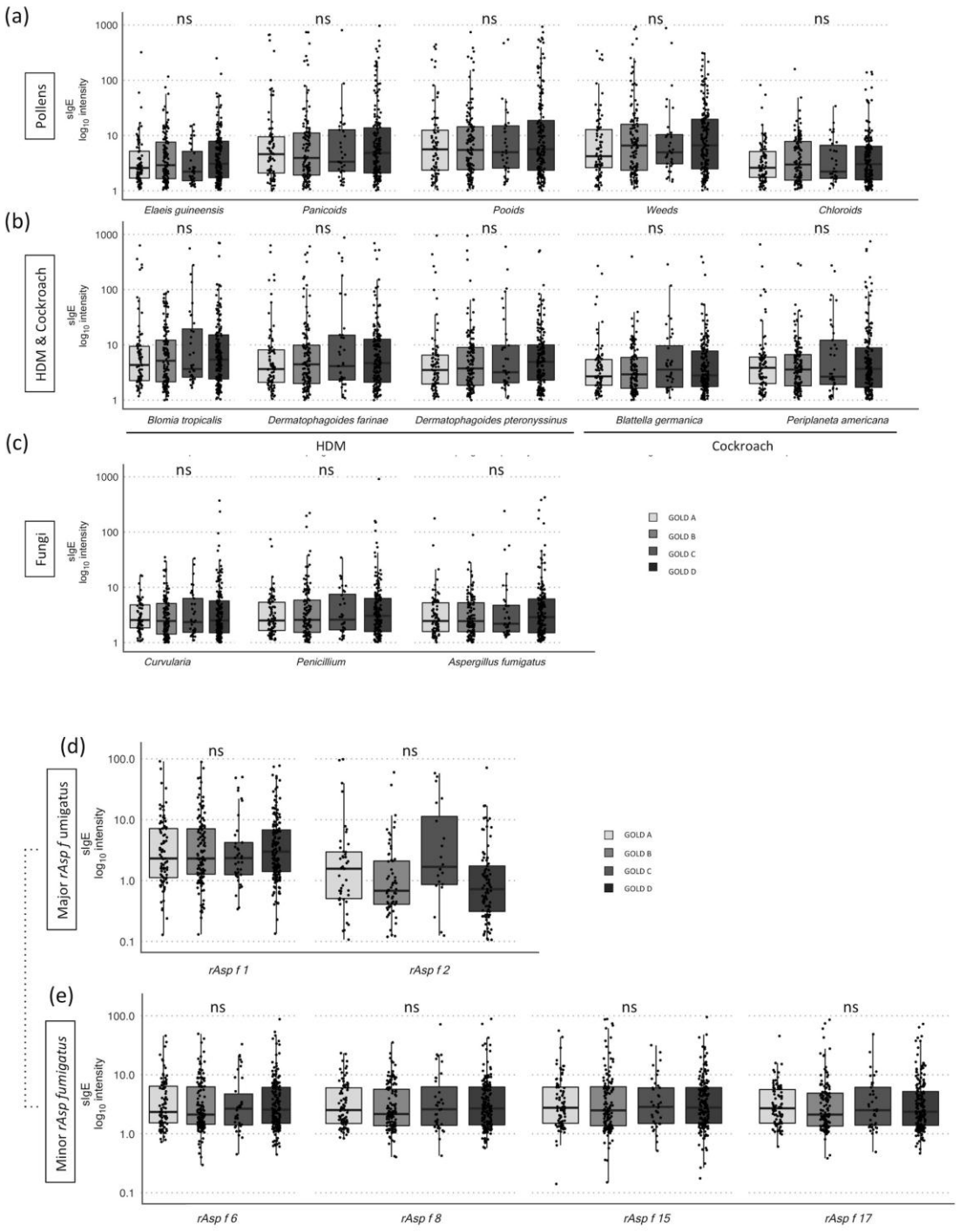
rAsp f 8
rAsp f 15
rAsp f 17

Figure E4


(a)
Pollens
sIgE log10 intensity
ns
Elaeis guineensis
Panicoids
Pooids
Weeds
Chloroids
(b)
HDM & Cockroach
Blomia tropicalis
Dermatophagoides farinae
Dermatophagoides pteronyssinus
Blattella germanica
Periplaneta americana
HDM
Cockroach
(c)
Fungi
Curvularia
Penicillium
Aspergillus fumigatus
GOLD A
GOLD B
GOLD C
GOLD D
(d)
Major rAsp f umigatus
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rAsp f 2
(e)
Minor rAsp fumigatus
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rAsp f 8
rAsp f 15
rAsp f 17

Figure E5

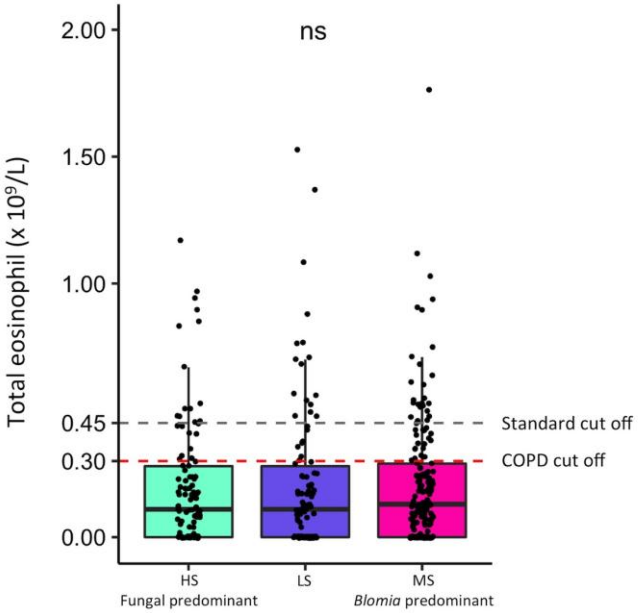
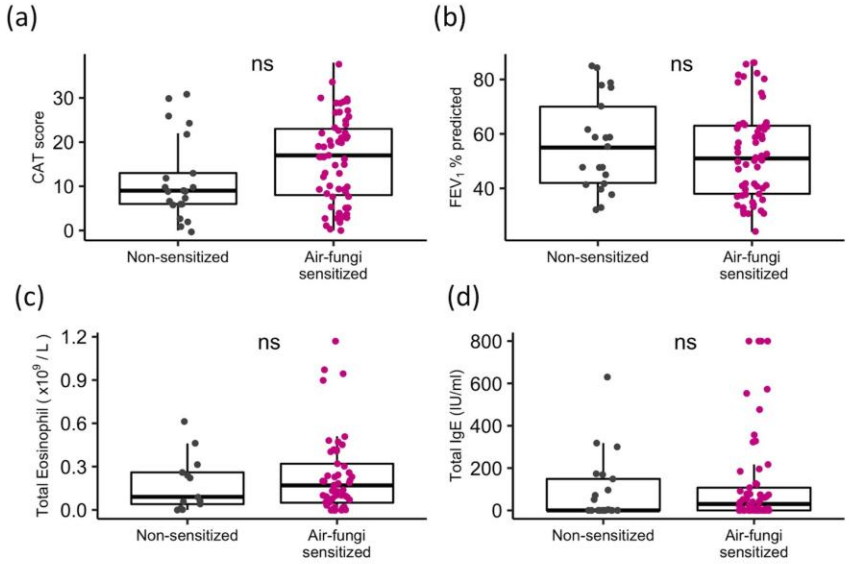

ns
2.00
1.50
1.00
0.45
0.30
0.00
Total eosinophil (x 10⁹/L)
Standard cut off
COPD cut off
HS
Fungal predominant
LS
MS
Blomia predominant

Figure E6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CAT score
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20
10
0
Non-sensitized
Air-fungi sensitized
(b)
ns
FEV1 % predicted
80
60
40
Non-sensitized
Air-fungi sensitized
(c)
ns
Total Eosinophil (x10^9 / L)
1.2
0.9
0.6
0.3
0.0
Non-sensitized
Air-fungi sensitized
(d)
ns
Total IgE (IU/ml)
800
600
400
200
0
Non-sensitized
Air-fungi sensitized