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

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Sensitisation to recombinant *Aspergillus fumigatus* allergens rAsp f 1, 3, 5 and 6 in COPD identifies a patient group with poor clinical outcomes missed by assessing for sensitisation to crude *Aspergillus fumigatus* allergens alone https://bit.ly/3zbZuFX

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Abstract

Background Variable clinical outcomes are reported with fungal sensitisation in chronic obstructive pulmonary disease (COPD), and it remains unclear which fungi and what allergens associate with the poorest outcomes. The use of recombinant as opposed to crude allergens for such assessment is unknown.

Methods A prospective multicentre 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 subgroups exist and their related clinical outcomes.

Results *Aspergillus fumigatus* sensitisation was associated with increased exacerbations in COPD. Unsupervised cluster analyses revealed two "fungal sensitisation" groups. The first was characterised by *Aspergillus* sensitisation and increased exacerbations, poorer lung function and worse prognosis. Polysensitisation in this group conferred even poorer outcome. The second group, characterised by *Cladosporium* sensitisation, was more symptomatic. Significant numbers of individuals demonstrated sensitisation responses to only recombinant (as opposed to crude) *A. fumigatus* allergens f 1, 3, 5 and 6, and exhibited increased exacerbations, poorer lung function and an overall worse prognosis. TDA validated these findings and additionally identified a subgroup within *Aspergillus*-sensitised COPD of patients with frequent exacerbations.

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 remains overlooked by assessment of only crude *Aspergillus* allergens.

Introduction

Fungal sensitisation is increasingly reported in individuals with chronic respiratory diseases, including asthma, chronic obstructive pulmonary disease (COPD) and bronchiectasis, where it is associated with persistent symptoms, increased disease severity and hospitalisations [1–7]. In COPD, our group and others

have demonstrated 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 colonisation and sensitisation to chronic infection and invasive aspergillosis, the latter associated with late diagnosis and high mortality [9–14]. Several individuals may also shift throughout their course of disease from one *Aspergillus*-associated disease to another depending on underlying host immunity. For instance, a sensitised COPD patient may have recurrent exacerbations that are treated with frequent oral corticosteroids, which dampens host immunity and subjects them to chronic or invasive consequences [15]. However, prior studies in COPD evaluating the relevance of fungal sensitisation, and in particular *Aspergillus* sensitisation, have reported differing outcomes, likely attributable to inherent variation in 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 routinely used in clinical practice to measure sensitisation responses; however, crude allergens lack specificity and remain highly variable between samples. Crude allergens are extracted from their natural source and, therefore, composition and quality are determined by the primary source, potential contamination and the 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 standardised, more reproducible and importantly can be used to differentiate cross-reactivity from co-sensitivity to other allergens, thus more accurately identifying a true triggering allergen of the sensitisation response [22, 23]. To date, 23 recombinant *A. 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 with the lack of data on 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 years with stable COPD diagnosed based on the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria with a forced expiratory volume in 1 s (FEV_1)/forced vital capacity (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.* dyspnoea, chronic cough or chronic sputum production, recurrent lower respiratory tract infections and wheeze) were prospectively recruited from five tertiary centres across three countries in Asia between 2012 and 2020 [26, 27]. Individuals with COPD were recruited from Singapore (three sites: Singapore General Hospital, Tan Tock Seng Hospital and Changi General Hospital), Malaysia (one site: University Malaya Medical Center, Kuala Lumpur) and Hong Kong (one 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 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 Working Group criteria were also excluded [28, 29]. A COPD exacerbation was defined as an acute worsening of respiratory symptoms with increased dyspnoea, cough, sputum purulence and/or volume or wheeze requiring treatment with antibiotics and/or corticosteroids. A non-diseased cohort of patients aged ⩾40 years with no respiratory symptoms and normal spirometry was 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 are presented in table 1. Clinical data collated included 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 never-smoker), exacerbations (as defined previously) and hospitalisation for COPD exacerbations in the preceding year including COPD treatment. Spirometry was performed according to American Thoracic Society/European Respiratory Society

TABLE 1 Demographics of the non-diseased and COPD cohorts

	Non-diseased	COPD
Subjects (n)	33	614
Age (years)	61 (58–63)	74 (68–79)
Male sex	11 (33.3)	589 (95.9)
BMI ($kg \cdot m^{-2}$)	23.6 (20.7–26.6)	21.9 (18.9–24.8)
Smoking status		
Current	3 (9.0)	364 (59.3)
Ex-smoker	2 (6.0)	246 (40.1)
Never-smoker	28 (85.0)	4 (0.6)
Smoking pack years	27 (15–33)	50 (40–68)
FEV_1 % predicted	93 (83–97)	47 (34–60)
FEV_1/FVC % predicted	83 (77–84)	51 (41–61)
Exacerbations in the past 12 months		
0–1	NA	400 (65.1)
>1	NA	214 (34.9)
Hospitalised exacerbation in the past 12 months		
Yes	NA	272 (44.3)
No	NA	342 (55.7)
CAT score [30]	NA	15 (10–22)
BODEx index [31]	NA	4 (2–6)
Blood eosinophil count ($\times 10^9 \cdot L^{-1}$)	NA	0.1 (0.0–0.3)
Total IgE ($IU \cdot mL^{-1}$)	NA	77.8 (17.6–291.1)
Treatment		
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 are presented as n (%) or median (IQR), unless otherwise indicated. COPD: chronic obstructive pulmonary disease; BMI: body mass index; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; CAT: COPD Assessment Test; BODEx: BMI (B), airway obstruction (as FEV_1 % predicted) (O), dyspnoea (as modified Medical Research Council score) (D) and exacerbations (Ex); SABA: short-acting β-agonist; SAMA: short-acting muscarinic antagonist; LABA: long-acting β-agonist; LAMA: long-acting muscarinic antagonist; ICS: inhaled corticosteroid; NA: not applicable.

guidelines [32]. A multidimensional prognostic index, using the BODEx index (B: body mass index (BMI); O: airway obstruction (as FEV_1 % predicted); D: dyspnoea (as modified Medical Research Council (mMRC) score); and Ex: exacerbations) was determined at recruitment [31, 33]. The number of COPD exacerbations was used to substitute for 6-min walk tests in the BODEx index because this has been shown to be of comparable prognostic value [31]. Frequent COPD exacerbators were defined as having two or more exacerbations in the year preceding study recruitment, and a hospitalised exacerbation was defined as a COPD exacerbation necessitating hospital admission [26, 34].

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 were non-normally distributed and are presented as medians with interquartile ranges (IQRs). 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, BMI and smoking status. Logistic regression was performed using the *glm* function in R. Unsupervised clustering was performed using Ward's minimum variance method for hierarchical clustering. Specific IgE values were 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 $p \leqslant 0.05$.

Topological data analysis (TDA) was performed using Mapper, a computational method to visualise the high-dimensional distribution or structure of input data with simplicial complexes [35]. 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 two 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 visualised as "percentage of positive class" (categorical) and the median (continuous) expression values for node colourings. Mapper was implemented using the KepplerMapper module in Python [36].

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

Results

Sensitisation to A. fumigatus *is associated with COPD exacerbations*

Demographics of the study cohorts are summarised in table 1. 614 individuals with COPD, recruited during clinical stability from five centres 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 was predominantly male (95.9%), with significant smoking rates (59.3%), a median FEV_1 % predicted of 47% and a median CAT score of 15. 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 (IQR): 1 (0–2), p=0.002) compared to house dust mite, pollen and cockroach sensitisation (figure 1a). To evaluate which fungi were specifically associated with COPD exacerbations, fungal sensitisation was categorised as *A. fumigatus* (n=130), non-fumigatus *Aspergillus* (n=373) or other fungi (n=157). Individuals with *A. fumigatus* sensitisation (IRR 3.48, 95% CI 1.38–8.75, p=0.01; median (IQR): 1 (0–2), p<0.001) (figure 1b), characterised predominantly by sensitisation responses to the major recombinant *A. fumigatus* (r Asp f) 1, 2 and 3 allergens (n=394), demonstrated the highest exacerbation frequency (IRR 1.62, 95% CI 1.17–2.23, p<0.001; median (IQR): 1 (0–3), p<0.001) (figure 1c). Taken together, these results 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

Because fungal sensitisation in COPD associates with increased exacerbations, we next evaluated if there are specific COPD patient groups, based on fungal sensitisation profile, 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 (tables 2 and 3 and figure 2a), where cluster 1 (n=335) demonstrated significantly increased exacerbations (IRR 1.59, 95% CI 1.24–2.04, p<0.001; median (IQR): 1 (0–3), p=0.001), hospitalised (severe) exacerbations (IRR 2, 95% CI 1.51–2.64, p<0.01; median (IQR): 1 (0–2), p<0.001), poorer lung function (median (IQR) FEV_1 % predicted: 41.2% (32.1–55.7%), p<0.001) and worse prognosis (median (IQR) BODEx index: 4 (3–6), p<0.001) but interestingly remained less

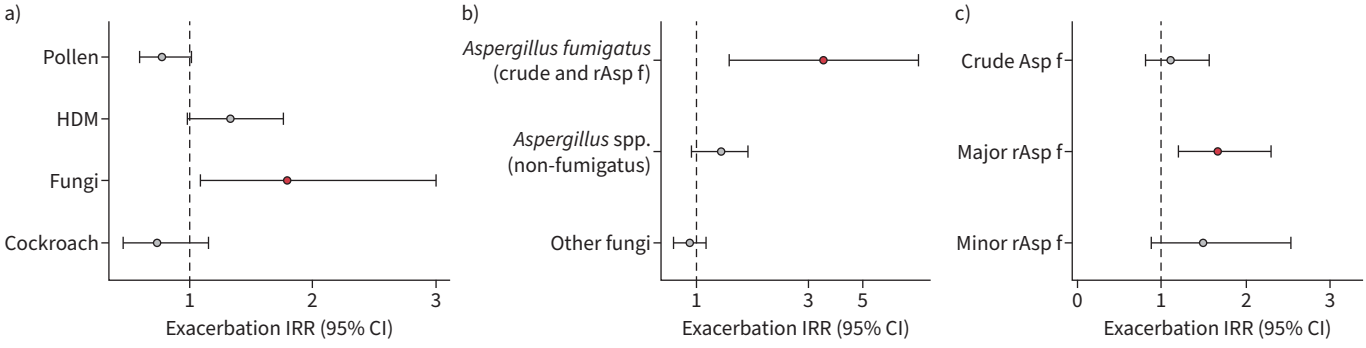

FIGURE 1 Fungal sensitisation, including sensitisation to major recombinant *Aspergillus fumigatus* allergens, significantly associates with a higher frequency of exacerbations in chronic obstructive pulmonary disease (COPD). Forest plots illustrating the incidence rate ratio (IRR) for COPD exacerbations with detectable sensitisation to a) key groups of established allergens, b) fungal allergens and c) *A. fumigatus* allergens. The error bars indicate the 95% confidence intervals and dots represent the IRR for COPD exacerbation. Red dots correspond to statistical significance (p<0.05). HDM: house dust mite; rAsp f: recombinant *Aspergillus fumigatus*.

TABLE 2 Panel of fungal allergens used in this study categorised as crude or recombinant

Crude allergens	Recombinant *A. fumigatus* (rAsp f) allergens	Recombinant *Aspergillus* (non-fumigatus) allergens
Aspergillus fumigatus	**rAsp f 1**	rAsp fl 1 (*A. flavus*)
A. terreus	**rAsp f 2**	rAsp n 14 (*A. niger*)
A. syndowii	**rAsp f 3**	rAsp n 25 (*A. niger*)
Trametes sanguineus	rAsp f 4	rAsp o 21 (*A. 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	

Major recombinant *A. fumigatus* allergens are indicated in bold. rAsp f: recombinant *Aspergillus fumigatus*.

symptomatic (median (IQR) CAT score: 14 (10–20), p=0.01) (figure 2b–d). Because prognosis by BODEx index differed between clusters, its contributing components were further assessed. This assessment revealed a significantly lower BMI in cluster 1 (p<0.001) but no difference in mMRC dyspnoea index (p=0.225) (supplementary figure E1). When patients were grouped by their GOLD stage (1–4) based on lung function (FEV_1 % predicted), increased exacerbation frequency was observed with decreasing lung function in both clusters (supplementary figure E2). Cluster 1, illustrating an adverse clinical profile, was characterised by increased sensitisation responses to *Aspergillus*-related allergens, specifically *A. terreus*; rAsp f 1, 3, 5 and 6; rAsp *niger* 14 and rAsp *oryza* 21, while cluster 2 (n=279), with higher symptomatic burden (median (IQR) CAT score: 16 (10–24), p=0.01) was 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 unrecognised specific recombinant fungal allergens that demonstrate clinical significance.

Polysensitisation to cluster 1-related allergens confers poorer clinical outcomes in COPD

Because cluster 1 demonstrated poor clinical outcomes from earlier analyses, 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 subgrouped by the number of cluster 1-related allergens to which the patient was sensitised as follows: non-sensitised (n=149), sensitised to one to two allergens (n=270) and sensitised to more than two allergens (n=198). The sensitisation profiles were then assessed in relation to clinical outcomes. Polysensitisation was associated with increased exacerbation frequency (IRR for one to two allergens 1.68, 95% CI 1.22–2.32, p=0.002; IRR for more than two allergens 1.87, 95% CI 1.33–2.62, p<0.001; figure 3a) (median (IQR) for non-sensitised: 0 (0–2); for one to two allergens: 1 (0–2); and for more than two allergens: 1 (0–3); p=0.001) and poorer lung function (median (IQR) FEV_1 % predicted for non-sensitised: 53% (38–68%); for one to two allergens: 46% (34–62%); and for more than two allergens: 42% (32–54%); p<0.001; figure 3b) relative to the non-sensitised group. Importantly, symptomatic burden did not relate to polysensitisation, with the highest CAT scores in the non-sensitised group (median (IQR) CAT score non-sensitised: 18 (10–25) *versus* one to two allergens: 15 (9–21) and more than two allergens: 15 (10–20); p=0.03; figure 3d). When prognosis (by BODEx index) was considered, sensitisation to at least one cluster 1-related allergen conferred a poorer prognosis and additional polysensitisation did not confer any additional risk (median (IQR) BODEx for non-sensitised: 3 (2–5) *versus* one to two allergens: 4 (3–6) and more than two allergens: 4 (3–6); p<0.001; figure 3c). Polysensitisation to cluster 1-related allergens therefore relates to poorer COPD outcomes, including higher exacerbation frequency and poorer lung function, but has no effect on symptoms or prognostic risk.

TABLE 3 Demographics of cluster 1 and cluster 2 fungal sensitisation groups

	Cluster 1	Cluster 2	p-value
Subjects (n)	335	279	
Age (years)	74 (68–79)	73 (69–79)	0.840
Male sex	325 (97.1)	264 (94.6)	0.198
BMI (kg·m^{-2})	21.3 (18.6–23.7)	22.5 (19.7–25.9)	<0.001
Smoking status			<0.001
Current	246 (73.4)	118 (42.3)	
Ex-smoker	86 (25.7)	160 (57.3)	
Never-smoker	3 (0.9)	1 (0.4)	
Smoking pack years	50 (38–65)	50 (40–80)	0.253
FEV_1 % predicted	41 (32–56)	52 (37–66)	<0.001
FEV_1/FVC % predicted	49 (40–59)	54 (45–65)	<0.001
Exacerbations in the past 12 months			0.001
0–1	204 (60.9)	196 (70.3)	
>1	131 (39.1)	83 (29.7)	
Hospitalised exacerbation in the past 12 months			0.002
Yes	167 (49.9)	175 (62.7)	
No	168 (50.1)	104 (37.3)	
CAT score [30]	14 (10–20)	16 (10–24)	0.010
BODEx index [31]	4 (3–6)	3 (2–5)	<0.001
Blood eosinophil count (×10^9·L^{-1})	0.1 (0.0–0.3)	0.1 (0.0–0.4)	0.128
Total IgE (IU·mL^{-1})	80.9 (19.2–250.6)	62.9 (11.3–408.6)	0.811
Anti-*Aspergillus* IgG (AU·mL^{-1})	0.22 (0.01–1.15)	0.03 (0–0.26)	0.008
Available HRCT thorax scans	55 (16.4)	190 (68.1)	
HRCT showing bronchiectasis	7 (12.7)	52 (27.4)	0.040
Treatment			<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)	

Data are presented as n (%) or median (IQR), unless otherwise indicated. BMI: body mass index; FEV_1: forced expiratory volume in the 1 s; FVC: forced vital capacity; CAT: COPD Assessment Test; BODEx: BMI (B), airway obstruction (as FEV_1 % predicted) (O), dyspnoea (as modified Medical Research Council score) (D) and exacerbations (Ex); Ig: immunoglobulin; HRCT: high-resolution computed tomography; SABA: short-acting β-agonist; SAMA: short-acting muscarinic antagonist; LABA: long-acting β-agonist; LAMA: long-acting muscarinic antagonist; ICS: inhaled corticosteroid.

Sensitisation to cluster 1-related recombinant *A. fumigatus* allergens rAsp f 1, 3, 5 and 6 uncovers significant clinical associations missed by only assessing the sensitisation response to crude *A. 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 *A. fumigatus* alone, the latter approach being the most frequently used in current clinical practice. Importantly, we uncovered a significant number of COPD patients without crude *A. fumigatus* sensitisation who demonstrated significant sensitisation responses to rAsp f 1, 3, 5 and/or 6 (n=309, 63.8%). To further investigate this phenomenon, we categorised patients into four groups based on sensitisation responses to crude and rAsp f 1, 3, 5 and/or 6 responses 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 *A. fumigatus* allergens was associated with 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 (IQR): 2 (0–3); and C^-R^+: IRR 1.55, 95% CI 1.15–2.10, p=0.004; median (IQR): 1 (0–2); relative to C^-R^-: median (IQR): 0 (0–2)). No significance difference in exacerbations was noted in the C^+R^- group (IRR 1.15, 95% CI 0.56–2.36, p=0.70; median (IQR): 1 (0–1)) relative to C^-R^- (figure 4a). Similar trends were observed for lung function (p<0.001) and prognosis (p<0.001), with the lowest lung function in recombinant *A. fumigatus*-sensitised groups independent of their crude sensitisation status (figure 4b, c). No significant differences were observed in relation to symptoms across all groups (figure 4d). Taken together, these data

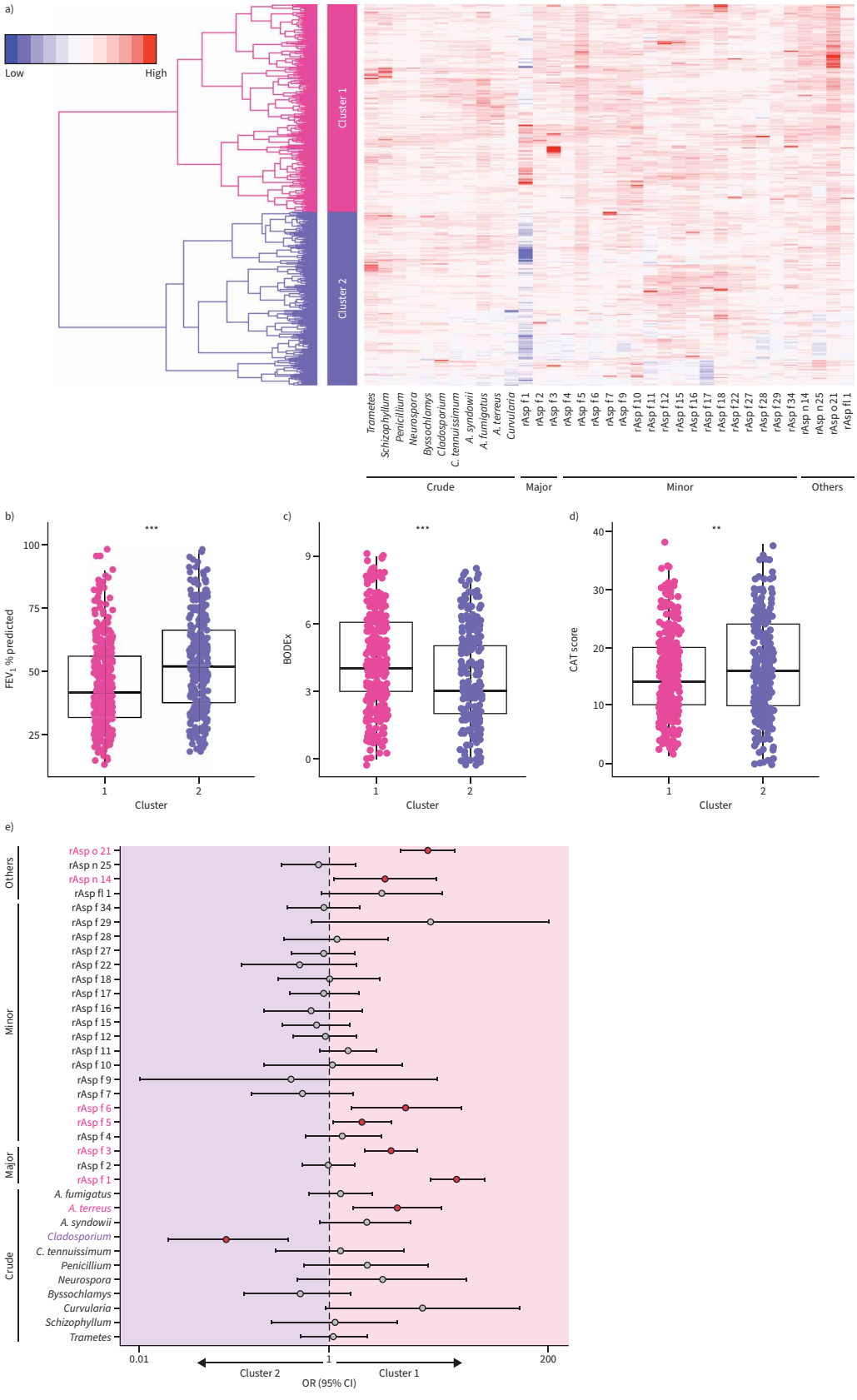

FIGURE 2 Unsupervised clustering of fungal allergens in chronic obstructive pulmonary disease revealed two clusters characterised by significant differences in lung function, symptom burden and disease prognosis.

a) Heatmap demonstrating two fungal sensitisation clusters indicated by blue and red colouration. b–d) Scatter box plots illustrating differences between clusters in lung function, as forced expiratory volume in 1 s (FEV_1) % predicted (b); prognosis, as BODEx index (body mass index (B), airway obstruction (as FEV_1 % predicted) (O), dyspnoea (as modified Medical Research Council score) (D) and exacerbations (Ex) (c); and symptom burden, as COPD Assessment Test (CAT) score (d). e) Forest plot illustrating the odds ratio for detecting a sensitisation response to each respective fungal allergen by cluster membership. Clusters 1 and 2 are indicated by pink and purple, respectively. Red dots correspond to statistical significance ($p<0.05$) and error bars indicate the 95% confidence intervals. rAsp f: recombinant *Aspergillus fumigatus*; n: *niger*; o: *oryzae*; fl: *flavus*. **: $p\leqslant0.01$; ***: $p\leqslant0.001$.

suggest the clinical importance of evaluating sensitisation responses to cluster 1-related recombinant *A. fumigatus* allergens rAsp f 1, 3, 5 and 6 in COPD. Significant numbers of patients appear to be missed by the practice of exclusively screening for sensitisation to crude *A. fumigatus*.

Validation of COPD fungal sensitisation clusters with TDA

Having identified two fungal-sensitisation COPD clusters by unsupervised approaches, we sought to independently validate these patient groups using an alternate analytical approach: 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 614 COPD patients studied. Each derived node represents a

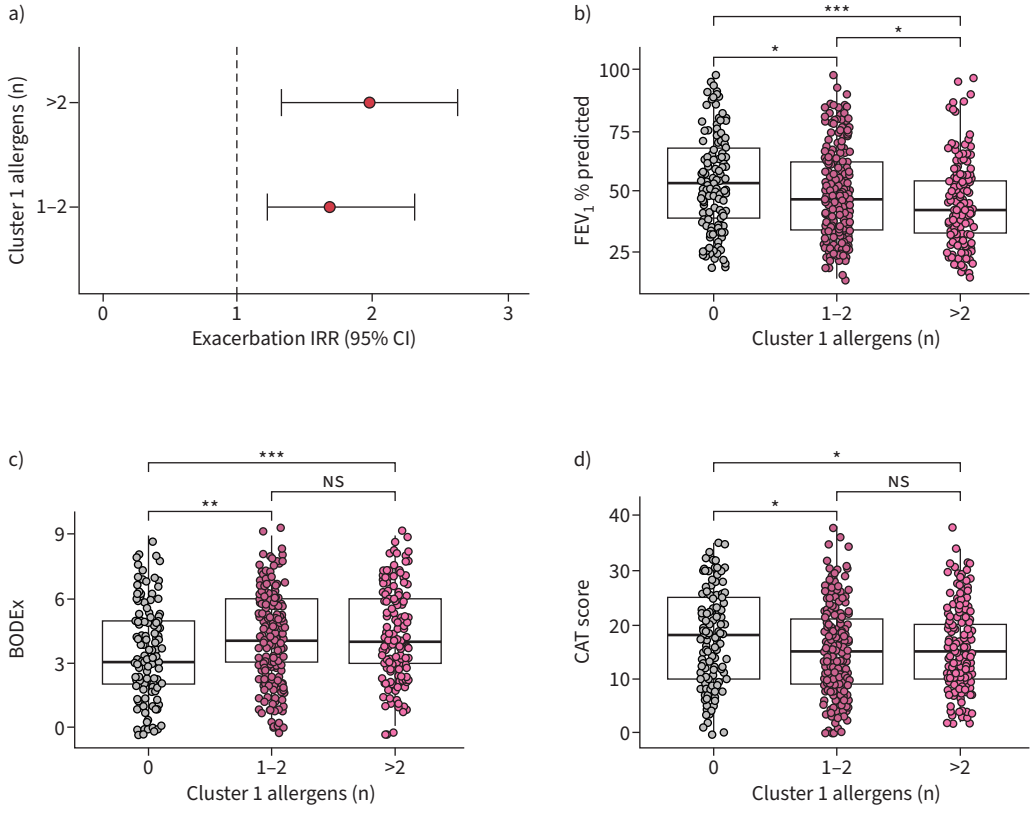

FIGURE 3 Polysensitisation to cluster 1-related allergens is associated with higher exacerbation frequency and poorer lung function in chronic obstructive pulmonary disease (COPD). a) Forest plot illustrating the incidence rate ratio (IRR) for COPD exacerbations in individuals demonstrating sensitisation to one to two or more than two 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. b–d) Scatter box plots illustrating differences in lung function, as forced expiratory volume in 1 s (FEV_1) % predicted (b); prognosis, as BODEx index (body mass index (B), airway obstruction (as FEV_1 % predicted) (O), dyspnoea (as modified Medical Research Council score) (D) and exacerbations (Ex) (c); and symptom burden, as COPD Assessment Test (CAT) score (d), between non-sensitised (*i.e.* no demonstrable sensitisation to any cluster 1-related allergens) and those polysensitised to either one to two or more than two cluster 1-related allergens. Error bars indicate 95% CI and dots represent IRR for a COPD exacerbation. NS: nonsignificant. *: $p\leqslant0.05$; **: $p\leqslant0.01$; ***: $p\leqslant0.001$.

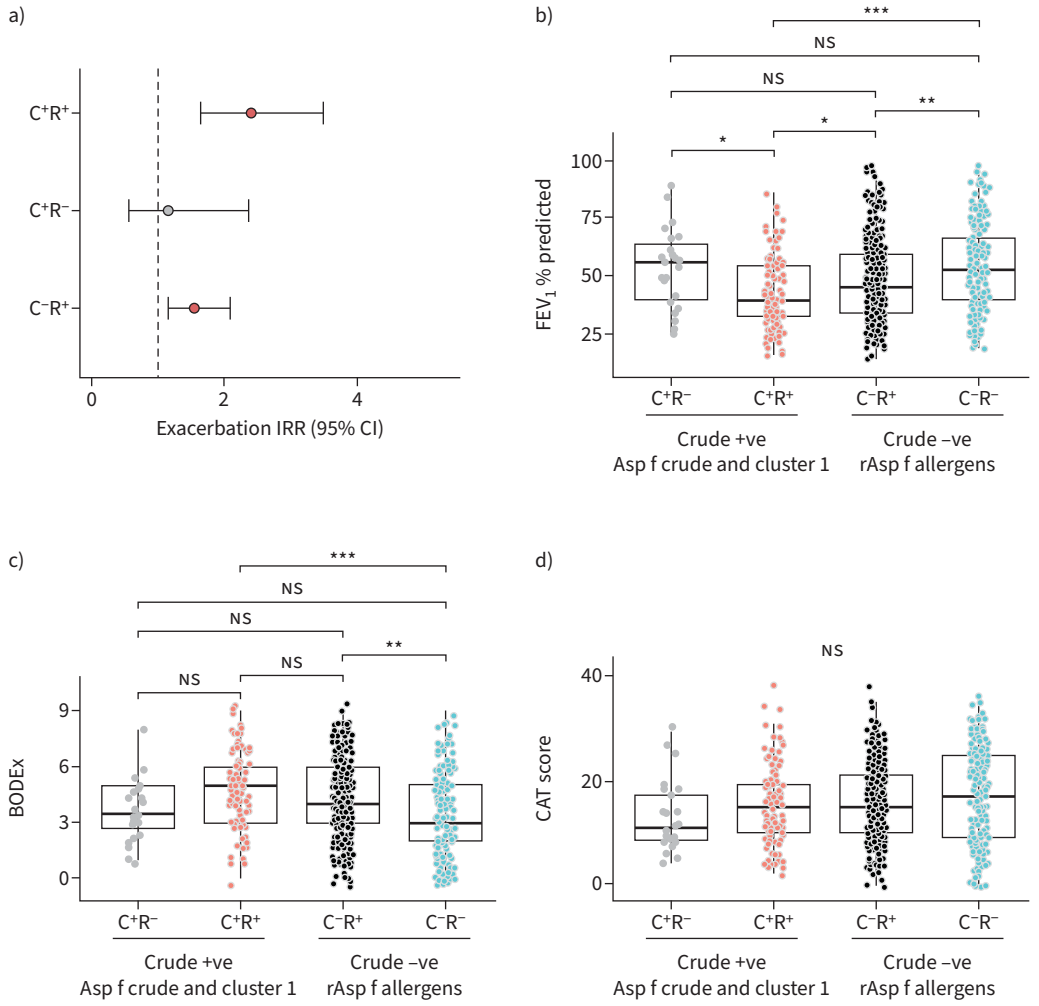

FIGURE 4 Sensitisation to cluster 1-related recombinant *Aspergillus fumigatus* allergens rAsp f 1, 3, 5 and 6 demonstrates clinical significance missed by assessment of the sensitisation response to only crude *A. fumigatus*. a) Forest plot illustrating the incidence rate ratio (IRR) for chronic obstructive pulmonary disease (COPD) exacerbations between individuals with demonstrable *A. fumigatus* sensitisation responses to crude allergens only (C^+R^-), recombinant allergens only (C^-R^+) and both (C^+R^+). b–d) Scatter box plots illustrating differences in lung function, as forced expiratory volume in 1 s (FEV_1) % predicted (b); prognosis, as BODEx index (body mass index (B), airway obstruction (as FEV_1 % predicted) (O), dyspnoea (as modified Medical Research Council score) (D) and exacerbations (Ex) (c); and symptom burden, as COPD Assessment Test (CAT) score (d), between combinations of individuals sensitised to crude (C) and recombinant (R) *A. fumigatus* allergens in the groups C^+R^-, C^+R^+, C^-R^+ and C^-R^-. Error bars indicate 95% CI and dots represent IRR for a COPD exacerbation where red colouration corresponds to statistical significance ($p<0.05$). rAsp f: recombinant *A. fumigatus* allergens; NS: nonsignificant. *: $p\leqslant0.05$; **: $p\leqslant0.01$; ***: $p\leqslant0.001$.

patient group, nodal size represents the patient number within that group, and lines between nodes correspond to the connections between patient groups. Colouration contrast was 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 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 circles). This subgroup demonstrated increased specific IgE sensitisation responses to rAsp f 3, 6 and 34.

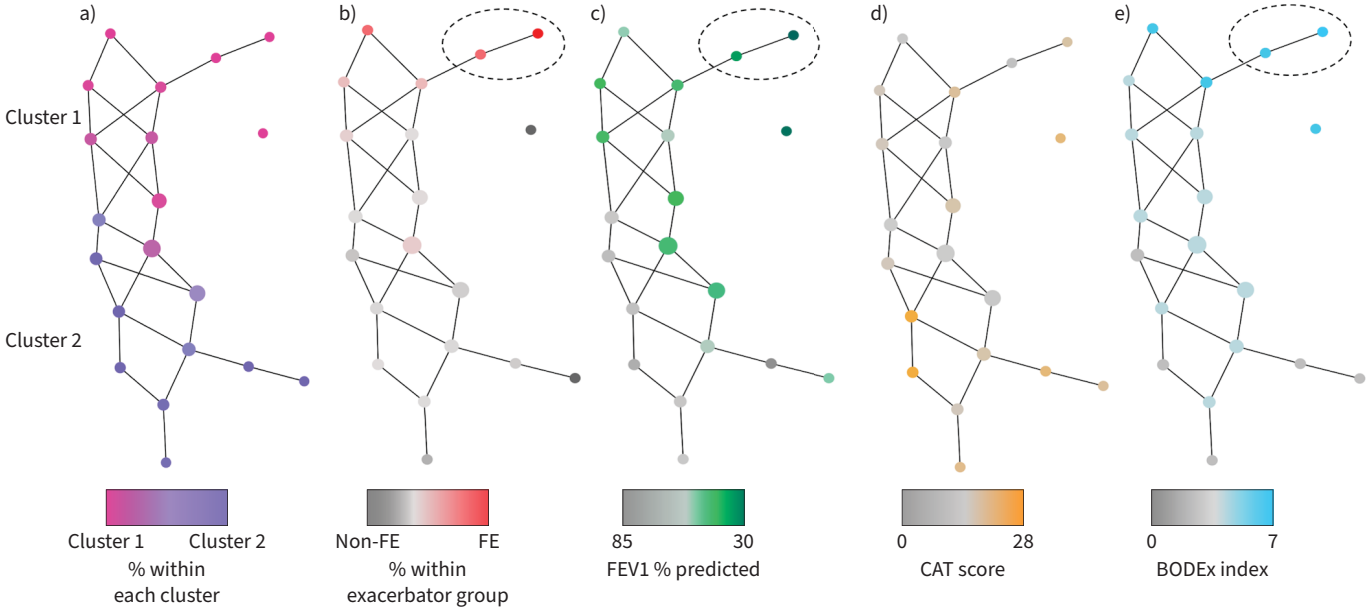

FIGURE 5 A topological data analysis (TDA) network including all crude and recombinant fungal allergens assessed in our comprehensive panel. The network illustrates connections between chronic obstructive pulmonary disease (COPD) patients based on their underlying sensitisation status and allergen pattern and coloured according to a) the established patient clusters (cluster 1: pink; cluster 2: purple); b) exacerbation frequency (grey to red for non-frequent exacerbators (FE) to FE); c) lung function, as forced expiratory volume in 1 s (FEV_1) % predicted (grey to green for high to low); d) symptom burden, as COPD Assessment Test (CAT) score (grey to orange for low to high); and e) prognosis, as BODEx index (body mass index (B), airway obstruction (as FEV_1 % predicted) (O), dyspnoea (as modified Medical Research Council score) (D) and exacerbations (Ex)) (grey to blue for low to high). Each node represents a group of COPD patients and nodal size represents 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 FE, lowest FEV_1 % predicted and highest BODEx index.

Discussion

Through our multicentre prospective study assessing a comprehensive panel of crude and recombinant fungal allergens, we evaluated the clinical relevance of fungal sensitisation, in particular *A. fumigatus* sensitisation in COPD. In addition, we assessed the clinical value of measuring sensitisation responses to recombinant *Aspergillus* allergens, in comparison to responses to crude allergens. Fungal sensitisation, and specifically *A. fumigatus* sensitisation, was associated with increased exacerbation frequency in COPD. Unsupervised analyses of the measured sensitisation responses revealed two "fungal sensitisation" groups. One group was characterised 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 only demonstrated sensitisation responses to recombinant *A. fumigatus* allergens f 1, 3, 5 and 6, and these patients exhibited increased exacerbations, poorer lung function and an overall worse prognosis. Therefore, even in the absence of a detectable sensitisation response to crude *Aspergillus* allergen, measuring responses to recombinant *A. fumigatus* allergens f 1, 3, 5 and 6 should be considered because they demonstrate clinical relevance in COPD. TDA corroborated our findings but additionally identified a further subgroup within *Aspergillus*-sensitised COPD that included an enrichment of frequent exacerbators with poorest lung function and prognosis. These individuals were characterised by increased sensitisation responses to two of the previously identified recombinant *A. fumigatus* allergens (rAsp f 3 and 6) but also the addition of a significant response to rAsp f 34. Taken together, our presented work, for the first time, reveals the importance of assessing sensitisation to recombinant *A. fumigatus* allergens in COPD.

Fungal sensitisation is associated with progressive and persistent symptoms, increased disease severity and reduced lung function in asthma, bronchiectasis and cystic fibrosis [3, 37–42]. 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, there is misalignment between the clinical fungal extracts available for assessment and the environmental fungi that have been detected, which potentially contributes to a likely under-diagnosis

of sensitisation response in respiratory disease and an underappreciation of its impact on clinical outcomes [43]. In COPD, and through next-generation sequencing of environmental samples including indoor and outdoor air, we have previously shown that a subgroup of COPD patients has measurable immunological responses to fungi extract within breathable air [1, 44]. There is a relationship between air fungi-sensitised COPD, including to *Aspergillus* species, and exacerbations, but the specific fungal types and optimal allergen screen to identify such patients remain unclear. In the current study, we advance specific fungi and provide a novel panel of recombinant allergens useful for screening that identifies 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 *A. fumigatus*, remains important because it is associated with negative clinical consequences in COPD.

Here, we describe two groups of fungal-sensitised COPD: one characterised by *Aspergillus* sensitisation and poor clinical outcomes and a second by *Cladosporium* sensitisation and significant symptomatic disease. In COPD, prevalence of *Aspergillus* sensitisation ranges from 7.9% to 18.3% with variable reported clinical outcomes [17]. Bafadhel *et al*. [45] report poor lung function in association with *Aspergillus* sensitisation, while Everaerts *et al*. [46] found no such association but a higher occurrence of bronchiectasis–COPD overlap. Studies in other Asian settings, specifically from China and India, similarly report no association between *Aspergillus* sensitisation and lung function decline in COPD [18, 19]. In our study, *Aspergillus* sensitisation occurred in 21% of individuals with COPD, which is slightly higher than the reported prevalence in the current literature. Importantly, we detected an association between *Aspergillus* sensitisation and higher exacerbation frequency in COPD, a feature absent in the non-sensitised cohort. Variations in earlier literature are likely attributable to cohort differences, which include geographic and potentially climatic factors. Furthermore, methodologies used to define a sensitisation response varied between previous studies with some focusing solely on a skin prick test response [47]. 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, because crude allergen extracts are viewed as being unstable, being easily contaminated and lacking in specificity, as well as having 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, 23 rAsp f allergens have been listed in the World Health Organization/International Union of Immunological Societies nomenclature; however, only eight (specifically rAsp f 1, 2, 3, 4, 6, 12, 15 and 17) have been previously assessed in respiratory disease [2, 8, 25, 48, 49]. Muthu *et al.* [49] report that use of crude *Aspergillus* allergens may lead towards misclassification of *Aspergillus* sensitisation and that rAsp f 1 and 2 are more specific for this diagnosis in asthmatic patients. Critically, rAsp f allergens are shown to distinguish *Aspergillus* sensitisation from ABPA across a number of respiratory diseases, including 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 are related to very frequent exacerbations (*i.e.* three or more COPD exacerbations annually) [1, 46, 50]. However, these prior works in COPD, while demonstrating clinical relevance, 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 because they associate with the poorest clinical outcomes, are not related to symptoms and cannot be identified by screening against crude *A. fumigatus* allergens alone.

TDA represents a set of mathematical methods used to study the shape and structure of high-dimensional datasets [51]. Mapper, a computational algorithm that forms part of the TDA suite of methodologies, uses dimensionality reduction followed by clustering to visualise high-dimensional datasets as networks [51, 52]. TDA has been used and validated across various clinical studies for the modelling and classification of patient subgroups and gene expression and for accessing genotype–phenotype relationships [51, 53]. 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, which was enriched for frequent COPD exacerbators with the poorest lung function and worst prognosis. These individuals were characterised 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 for 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. Because only cross-sectional assessment was performed, we were unable to ascertain longitudinal changes to both sensitisation state and clinical outcomes. While a higher number of *Aspergillus*-sensitised individuals were found to be using inhaled corticosteroids in our study, we were unable to ascertain from this dataset whether this benefited the sensitisation response or contributed to further fungal colonisation of the airway. Because computed tomography chest scanning was not standardised across centres 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 was male, and while characteristic of the demographics observed from prior Asian studies in COPD, this makes our findings less generalisable to female cohorts. The male predominance in Asian COPD cohorts may be a consequence of under-diagnosis of COPD in Asian women. All patients were recruited from tertiary specialist centres 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 to be a treatable trait in COPD. Measuring sensitisation responses to recombinant *Aspergillus* allergens identifies important subgroups of patients with poor COPD outcomes who remain overlooked by assessing only crude *Aspergillus* allergens. Screening of recombinant *Aspergillus* allergens f 1, 3, 5, 6 and 34 at an early disease stage may be helpful in identifying future high-risk COPD patients who will potentially experience frequent exacerbations and a 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 pathway will allow for the early identification of high-risk COPD phenotypes that will over the longer term reduce morbidity and mortality 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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Conflict of interest: P.Y. Tiew received honoraria for lectures and advisory board meetings paid to her hospital (Singapore General Hospital) from AstraZeneca, GlaxoSmithKline and Boehringer Ingelheim, outside the submitted work. M.S. Koh received research grant support from AstraZeneca, and honoraria for lectures and advisory board meetings paid to her hospital (Singapore General Hospital) from GlaxoSmithKline, AstraZeneca, Novartis, Sanofi and Boehringer Ingelheim, outside the submitted work. K. Tsaneva-Atanasova reports EPSRC grant EP/T017856/1. F.T. Chew reports personal fees for consultancy from Sime Darby Technology Centre, First Resources Ltd, Genting Plantation and Olam International, outside the submitted work. S.H. Chotirmall is on advisory boards for CSL Behring, Pneumagen Ltd and Boehringer Ingelheim, serves on a data and safety monitoring board for Inovio Pharmaceuticals Inc. and Imam Abdulrahman Bin Faisal University, and has received personal fees from AstraZeneca, all outside of the submitted work. All other authors have no conflicts of interest to declare.

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