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Sensitisation to recombinant *Aspergillus fumigatus* allergens and clinical outcomes in COPD

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Take home message: Sensitisation to recombinant *Aspergillus fumigatus* allergens rAsp f1, f3, f5 and f6 in COPD identifies a patient group with poor clinical outcomes missed by assessing for sensitisation to crude *Aspergillus fumigatus* allergens alone.

Abstract

Background: Variable clinical outcomes are reported with fungal sensitisation in COPD, and it remains unclear which fungi and what allergens associate with poorest outcomes. The use of recombinant as opposed to crude allergens for such assessment is unknown. **Methods:** A prospective multicenter assessment of stable COPD (n=614) was undertaken in five hospitals across three countries: Singapore, Malaysia, and Hong Kong. Clinical and serological assessment was performed against a panel of 35 fungal allergens including crude and recombinant *Aspergillus* and non-*Aspergillus* allergens. Unsupervised clustering and Topological Data Analysis (TDA) approaches were employed using the measured sensitisation responses to elucidate if sensitisation sub-groups exist and their related clinical outcomes. **Results**: *Aspergillus fumigatus* sensitisation associates with increased exacerbations in COPD. Unsupervised cluster analyses reveal two 'fungal sensitisation' groups, one characterized by *Aspergillus* sensitisation and increased exacerbations, poorer lung function and worse prognosis. Polysensitisation in this group confers even poorer outcome. The second group, characterized by *Cladosporium* sensitisation is more symptomatic. Significant numbers of individuals demonstrate sensitisation responses to only recombinant (as opposed to crude) *Aspergillus fumigatus* allergens 1, 3, 5, and 6, and exhibit higher exacerbations, poorer lung function and an overall worse prognosis. TDA validated these findings and additionally identified a sub-group within '*Aspergillus* sensitised COPD' enriched for frequent exacerbators. **Conclusion**: *Aspergillus* sensitisation is a treatable trait in COPD. Measuring sensitisation responses to recombinant *Aspergillus* allergens identifies an important patient subgroup with poor COPD outcomes that remain overlooked by assessment of only crude *Aspergillus* allergens.

Keywords: COPD, *Aspergillus*, sensitisation, fungi, recombinant allergens

Introduction

Fungal sensitisation is increasingly reported in individuals with chronic respiratory disease including asthma, chronic obstructive pulmonary disease (COPD) and bronchiectasis where it is associated to persistent symptoms, increased disease severity, and hospitalizations [1-7]. In COPD, our group and others demonstrate that fungal sensitisation occurs at high frequencies, associates with exacerbations, relates to indoor and outdoor environments and can be linked to bronchiectasis-COPD overlap [1, 8]. *Aspergillus* species themselves exhibit a diverse spectrum of disease, ranging from colonization and sensitisation to chronic infection and invasive aspergillosis, the latter associated with late diagnosis and high mortality [9-14]. Several individuals, throughout their course of disease may also shift from one *Aspergillus*-associated disease to another depending on underlying host immunity. For instance, a sensitised COPD patient may have recurrent exacerbations whose treatment includes the frequent use of oral corticosteroids which dampens host immunity subjecting them to chronic or invasive consequences [15]. Prior studies in COPD evaluating the relevance of fungal sensitisation, and in particular *Aspergillus* sensitisation have however reported differing outcomes, likely attributable to inherent variation to the studied cohorts, geographical differences and critically the fungal extracts used to define the sensitisation response [16-19]. Importantly, no study to date has systematically evaluated the value and clinical correlates of measuring the sensitisation response to crude and recombinant *Aspergillus* allergens in COPD.

Crude allergens are used routinely in clinical practice to measure sensitisation responses, however, crude allergens lack specificity and between samples remain highly variable. Crude allergens are derived by extraction from their natural source and therefore composition and quality are determined by the primary source, potential contamination and protocols used for processing, extraction and storage [20, 21]. Taken together, these issues can result in batch variation, with instability and poor immunogenicity recorded for particular allergens including fungi [20]. With advances in available molecular technologies, recombinant allergens are increasingly being employed, particularly to overcome the weaknesses associated with crude allergens. Recombinant allergens, produced by DNA cloning and protein purification are better standardized, more reproducible, and importantly can be used to differentiate cross-reactivity from co-sensitivity to other allergens, and therefore more accurately identify a true triggering allergen of the sensitisation response [22, 23]. To date, twenty-three recombinant *Aspergillus fumigatus* (rAsp f) allergens have been identified, however only eight so far have been evaluated for a sensitisation response across the various chronic respiratory disease states including COPD [2, 22, 24, 25].

Based on the relationship between fungal sensitisation and poorer COPD outcomes, coupled to the lack of study of the sensitisation response to recombinant *Aspergillus* allergens, we sought to assess the role of fungal sensitisation in COPD by assessing the sensitisation response to an extensive panel of crude and recombinant fungal allergens to better understand their clinical value, if any in relation to COPD outcomes.

Methods

Study Cohort : Individuals aged 40 and above with stable COPD diagnosed based on the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria with an FEV/FVC ratio <0.7, risk factor exposure (i.e. ≥ 15 pack years smoking history, history of heavy and prolonged exposure to burning fossil fuels in an enclosed space, or high exposure to dust in an occupational setting) and/or COPD symptoms (i.e. dyspnea, chronic cough or chronic sputum production, recurrent lower respiratory tract infections and wheeze) were prospectively recruited from five tertiary centers across three countries in Asia between 2012 and 2020 [26, 27]. Individuals with COPD were recruited from Singapore (3 sites: Singapore General Hospital, Tan Tock Seng Hospital and Changi General Hospital), Malaysia (1 site: University Malaya Medical Center, Kuala Lumpur) and Hong Kong (1 site: Prince of Wales Hospital). Individuals receiving long-term immunosuppression including oral corticosteroids for the past year, those with recent exacerbation (within 6 weeks) prior to recruitment and those with a diagnosis of asthma based on the Global Initiative for Asthma (GINA) guidelines (i.e. variable symptoms and documented expiratory flow limitation) were excluded. Individuals with a diagnosis of allergic bronchopulmonary aspergillosis (ABPA) based on the International Society of Human and Animal Mycology (ISHAM)-working group criteria were also excluded [28, 29]. A COPD exacerbation was defined as an acute worsening of respiratory symptoms with increased dyspnea, cough, sputum purulence and/or volume or wheeze requiring treatment with antibiotics and/or corticosteroids. A non-diseased cohort aged 40 and above with no respiratory symptoms and normal spirometry were prospectively recruited at Nanyang Technological University (Singapore) as a control group to determine the cut off values of specific IgE against the various tested allergens. Blood (plasma) and clinical data were collected at recruitment and presented in Table 1. Clinical data collated include individual demographics, pulmonary function, symptoms (as COPD assessment test (CAT) score), smoking status (as current, ex-smoker: defined as having quit in the last 6 months and life-long non-smoker), exacerbations (as defined previously) and hospitalization for COPD exacerbations in the preceding year including COPD treatment. Spirometry was performed according to ATS/ERS guidelines [30]. Multidimensional prognostic index as the BODEx index (i.e. B: BMI, O: airway obstruction (as FEV_1 % predicted), D: dyspnea (as mMRC score) and Ex: exacerbations) was determined at recruitment [31, 32]. Number of COPD exacerbations was used to substitute for 6

minute- walk tests (6MWT) in the BODEx index calculated as this has been shown to be of comparable prognostic value [31]. Frequent COPD exacerbators were defined as 2 or more exacerbations in the year preceding study recruitment and a hospitalized exacerbation defined as a COPD exacerbation necessitating hospital admission [26, 33].

Statistical analysis: Statistical analysis was performed using R (version 3.6.1, R Foundation for Statistical Computing, Vienna, Austria.). Data distribution was assessed using the Shapiro-Wilk test. All continuous data was non-normally distributed and presented as medians with interquartile ranges (IQR). Between group comparisons were performed by Mann-Witney U or Kruskal-Wallis testing with Benjamin Hochberg correction applied for multiple comparisons. COPD exacerbation rate was assessed using negative binomial regression adjusted for age, gender, body mass index (BMI) and smoking status. Logistic regression was performed using the *glm* function in R. Unsupervised clustering was performed using hierarchical clustering Ward's minimum variance method. Specific IgE values determined with immune-dot-blot assay for crude and recombinant fungal allergens and included for cluster analyses. The optimal number of clusters was assessed using the R "NbClust" package and cluster stability was demonstrated and computed using a Jaccard similarities index with bootstrapping over 100 iterations. The mean Jaccard value was 0.7 suggesting stability of the identified clusters. Statistical significance was defined as $p \leq 0.05$.

Topological data analysis (TDA) was performed using mapper, a computational method to visualize the high dimensional distribution or structure of input data with simplicial complexes [34]. The mapper algorithm was implemented on the crude and recombinant allergen expression data with dimensionality reduction performed using the projection lens with "L2 norm", clustering using kmeans with 2 clusters and the cover parameters as follows: number of cubes = 10 and percentage overlap= 25%. The resulting TDA network was imported into 'Cytoscape' for visualisation, using custom scripts in python. The network was visualized as 'percentage of positive class' (categorical) and the median (continuous) expression values for node colourings. Mapper was implemented using the 'KepplerMapper' module in python [35].

Further details on the ethics approvals, blood sampling, the allergen panel and immune-dot-blot assays can be found in the supplementary methods.

Results

Sensitisation to *Aspergillus fumigatus* associates with COPD exacerbations. Demographics of the study cohorts are summarized in Table 1. N=614 individuals with COPD, recruited during clinical stability from five centers across three countries were evaluated for sensitisation responses to house dust mite, pollen, cockroach and fungal allergens in relation to clinical COPD outcomes. The COPD cohort were predominant male (95.9%), with significant smoking rates (59.3%) and median FEV_1 % predicted and CAT score of 47% and 15 respectively. Patients demonstrating fungal sensitisation experienced a significantly increased exacerbation frequency (Incidence Rate Ratio (IRR): 1.80; 95% CI: 1.09-2.99, p=0.02) (median 1; IQR 0-2, p=0.002) compared to house dust mite, pollen, and cockroach sensitisation (Figure 1a). To next evaluate which fungi specifically associate with COPD exacerbations, fungal sensitisation was categorized into *Aspergillus fumigatus* (n=130), *Aspergillus* (non-fumigatus) (n=373) or other fungi (n=157) sensitisation groups respectively. Individuals with *Aspergillus fumigatus* sensitisation (IRR: 3.48; 95% CI: 1.38-8.75, p=0.01) (Figure 1b) (median 1; IQR 0-2, p<0.001), characterized predominantly by sensitisation responses to the major recombinant *Aspergillus fumigatus* (n=394)(r Asp f) 1, 2 and 3 allergens demonstrate highest exacerbation frequency (IRR: 1.62; 95% CI: 1.17-2.23, p<0.001) (Figure 1c) (median 1; IQR 0-3, p<0.001). Taken together, this suggests that fungal sensitisation in COPD, specifically major rAsp f sensitisation is important in identifying patients at high risk of increased exacerbations.

Unsupervised clustering of fungal allergens in COPD reveals two patient groups with variable clinical outcomes. As fungal sensitisation in COPD associates with increased exacerbations, we next evaluated if specific COPD patient groups, based on fungal sensitisation profile exist and their associated clinical risk. Unsupervised clustering of specific IgE responses to a broad panel of crude and recombinant fungal allergens revealed two patient clusters (Table 2, Table 3 and Figure 2a) where cluster 1 (n=335) demonstrates significantly increased exacerbations (IRR: 1.59; 95% CI:1.24-2.04, p<0.001) (median 1; IQR 0-3, p=0.001) and hospitalized (severe) exacerbations (IRR: 2; 95%CI: 1.51-2.64, p<0.01) (median 1; IQR 0-2, p<0.001), poorer lung function (median FEV_1% predicted: 41.2; IQR: 32.1-55.7, p<0.001) and worse prognosis (median BODEx index: 4; IQR: 3-6, p<0.001) but interestingly remain less symptomatic (median CAT score: 14; IQR 10-20, p=0.01) (Figure 2b-d). As prognosis by BODEx index differed between clusters, further assessment of its contributing components revealed a significantly lower body mass index (BMI) in cluster 1 (p<0.001) but no differences in mMRC dyspnea index (p=0.225) (Figure E1). When patients were grouped by their GOLD stage (1 to 4) based on lung function (FEV_1% predicted), increased exacerbation frequency is observed with decreasing lung function in both clusters (Figure E2). Cluster 1, illustrating an adverse clinical profile, was characterized by increased sensitisation responses to *Aspergillus-related* allergens, specifically *Asp. terreus*, rAsp f 1, 3, 5 ,6, rAsp. *niger* 14 and rAsp. *oryza* 21, while cluster

2 (n=279), with higher symptomatic burden (median CAT score: 16; IQR 10-24, p=0.01) is distinguished by significantly elevated sensitisation responses to *Cladosporium* (Figure 2e). Taken together, our unbiased clustering approach further highlights the importance of *Aspergillus* sensitisation in COPD but additionally identifies previously unrecognized specific recombinant fungal allergens that demonstrate clinical significance.

Polysensitisation to cluster 1 related allergens confers poorer clinical outcomes in COPD. As cluster 1 demonstrates poor clinical outcomes from earlier analysis, we next sought to understand the significance, if any, of polysensitisation to cluster 1 related allergens among the COPD cohort. To do this, sensitisation profiles were sub-grouped by the number of cluster 1 related allergens to which patient was sensitised as follows: non-sensitised (n=149), sensitised to 1-2 (n=270) and >2 allergens (n=198) respectively and assessed in relation to clinical outcomes. Increased exacerbation frequency (IRR for 1-2 allergens: 1.68; 95% CI:1.22-2.32, p=0.002 and >2 allergens: 1.87; 95% CI: 1.33-2.62 respectively, p<0.001; Figure 3a) (median for non-sensitised: 0 (IQR:0-2), 1-2 allergens: 1 (IQR: 0-2) and >2 allergens: 1 (IQR:0-3), p=0.001) relative to the non-sensitised group and poorer lung function (median FEV1% predicted for non-sensitised: 53% (IQR: 38-68%), 1-2 allergens: 46% (IQR: 34-62%) and >2 allergens: 42% (IQR: 32%-54%) respectively, p<0.001) (Figure 3b) was observed with polysensitisation. Importantly, symptomatic burden did not relate to polysensitisation, with highest CAT scores in the non-sensitised group (median CAT score non-sensitised: 18 (IQR: 10-25) versus 1-2 allergens: 15 (IQR: 9-21) and >2 allergens: 15 (IQR: 10-20) respectively, p=0.03) (Figure 3c). When prognosis (by BODEx index) is considered, sensitisation to at least one cluster 1 related allergen conferred a poorer prognosis and additional polysensitisation does not appear to confer any additional risk (median BODEx for non-sensitised: 3 (IQR: 2-5) versus 1-2 allergens: 4 (IQR: 3-6) and >2 allergens: 4 (IQR: 3-6) respectively, p<0.001) (Figure 3d). Polysensitisation to cluster 1 related allergens therefore relate to poorer COPD outcomes including higher exacerbation frequency and poorer lung function but have no effect on symptoms or prognostic risk.

Sensitisation to cluster 1 related recombinant *Aspergillus fumigatus* allergens rAsp f1, f3, f5 and f6 uncovers significant clinical associations missed by assessment of the sensitisation response to only crude *Aspergillus fumigatus*. Having identified the importance and clinical relevance of specific rAsp f allergens (rAsp f 1, 3, 5, 6) through our unsupervised clustering approach, we next evaluated their importance in relation to sensitisation responses to crude *Aspergillus fumigatus* alone, the latter approach used most frequently in current clinical practice. Importantly, we uncovered a significant number of COPD patients without crude *Aspergillus fumigatus* sensitisation which demonstrate significant sensitisation responses to rAsp f 1, 3, 5 and/or 6 (n=309, 63.8%). To further investigate this phenomenon, we categorized patients into four groups based on sensitisation responses to crude

and rAsp f 1, 3, 5 and/or 6 responses respectively as (1) sensitised to crude allergens only (C+R-)(n=24); (2) sensitised to both crude and recombinant allergens (C+R+) (n=106); (3) sensitised to recombinant allergens only (C-R+) (n=309) and (4) non-sensitised (C-R-) (n=175). Sensitisation to recombinant *Af* allergens demonstrates a significantly increased exacerbation frequency irrespective of crude sensitisation status: C+R+ (IRR: 2.40; 95% CI: 1.64-3.49, p<0.001) (median: 2; IQR 0-3) and C-R+ (IRR: 1.55; 95% CI: 1.15-2.10, p=0.004) (median: 1; IQR 0-2) relative to C-R- (median: 0; IQR 0-2). No significance difference in exacerbations were noted in the C+R- group (IRR: 1.15; 95% CI: 0.56-2.36, p=0.70) (median: 1; IQR 0-1) relative to C-R- (Figure 4a). Similar trends were observed for lung function (p<0.001) and prognosis (p<0.001) with lowest lung function in recombinant *Af* sensitised groups independent of their crude sensitisation status (Figure 4b and 4c). No significant differences were observed in relation to symptoms across all groups (Figure 4d). Taken together, these data suggest the clinical importance of evaluating sensitisation responses to cluster 1 related recombinant *Aspergillus fumigatus* allergens rAsp f 1, 3, 5, and 6 in COPD. Significant numbers of patients appear missed by the practice of exclusively screening for sensitisation to crude *Aspergillus fumigatus*.

Validation of COPD fungal sensitisation clusters with Topological Data Analysis (TDA). Having identified two fungal sensitisation COPD clusters by unsupervised approaches, we sought to independently validate these patient groups using an alternate analytical approach: Topological Data Analysis (TDA), a data structure-based inference method. TDA mapper networks were generated using all crude and recombinant fungal allergens outlined in Table 2 from all n=614 COPD patients studied. Each derived node represents a patient group, nodal size the patient number within that group, and lines between nodes correspond to the connections between patient groups. Coloration contrast is used to illustrate specific predefined median feature scores across the TDA network. TDA validated our previously identified clusters, with clear separation of the two previously identified groups (Figure 5a). Interestingly, cluster 1 patients were grouped into an area of the TDA network structure with higher occurrence of frequent exacerbators (Figure 5b in red), poorer lung function (Figure 5c in green) and worse prognosis (Figure 5e in blue) in agreement with our prior findings (Figure 2). Cluster 2 patients had higher symptom scores (Figure 5d in orange) again corroborating our previous findings (Figure 2). In addition to validating our earlier findings (Figure 2a), TDA further identified an additional subgroup of patients within cluster 1 with the highest proportions of frequent exacerbators, poorest lung function and worst prognosis (Figure 5 dotted circle). This subgroup demonstrates increased specific IgE sensitisation responses to rAsp f 3, 6 and 34.

Discussion

Through our multicentre prospective study assessing a comprehensive panel of crude and recombinant fungal allergens, we evaluate the clinical relevance of fungal sensitisation and in particular *Aspergillus fumigatus* sensitisation in COPD. In addition, we assess the clinical value of measuring sensitisation responses to recombinant *Aspergillus* allergens comparing this to responses to crude allergens. Fungal sensitisation, and specifically *Aspergillus fumigatus* sensitisation associates with an increased exacerbation frequency in COPD. Unsupervised analyses of the measured sensitisation responses reveal two 'fungal sensitisation' groups: one characterized by *Aspergillus* sensitisation and increased exacerbations, poorer lung function and worse prognosis. Interestingly, this group was less symptomatic and polysensitisation conferred further increased risks of exacerbations and lower lung function. Significant numbers of individuals with COPD demonstrate sensitisation responses only to recombinant *Aspergillus fumigatus* allergens 1, 3, 5, and 6, and these patients exhibit higher exacerbations, poorer lung function and an overall worse prognosis. Therefore, even in the absence of a detectable sensitisation response to crude *Aspergillus* allergen, measurement of responses to recombinant *Aspergillus fumigatus* allergens 1, 3, 5, and 6 should be considered as they demonstrate clinical relevance in COPD. TDA corroborated our findings but additionally identified a further sub-group within *Aspergillus* sensitised COPD that included an enrichment of frequent exacerbators with poorest lung function and prognosis. These individuals were characterized by increased sensitisation responses to two of the previously identified recombinant *Aspergillus fumigatus* allergens (rAsp f 3 and 6) but also the addition of a significant response to rAsp 34. Taken together, our presented work, for the first time, reveals the importance of assessing sensitisation to recombinant *Aspergillus fumigatus* allergens in COPD.

Fungal sensitisation is associated with progressive and persistent symptoms, disease severity and reduced lung function in asthma, bronchiectasis and cystic fibrosis [3, 36-41]. In COPD, our group has previously reported poorer clinical outcomes in patients demonstrating fungal sensitisation when compared to house dust mite sensitisation [1]. Environmental exposure remains a key source of contact with fungal allergens, however mis-alignment in the available clinical fungal extracts for assessment and environmental fungi that have been detected are reported which potentially contributes to a likely under-diagnosis of sensitisation response in respiratory disease and an underappreciation of their impact on clinical outcomes [42]. In COPD, and through next-generation sequencing of environmental samples including indoor and outdoor air, we have previously shown that a sub-group of COPD patients have measurable immunological responses to fungi extract within breathable air [1, 43]. Air-fungi sensitised COPD including that to *Aspergillus* species relates to

exacerbations, however, the specific fungal types and optimal allergen screen to identify such patients remains unclear. Here, in the current study we advance specific fungi and provide a novel panel of recombinant allergens useful for screening that identify patients demonstrating poor clinical COPD outcomes. By assessing a broad range of fungal allergens including both crude and recombinant forms, we have performed the largest and most comprehensive fungal allergen screen available in COPD to date. Our presented results suggest that fungal sensitisation, specifically to *Aspergillus fumigatus* remains important as it associates with negative clinical consequence in COPD.

Here, we describe two groups of 'fungal sensitised' COPD: one characterized by *Aspergillus* sensitisation and poor clinical outcomes and a second by *Cladosporium* sensitisation and significant symptomatic disease. In COPD, prevalence of *Aspergillus* sensitisation (AS) ranges from 7.9% to 18.3% with variable reported clinical outcomes [17]. Bafadhel *et al* report poor lung function in association with AS, while Everaerts *et al.* found no such association but a higher occurrence of bronchiectasis-COPD overlap [44, 45]. Studies in other Asian settings, specifically from China and India similarly report no association between AS and lung function decline in COPD [18, 19]. In our study, AS occurred in 21% of individuals with COPD, slightly higher than the reported prevalence in the current literature. Importantly, we detect an association between AS and higher exacerbation frequency in COPD, a feature absent in the non-sensitised. Variations in the prior literature is likely attributable to cohort differences which include geographic and potentially climatic factors. Furthermore, methodologies used to define a sensitisation response varied between prior studies with some focusing solely on a skin prick test response [46]. We build on this by including, for the first time, a comprehensive panel of recombinant *Aspergillus* allergens most of which have not previously been studied in COPD.

The representativeness of crude allergen extracts for the screening of sensitisation responses has been challenged. Viewed as being unstable, easily contaminated, lacking specificity, poor demonstrable immunogenicity and potential cross-reactivity with other allergens, recombinant allergens have been proposed as alternatives [20]. With improved technologies, recombinant allergens can now be produced in significant quantities and at high reproducible quality. To date, twenty-three rAsp f allergens have been listed in the WHO/IUIS nomenclature, however only eight (specifically rAsp f 1, 2, 3, 4, 6, 12, 15 and 17) have been previsouly assessed in respiratory disease [2, 8, 25, 47, 48]. Muthu and colleagues report that use of crude *Aspergillus* allergens portend toward misclassification of AS and that rAsp f 1 and 2 are more specific for this diagnosis in asthmatics [48]. Critically, rAsp f allergens are shown to distinguish AS from allergic bronchopulmoary aspergillosis (ABPA) across a number of respiratory diseases which include rAsp f 2, 4, and 6 in asthma, rAsp f 3 and 4 in cystic fibrosis and rAsp f 17 in bronchiectasis [2, 24, 25]. In COPD, rAsp f 1 and 3 appear to associate with

bronchiectasis-COPD overlap while rAsp f 1, 15 and 17 relate to very frequent exacerbators (i.e. ≥3 COPD exacerbations annually) [1, 45, 49]. These prior works in COPD, while demonstrating clinical relevance however only include a small, restricted and select number of rAsp f allergens for evaluation necessitating a broader understanding of their use and relevance in clinical COPD. Using the most comprehensive recombinant *Aspergillus* allergen panel to date, our work suggests that sensitisation to rAsp f 1, 3, 5 and 6 is of key clinical relevance in COPD as they associate with poorest clinical outcomes, are not related to symptoms and cannot be identified by screening against crude *Aspergillus fumigatus* allergens alone.

Topological Data Analysis (TDA) represents a set of mathematical methods used to study the "shape" and "structure" of high-dimensional datasets [50]. Mapper, a computational algorithm which forms part of the TDA suite of methodologies uses dimensionality reduction followed by clustering to visualise high-dimensional datasets as networks [50, 51]. TDA has been used and validated across various clinical studies for the modelling and classification of patient subgroups, gene expression and accessing genotype-phenotype relationships [50, 52]. Here, we employ TDA as a second confirmatory statistical approach to unsupervised hierarchical clustering to independently validate the presence of our two described ‘fungal sensitised groups’ and their relationship to key clinical outcomes in COPD. Through TDA, an additional subgroup of patients (within the *Aspergillus* sensitised group) was identified, enriched for frequent COPD exacerbators and with the poorest lung function and worst prognosis. These individuals were characterized by sensitisation responses to rAsp f 3, 6 and 34.

Our study is the first to comprehensively evaluate recombinant *Aspergillus* allergens in COPD and illustrate their importance and relevance to identifying patients with poorer clinical outcomes. This suggests that a wider panel of fungal allergens including a select group of recombinant allergens may be useful for the clinical evaluation of fungal sensitisation in COPD and to identify those at highest clinical risk. Despite its strengths and novelty, our study does have important limitations. As only cross sectional assessment was performed, we are unable to ascertain longitudinal changes to both sensitisation state and clinical outcomes. While a higher number of *Aspergillus* sensitized individuals were found to be using inhaled corticosteroids in our study, we are unable to ascertain from this dataset whether this benefited the sensitization response or contributed to further fungal colonization of the airway. As computed tomography (CT) chest scanning was not standardized across centers nor available in all recruited individuals, we were unable to make strong evaluations regarding association of sensitisation with bronchiectasis or bronchiectasis-COPD overlap. Over 90% of our recruited COPD cohort were male, and while characteristic of the demographics observed from prior Asian studies in COPD, this makes our findings less generalizable to female cohorts. The male predominance in Asian COPD cohorts may be a consequence of under-diagnosis of COPD in Asian

females. All patients were recruited from tertiary specialist centers and therefore the role of sensitisation in milder COPD phenotypes was not assessed. Finally, we did not perform formal comprehensive assessments for ABPA and while all COPD patients were recruited during periods of clinical stability, making ABPA unlikely, any association of recombinant *Aspergillus* allergens and ABPA cannot be made from this dataset and is beyond the scope of this study.

Aspergillus sensitisation appears a treatable trait in COPD. Measuring sensitisation responses to recombinant *Aspergillus* allergens identifies important patient subgroups with poor COPD outcomes that remain overlooked by assessment of only crude *Aspergillus* allergens. Screening of recombinant *Aspergillus* allergens 1, 3, 5, 6 and 34 at an early disease stage may be helpful in identifying future 'high-risk' COPD patients that will potentially experience frequent exacerbations and greater impact on lung function. While additional costs and expertise will be involved in establishing a clinical assessment pathway using recombinant *Aspergillus* allergens rather than traditional crude allergens, this allows for the early identification of high-risk COPD phenotypes that will over the longer-term reduce morbidity, mortality for patients and potentially provide cost savings to healthcare systems. Use of recombinant *Aspergillus* allergens in screening COPD patients demonstrating fungal sensitisation should be considered and may permit closer monitoring and appropriate intervention of such high-risk individuals.

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Table 1: Demographic table summarizing the non-diseased and COPD cohorts.

	Non-diseased	**COPD**
Subject number (n)	33	614
Age (years), Median (IQR)	61 (58-63)	74 (68-79)
Male Sex, n (%)	11 (33.3)	589 (95.9)
BMI (kg/m^2), Median (IQR)	23.6 (20.7-26.6)	21.9 (18.9-24.8)
Smoking status, n (%)		
Current	3 (9.0)	364 (59.3)
Ex-smoker	2 (6.0)	246 (40.1)
Never	28 (85.0)	4 (0.6)
Smoking pack years, Median (IQR)	27 (15-33)	50 (40-68)
FEV1 % predicted, Median (IQR)	93 (83-97)	47 (34-60)
FEV1/FVC (% predicted), Median (IQR)	83 (77-84)	51 (41-61)
No. of Exacerbations in the past 12 months		
0-1	NA	400 (65.1)
>1	NA	214 (34.9)
Hospitalized exacerbation in the past 12 months		
Yes	NA	272 (44.3)
No	NA	342 (55.7)
COPD assessment test (CAT) score [53], Median (IQR)	NA	15 (10-22)
BODEx index [31]	NA	4 (2-6)
Blood eosinophil count ($x10^9/L$), Median (IQR)	NA	0.1 (0.0-0.3)
Total IgE (IU/ml), Median (IQR)	NA	77.8 (17.6-291.1)
Treatment, n (%)		
SABA/SAMA	NA	93 (15.2)
LABA	NA	5 (0.8)
LAMA	NA	50 (8.2)
LAMA/LABA	NA	112 (18.2)
LAMA/ICS	NA	37 (6.0)
LABA/ICS	NA	147 (23.9)
LAMA/LABA/ICS	NA	170 (27.7)

Data is presented as the number of subjects (n) and 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 1^{st} second, FVC: Forced Vital Capacity, IQR: Interquartile Range, SABA: Short-acting beta-agonist, SAMA: Short-acting muscarinic antagonist, LABA: Long-acting beta-agonist, LAMA: Long-acting muscarinic antagonist, ICS: Inhaled corticosteroid. BODEx: B: BMI, O: airway obstruction (as FEV_1 % predicted), D: dyspnea (as mMRC score) and Ex: exacerbations.

Table 2: Panel of fungal allergens used in this study categorized as crude or recombinant.

Crude Allergens	Recombinant *Aspergillus fumigatus* (rAsp f) allergens	Recombinant *Aspergillus* (non-fumigatus) allergens
Aspergillus fumigatus	rAsp f 1	rAsp fl 1 (*Asp. flavus*)
Aspergillus terreus	rAsp f 2	rAsp n 14 (*Asp. niger*)
Aspergillus syndowii	rAsp f 3	rAsp n 25 (*Asp. niger*)
Trametes sanguineus	rAsp f 4	rAsp o 21 (*Asp. oryzae*)
Schizophyllum commune	rAsp f 5	
Curvularia spp.	rAsp f 6	
Cladosporium tenuissimum	rAsp f 7	
Cladosporium spp.	rAsp f 9	
Byssochlamys spectabilis	rAsp f 10	
Neurospora spp.	rAsp f 11	
Penicillium spp.	rAsp f 12	
	rAsp f 15	
	rAsp f 16	
	rAsp f 17	
	rAsp f 18	
	rAsp f 22	
	rAsp f 27	
	rAsp f 28	
	rAsp f 29	
	rAsp f 34	

rAsp f: recombinant Aspergillus fumigatus, fl: flavus, n: niger, o: oryzae. Major recombinant *Aspergillus fumigatus* allergens are indicated in bold.

Table 3: Demographic table summarizing cluster 1 and cluster 2 fungal sensitization groups

	Cluster 1	**Cluster 2**	**p-value**
Subject number (n)	335	279	
Age (years), Median (IQR)	74 (68-79)	73 (69-79)	0.840
Male Sex, n (%)	325 (97.1)	264 (94.6)	0.198
BMI (kg/m^2), Median (IQR)	21.3 (18.6-23.7)	22.5 (19.7-25.9)	<0.001
Smoking status, n (%)			<0.001
Current	246 (73.4)	118 (42.3)	
Ex-smoker	86 (25.7)	160 (57.3)	
Never	3 (0.9)	1 (0.4)	
Smoking pack years, Median (IQR)	50 (38-65)	50 (40-80)	0.253
FEV1 % predicted, Median (IQR)	41 (32-56)	52 (37-66)	<0.001
FEV1/FVC (% predicted), Median (IQR)	49 (40-59)	54 (45-65)	<0.001
No. of Exacerbations in the past 12 months			0.001
0-1	204 (60.9)	196 (70.3)	
>1	131 (39.1)	83 (29.7)	
Hospitalized exacerbation in the past 12 months			0.002
Yes	167 (49.9)	175 (62.7)	
No	168 (50.1)	104 (37.3)	
COPD assessment test (CAT) score [53], Median (IQR)	14 (10-20)	16 (10-24)	0.010
BODEx index [31]	4 (3-6)	3 (2-5)	<0.001
Blood eosinophil count ($x10^9/L$), Median (IQR)	0.1 (0.0-0.3)	0.1 (0.0-0.4)	0.128
Total IgE (IU/ml), Median (IQR)	80.9 (19.2-250.6)	62.9 (11.3-408.6)	0.811
Anti-*Aspergillus* IgG, AU/ml, Median (IQR)	0.22 (0.01-1.15)	0.03 (0-0.26)	0.008
Available HRCT Thorax scans, n (%)	55 (16.4)	190 (68.1)	
Numbers of HRCT showing bronchiectasis, n (%)	7 (12.7)	52 (27.4)	0.040
Treatment, n (%)			<0.001
SABA/SAMA	57 (17.0)	36 (12.9)	
LABA	1 (0.3)	4 (1.4)	
LAMA	14 (4.2)	36 (12.9)	
LAMA/LABA	22 (6.6)	90 (32.3)	
LAMA/ICS	30 (9.0)	7 (2.5)	
LABA/ICS	106 (31.6)	41 (14.7)	
LAMA/LABA/ICS	105 (31.3)	65 (23.3)	

SABA: short-acting β2-agonists; SAMA: short-acting muscarinic antagonists; LABA: Long-acting β2-agonists; LAMA: Long-acting muscarinic antagonists; ICS: Inhaled corticosteroids

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Figure Legends

Figure 1: Fungal sensitisation, including sensitisation to major recombinant *Aspergillus fumigatus* allergens significantly associates with a higher frequency of exacerbations in COPD. Forest plots illustrating the Incidence Rate Ratio (IRR) for COPD exacerbations with detectable sensitisation to (a) key groups of established allergens (b) fungal allergens and (c) *Aspergillus fumigatus* allergens. The error bar indicates the 95% confidence interval (CI) and dots represent the IRR for COPD exacerbation. Red dots correspond to statistical significance ($p<0.05$).

Figure 2: Unsupervised clustering of fungal allergens in COPD reveals two clusters characterized by significant differences in lung function, symptom burden and disease prognosis. (a) Heatmap demonstrating two fungal sensitisation clusters indicated by blue and red coloration respectively. Scatter box plots illustrating differences in (b) lung function (as FEV_1 % predicted) (c) prognosis (as BODEx index) and (d) symptom burden (as CAT score) between clusters. (e) Forest plot illustrating the odds ratio (OR) for detection of a sensitisation response to each respective fungal allergen by cluster membership. Clusters 1 and 2 are indicated by pink and purple colouration respectively. Red dots correspond to statistical significance ($p<0.05$) and error bars indicate the 95% confidence interval (CI). rAsp f: recombinant *Aspergillus fumigatus* allergen. *$p\leq0.05$, ***$p\leq0.001$. COPD: Chronic Obstructive Pulmonary Disease, FEV_1: Forced Expiratory Volume in the 1st second, BODEx: B: Body mass index, O: airway obstruction (as FEV_1 % predicted), D: dyspnea (as mMRC score) and Ex: exacerbations, CAT: COPD assessment test, cla: *Cladosporium* rAsp f: recombinant *Aspergillus fumigatus*, fl: *flavus*, n: *niger*, o: *oryzae*.

Figure 3: Polysensitisation to cluster 1 related allergens associates with higher exacerbation frequency and poorer lung function in COPD. (a) Forest plot illustrating the Incidence Rate Ratio (IRR) for COPD exacerbations in individuals demonstrating sensitisation to 1-2 or >2 cluster 1 related allergens. This is presented relative to individuals with COPD from the study cohort demonstrating no sensitisation to any cluster 1 related allergens. Scatter box plots illustrating differences in (b) lung function (as FEV_1 % predicted) (c) symptom burden (as CAT score) and (d) prognosis (as BODEx index) between non-sensitised (i.e. 0: no demonstrable sensitisation to any cluster 1 related allergens) and those polysensitised to either 1-2 or >2 cluster 1 related allergens. Error bars indicate 95% confidence intervals (CI) and dots represent IRR for a COPD exacerbation. ns: non-significant, *$p\leq0.05$, ***$p\leq0.001$. COPD: Chronic Obstructive Pulmonary Disease, FEV_1: Forced Expiratory Volume in the 1st second, CAT: COPD assessment test, BODEx: B: Body mass index, O: airway obstruction (as FEV_1 % predicted), D: dyspnea (as mMRC score) and Ex: exacerbations.

Figure 4: Sensitisation to cluster 1 related recombinant *Aspergillus fumigatus* allergens rAsp f1, f3, f5 and f6 demonstrates clinical significance missed by assessment of the sensitisation response to only crude *Aspergillus fumigatus*. (a) Forest plot illustrating the Incidence Rate Ratio (IRR) for COPD exacerbations between individuals with demonstrable Af sensitisation responses to crude allergens only (C+R-), recombinant allergens only (C-R+) and both (C+R+). Scatter box plots illustrating differences in (b) lung function (as FEV_1 % predicted) (c) prognosis (as BODEx index) and (d) symptom burden (as CAT score) between combinations of individuals sensitised to crude (C) and recombinant (R) Af allergens in the following groups: C+R-; C+R+; C-R+ and C-R-. Error bars indicate 95% confidence intervals (CI) and dots represent incidence rate ratio for a COPD exacerbation where red colouration corresponds to statistical significance ($p<0.05$). ns: non-significant, *$p\leq0.05$, **$p\leq0.01$, ***$p\leq0.001$. COPD: Chronic Obstructive Pulmonary Disease, FEV_1: Forced Expiratory Volume in the 1st second, BODEx: B: Body mass index, O: airway obstruction (as FEV_1 % predicted), D: dyspnea (as mMRC score) and Ex: exacerbations, CAT: COPD assessment test, rAsp f: recombinant *Aspergillus fumigatus* allergens, +ve: positive, -ve: negative.

Figure 5: A Topological Data Analysis (TDA) network including all crude and recombinant fungal allergens assessed in our comprehensive panel. The network illustrates connections between COPD patients based on their underlying sensitisation status, allergen pattern and coloured according to (a) the established patient clusters (cluster 1: pink, cluster 2: purple), (b) exacerbation frequency as frequent exacerbators: red and non-frequent exacerbators: grey, (c) lung function (as FEV_1 % predicted) (high: grey to low: green), (d) symptom burden (as CAT score) (high: orange to low: grey) and (e) prognosis (as BODEx index) (high: light-blue to low: grey). Each node represents a group of COPD patients, and nodal size the relative number of individuals within that group. Lines between nodes indicate overlapping individuals between groups and dotted circular lines represent nodes with the highest proportion of frequent exacerbators, lowest FEV_1 % predicted and highest BODEx index. COPD: Chronic Obstructive Pulmonary Disease, FEV_1: Forced Expiratory Volume in the 1st second, CAT: COPD assessment test, BODEx: B: Body mass index, O: airway obstruction (as FEV_1 % predicted), D: dyspnea (as mMRC score) and Ex: exacerbations.

Online supplement

Sensitisation to recombinant *Aspergillus fumigatus* allergens and clinical outcomes in COPD

Pei Yee Tiew[1], Jayanth Kumar Narayana[2], Marilynn Swee Li Quek[3] , Yan Ying Ang[3], Fanny Wai San Ko[4], Mau Ern Poh[5], Tavleen Kaur Jaggi[2], Huiying Xu[6], Kai Xian Thng[2], Mariko Siyue Koh[1], Augustine Tee[7], David Shu Cheong Hui[4], John Arputhan Abisheganaden[2,6], Krasimira Tsaneva-Atanasova [8,9], Fook Tim Chew[3], Sanjay H.Chotirmall[2,6]

SUPPLEMENTARY MATERIALS AND METHODS

Ethics approval: Written informed consent was obtained from all recruited participants and Institutional ethics approval from each site obtained as follows: CIRB 2017/2933 and 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 (control) recruitment was approved by the Nanyang Technological University (NTU) Institutional Review Board under IRB-2017-12-010 (Singapore).

Blood sampling: Venous blood was collected from each participant at recruitment following informed consent. Plasma was isolated with centrifugation at 1300g for 10 minutes at 18°C and stored at -80 degrees prior to immune-dot-blot assay assessment as described. All specimens from clinical sites were transported (temperature controlled) and processed centrally in Singapore to ensure consistency and standardization of all assessments.

Allergen panel: House dust mite (*Dermatophagoides farinae* (Der f); *Dermatophagoides pteronyssinus* (Der p); *Blomia tropicalis* (Blo t)), pollens (*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*) and cockroach (*Blattella germanica; Periplaneta Americana)* were included. A comprehensive panel of crude and recombinant fungal allergens included are summarized in Table 2.

Immune-dot-blot assay: Specific IgE to crude and recombinant allergens from the allergen panel were assessed using immune-dot-blot assays as previously described [1-3]. Crude allergens included: *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*), *A. terreus, A. sydowii*, *Cladosporium tenuissimum*, *Cladosporium* spp., *Neurospora* spp., *Byssochlamys spectabilis*, *Trametes sanguinea* and *Schizophyllum commune*. Recombinant allergens included Asp f 1 (M83781), Asp f 2 (U56938), Asp f 3 (U58050) ,Asp f 4 (AJ001732), Asp f 5 (Z30424), Asp f 6 (U53561), Asp f 7 (AJ223315), Asp f 9 (AJ223327), Asp f 10 (X85092), Asp f 11 (AJ006689), Asp f 12 (U92465), Asp f 13 (Z11580), Asp f 15 (AJ002026), Asp f 16 (AF062651), Asp f 17 (AJ224865), Asp f 18 (Y13338), Asp f 22 (AF284645), Asp f 27 (AJ937743), Asp f 28 (AJ937744), Asp f 29 (AJ937745), Asp f 34 (AM496018), Asp n 14 (AF108944) Asp n 25 (L20567), Asp o 21 (M33218). Briefly, each allergen was blotted onto a nitrocellulose membrane in duplicate with PBS and bovine serum albumin (BSA) as controls. The membranes were incubated with 0.1% PBS-Tween-20 followed by plasma in 1:8 dilution with PBS. After 16-hours, membranes were washed on three

separate occasions with 0.05% PBS-Tween-20, first for 15-minutes, followed by 7-minutes (twice) subsequently. After the washing steps, anti-human IgE antibody conjugated with alkaline phosphatase was added and incubated for 2h. Membranes were subsequently analyzed using Syngene imaging software with inter and intra-assay reproducibility above 90%. A sensitisation response was defined as a specific IgE binding intensity above the 95th percentile of the non-diseased control group for each respective allergen.

Anti-Aspergillus IgG: Platella anti-*Aspergillus* IgG (Bio-rad #62783) was performed according to manufacturer's instructions. Ten microliters of serum (in duplicate) were used for assays. Samples with concentrations of >5UA/ml were considered positive for the presence of IgG antibody to *Aspergillus.*

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1. Mac Aogain M, Tiew PY, Lim AYH, Low TB, Tan GL, Hassan T, Ong TH, Pang SL, Lee ZY, Gwee XW, Martinus C, Sio YY, Matta SA, Ong TC, Tiong YS, Wong KN, Narayanan S, Bijin Au V, Marlier D, Keir HR, Tee A, Abisheganaden JA, Koh MS, Wang Y, Connolly JE, Chew FT, Chalmers JD, Chotirmall SH. Distinct 'Immuno-Allertypes' of Disease and High Frequencies of Sensitisation in Non-Cystic-Fibrosis Bronchiectasis. *American journal of respiratory and critical care medicine* 2018.

2. Tiew PY, Ko FWS, Pang SL, Matta SA, Sio YY, Poh ME, Lau KJX, Mac Aogain M, Jaggi TK, Ivan FX, Gaultier NE, Uchida A, Drautz-Moses DI, Xu H, Koh MS, Hui DSC, Tee A, Abisheganaden JA, Schuster SC, Chew FT, Chotirmall SH. Environmental fungal sensitisation associates with poorer clinical outcomes in COPD. *Eur Respir J* 2020: 56(2).

3. Mac Aogain M, Chandrasekaran R, Lim AYH, Low TB, Tan GL, Hassan T, Ong TH, Hui Qi Ng A, Bertrand D, Koh JY, Pang SL, Lee ZY, Gwee XW, Martinus C, Sio YY, Matta SA, Chew FT, Keir HR, Connolly JE, Abisheganaden JA, Koh MS, Nagarajan N, Chalmers JD, Chotirmall SH. Immunological corollary of the pulmonary mycobiome in bronchiectasis: the CAMEB study. *Eur Respir J* 2018: 52(1).

Supplementary Figure Legends

Figure E1: Scatter box plots illustrating differences in (a) Body mass index (BMI) and (b) mMRC dyspnea score between clusters. ns: non-significant, ***$p \leq 0.001$. mMRC: Modified Medical Research Council.

Figure E2: Scattered box plots illustrating exacerbation frequency in relation to COPD gold stage (FEV_1 group) within each cluster. FEV_1: Forced Expiratory Volume in the 1^{st} second. * $p \leq 0.05$, **$p \leq 0.01$

Figure 1

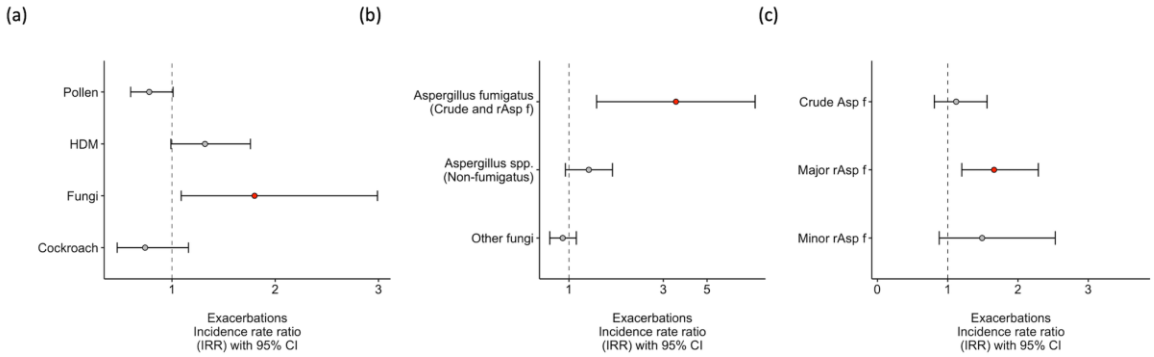


Figure 1: Fungal sensitisation, including sensitisation to major recombinant Aspergillus fumigatus allergens significantly associates with a higher frequency of exacerbations in COPD. Forest plots illustrating the Incidence Rate Ratio (IRR) for COPD exacerbations with detectable sensitisation to (a) key groups of established allergens (b) fungal allergens and (c) Aspergillus fumigatus allergens. The error bar indicates the 95% confidence interval (CI) and dots represent the IRR for COPD exacerbation. Red dots correspond to statistical significance ($p<0.05$).

Figure 2

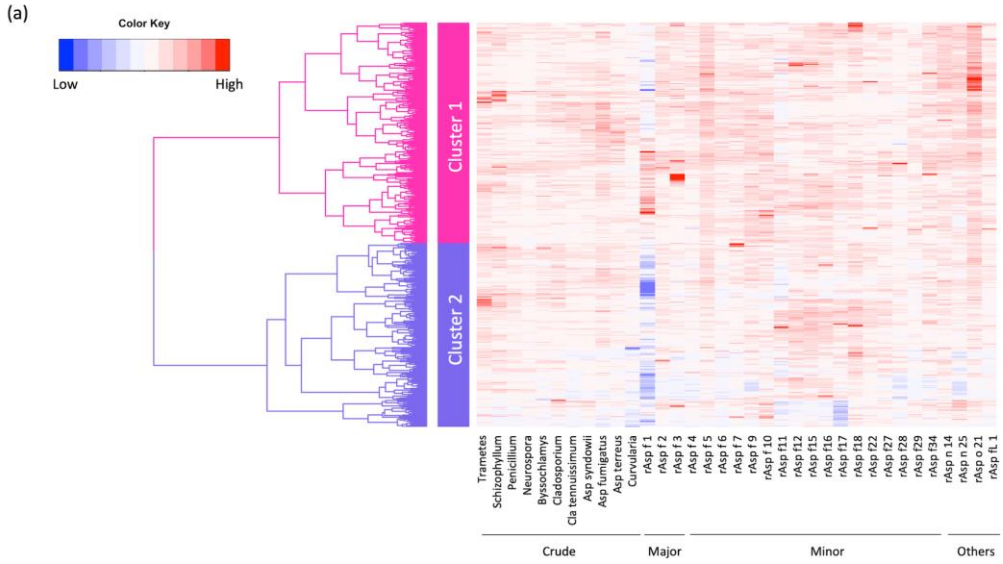
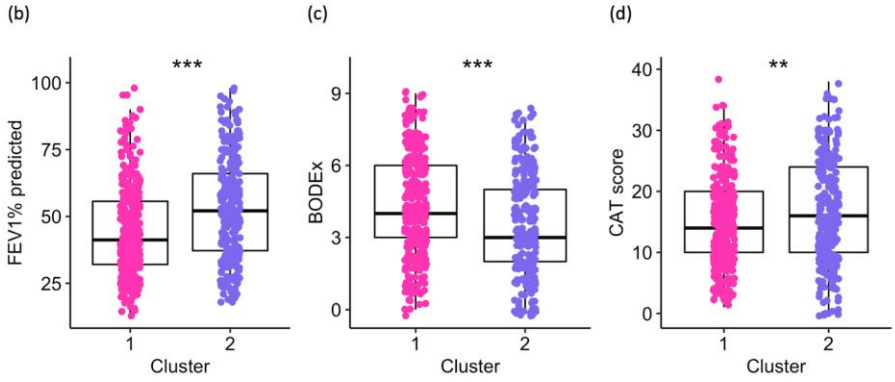


Figure 2: Unsupervised clustering of fungal allergens in COPD reveals two clusters characterized by significant differences in lung function, symptom burden and disease prognosis. (a) Heatmap demonstrating two fungal sensitisation clusters indicated by blue and red coloration respectively. Scatter box plots illustrating differences in (b) lung function (as FEV1 % predicted) (c) prognosis (as BODEx index) and (d) symptom burden (as CAT score) between clusters. (e) Forest plot illustrating the odds ratio (OR) fordetection of a sensitisation response to each respective fungal allergen by cluster membership. Clusters 1 and 2 are indicated by pink and purple colouration respectively. Red dots correspond to statistical significance ($p<0.05$) and error bars indicate the 95% confidence interval (CI). rAsp f: recombinant Aspergillus fumigatus allergen. *$p\leq0.05$, ***$p\leq0.001$. COPD: Chronic Obstructive Pulmonary Disease, FEV1: Forced Expiratory Volume in the 1st second, BODEx: B: Body mass index, O: airway obstruction (as EV1 % predicted), D: dyspnea (as mMRC score) and Ex: exacerbations, CAT: COPD assessment test, cla: Cladosporium rAsp f: recombinant Aspergillus fumigatus, fl: flavus, n: niger, o: oryzae.



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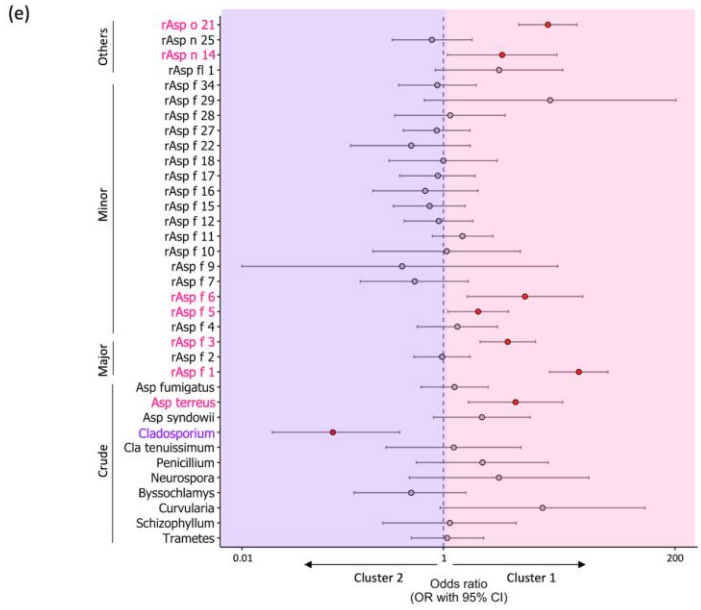

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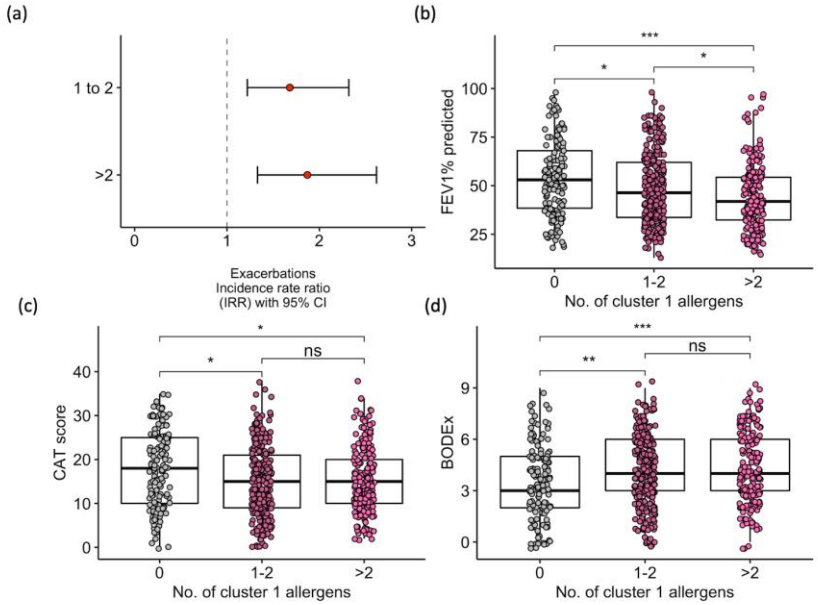

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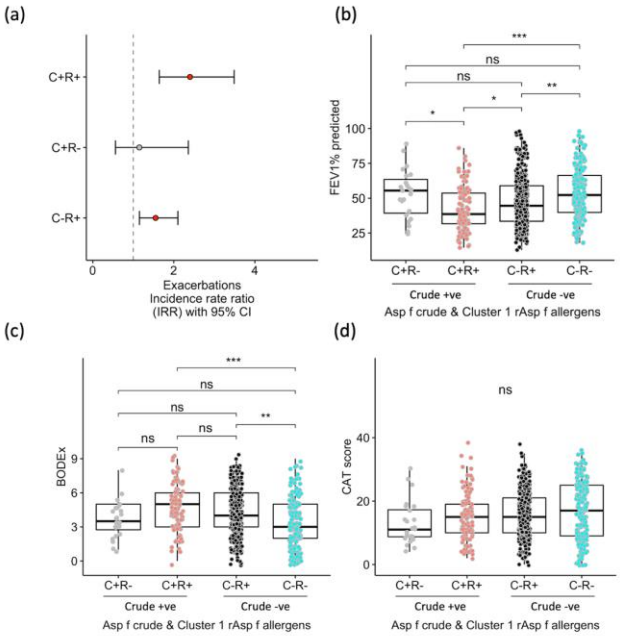

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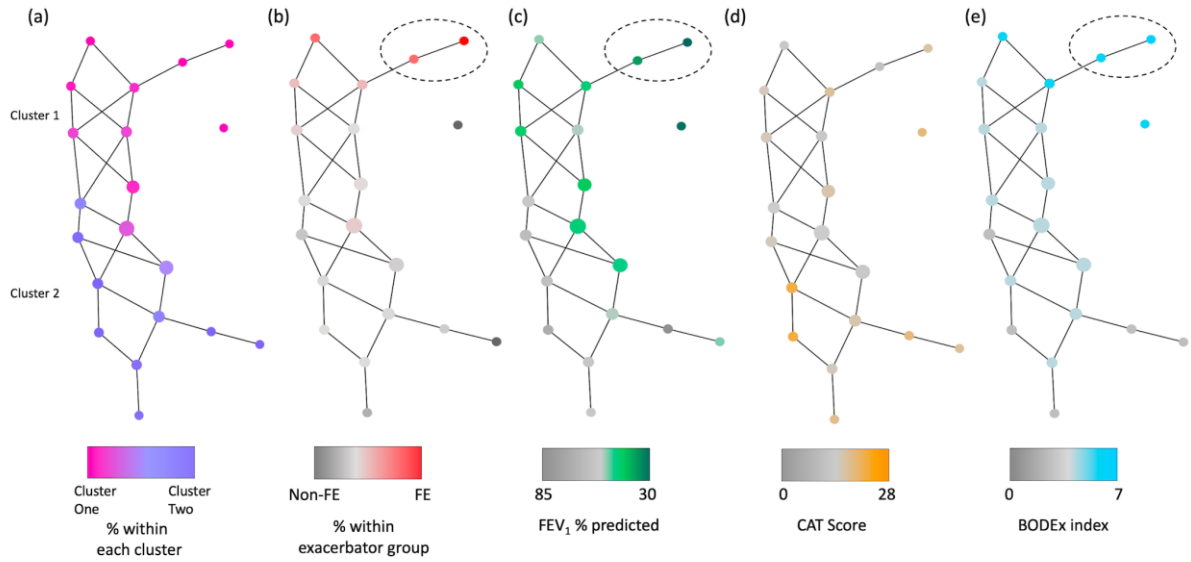

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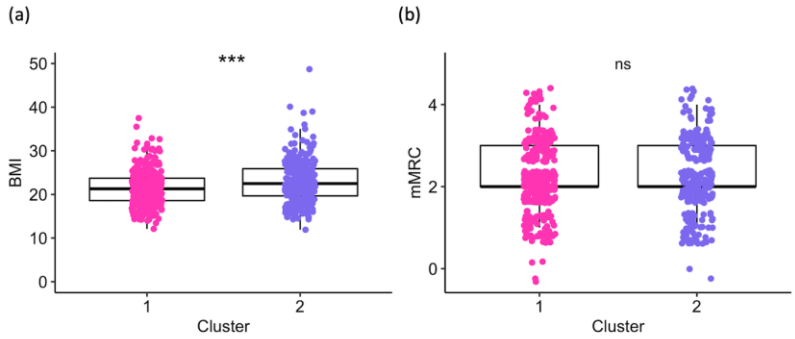

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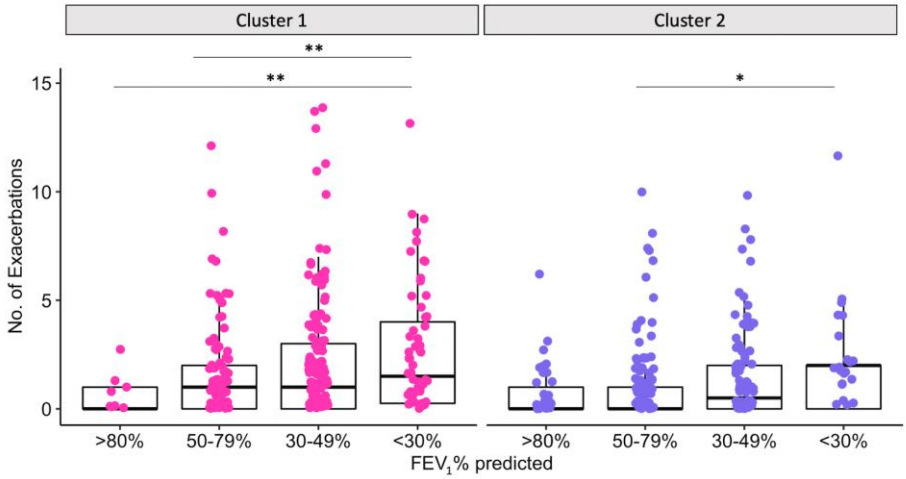

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