The relationship between biomarkers of fungal allergy and lung damage in asthma.

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At a Glance Commentary: Sensitisation to fungal allergens is common in a severe asthma population. In this paper we demonstrate that IgE sensitisation to the thermotolerant filamentous fungi *A. fumigatus* and *P.chrysogenum* is associated with fixed airflow obstruction and radiological markers of lung damage, but a threshold level for total IgE was of limited value adding support for the argument to move away from the restrictive term of ABPA.

Abstract

Background: Immunological biomarkers are key to the diagnosis of allergic bronchopulmonary aspergillosis (ABPA) and fungal sensitisation, but how these relate to clinically relevant outcomes is unclear.

Objectives: To assess how fungal immunological biomarkers are related to fixed airflow obstruction and radiological abnormalities in moderate to severe asthma.

Methods: Cross-sectional study of 431 asthmatics. Inflammatory biomarkers, lung function and an IgE fungal panel to colonising filamentous fungi, yeasts and fungal aeroallergens were measured. CT scans were scored for the presence of radiological abnormalities. Factor analysis informed the variables used in a k-means cluster analysis. Fixed airflow obstruction and radiological abnormalities were then mapped to these immunological variables in the cluster analysis.

Results: 329 (76.3%) subjects were sensitised to ≥1 fungi. Sensitisation to *A. fumigatus* and/or *P.chrysogenum* was associated with a lower post-bronchodilator FEV_1 compared to those not sensitised to fungi ((73.0 (95%CI 70.2-76)vs 82.8 (95%CI 78.5-87.2) % predicted, p<0.001), independent of atopic status (p=0.005)), and an increased frequency of bronchiectasis (54.5%, p<0.001), tree-in-bud (18.7%, p<0.001) and collapse/consolidation (37.5%, p=0.002). Cluster analysis identified three clusters: (i) hypereosinophilic (n=71, 16.5%), (ii) high immunological biomarker load and high frequency of radiological abnormalities (n=34, 7.9%), (iii) low levels of fungal immunological biomarkers (n=326, 75.6%).

Conclusions: IgE sensitisation to thermotolerant filamentous fungi, in particular *A. fumigatus* but not total IgE, is associated with fixed airflow obstruction and a number of

radiological abnormalities in moderate to severe asthma. All patients with IgE sensitisation to *A. fumigatus* are at risk of lung damage irrespective of whether they meet the criteria for ABPA.

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Key words: asthma; lung damage, *Aspergillus fumigatus,* allergic fungal airway disease, ABPA.

Introduction.

Allergy to fungi is associated with asthma, particularly in its more severe manifestations (1). Thermotolerant fungi, typified by the filamentous *A. fumigatus* and the yeast *C. albicans*, can colonise the airways whilst fungi that are rarely able to grow at 37^{o}C such as *Alternaria alternata* and *Cladosporium herbarum*, primarily exacerbate asthma by acting as aeroallergens. While the health effects of these non-thermotolerant fungi are predictable and directly related to outdoor spore concentrations, the effects of thermotolerant fungi are more complex, heterogeneous and far reaching (2) . The most well described syndrome associated with fungal allergy is allergic bronchopulmonary aspergillosis (ABPA) (or mycosis [ABPM], when other fungal genera are considered to be causal). The term ABPA was first used by Hinsen et al to describe three cases of severe lung disease associated with allergy to *A. fumigatus* (3). A number of case series in the 1970s then described the clinical and immunological features of the condition (4). These included a diagnosis of asthma or cystic fibrosis, evidence of fleeting lung shadows on chest x-rays, a raised specific serum IgE (sIgE) and IgG to *A. fumigatus,* a high total IgE (>1000ng/ml or 417 IU/ml), proximal bronchiectasis and a blood eosinophilia. These anecdotal observations, without statistical underpinning, became established as a set of diagnostic criteria for ABPA (5-7). Although common in severe asthma only ~10% of asthmatics that are IgE sensitised to *A. fumigatus* fulfil the diagnostic criteria for ABPA. There is limited evidence that the criteria used to define ABPA appropriately select those at risk of disease progression or identify those who will respond to treatment. Total IgE has been given considerable prominence in the diagnosis of ABPA and a cut off of 1000 IU/l has been used to increase specificity, although the basis for this precise figure is not clear. The term severe asthma with fungal sensitization (SAFS) was defined to capture patients with asthma and fungal allergy who did not meet the criteria for

ABPA mainly because they had a total IgE <1000 IU/L. (8). Recent attempts have been made to revise the ABPA criteria to make them more clinically relevant (9). However these studies have generally started with the premise that ABPA represents a distinct subset of disease, which can be separated from the body of fungal allergy, rather than there being a continuous spectrum of disease with ABPA at the florid end. In the absence of a gold standard there is a danger of this approach being circular (10). The question that needs addressing is whether fungal allergy leads to distinct clinical problems, and if so, whether these are responsive to specific treatment(s). We, and others, have shown an association between IgE sensitization to *A. fumigatus*, fixed airflow obstruction and bronchiectasis (11, 12). In addition other radiological abnormalities including fleeting shadows, high attenuation mucus and lung fibrosis are a consistent feature of the clinical descriptions of fungal allergy in asthma (13-15). It remains unclear how the immunological biomarkers that are used to diagnose ABPA relate to specific disease outcomes, in terms of lung damage, and whether they clearly identify those with fungal allergy that are at risk of disease progression. We have therefore recruited a large cohort of asthmatics enriched for IgE-sensitisation to fungi to determine the relationship between immunological biomarkers of fungal allergy and evidence of lung damage in asthma, as defined by fixed airflow obstruction and radiological evidence of structural abnormalities.

Methods

Subjects

Data was collected from patients attending secondary and tertiary asthma clinics at Glenfield Hospital (Leicester, UK) from January 2011 to August 2015. Eligibility was based on a clinical diagnosis of asthma and evidence of variable airflow obstruction with either a positive

methacholine challenge test (20% drop in FEV_1 caused by <8mg/ml concentration of methacholine) or a >12% variability in FEV_1 spontaneously between clinic visits or following 200 µg of inhaled salbutamol. Asthma severity was graded using the Global Initiative for Asthma (GINA) 2012 criteria (www.ginasthma.org). Patients with a greater than 20 pack year smoking history were excluded from the study, as were patients receiving anti-fungal or immunomodulatory treatment (such as omalizumab), with the exception of glucocorticoids. Subjects were grouped as being sensitised to fungi if they had a positive specific IgE (>0.35kU/L; UniCAP250 system (Pharmacia, Milton Keynes, United Kingdom)) to any of the following fungal panel: *Aspergillus fumigatus*, *Penicillium chrysogenum*, *Candida albicans*, *Alternaria alternata* and *Cladosporium herbarum*. *Aspergillus* and *Penicillium* species were treated as thermotolerant filamentous fungi (many of these species readily grow at 37°C), and *Alternaria* and *Cladosporium* as non-thermotolerant filamentous fungi (most species will not grow at 37°C). Patterns of fungal sensitisation were grouped as follows: Group 1 = thermotolerant filamentous fungi (*A. fumigatus*, *P. chrysogenum*), Group 2 = thermotolerant yeasts (*C. albicans*), Group 3 = non-thermotolerant fungi (*A. alternata, C. herbarum*); Group 4 = not sensitised to fungi.

Atopic status was based on either a positive skin prick test (>3mm; ALK-Abelló, Høsholm, Denmark) or a positive specific IgE (>0.35kU/L) to *Dermatophagoides pteronyssinus*, dog, cat, grass pollen or tree pollen. Skin prick test data against common aeroallergens is regarded as the gold standard for measurement of atopic status but is relatively insensitive and poorly quantitative for detection of sensitisation to fungal allergens. Thus, specific IgE was used to designate sensitisation for those allergic to fungi, whereas atopic status was taken from skin prick test data unless this was not available, for example if a subject was taking antihistamines. All subjects within the study gave written informed consent for the use of

the clinical data for research purposes (REC reference 13/EM/0287) or were part of a service improvement project approved by University Hospitals of Leicester Research and Development Office (registration number: 7642e).

Clinical assessment

Demographic details (age, sex, BMI) and clinical data (age of asthma diagnosis, asthma duration, smoking status and pack year history, Asthma Control Questionnaire-6 scores (16), methacholine challenge test results and GINA status) were collected during a stable visit (at least six weeks post exacerbation). In addition, each subject was classified by their clinical asthma endotype (early-onset atopic, obese non-eosinophilic, early-onset symptom predominant, late onset inflammation predominant)(17). Spirometry was undertaken according to standardised methods by a trained physiologist using a dry bellows spirometer (Vitalograph Ltd, Maids Moreton, UK)(18). Post-bronchodilator FEV_1 was recorded as percentage predicted. Sputum induction was carried out in accordance with published methods (19). Induced sputum was used for cytospins to obtain differential cell counts but was not collected for fungal culture.

Radiological imaging

High resolution computerised tomography (HRCT) scans were undertaken in those with severe and/or difficult to treat asthma. Scans of the whole thorax (including both inspiratory and expiratory phases) were obtained using a Siemens Somaton Definition AS plus spiral scanner (Siemens Healthcare, Knoxville, TN). Scans were performed in a caudio-cranial direction using 1mm thickness slices at 0.7mm intervals.

The clinical radiology report was scored by one of the investigators (KW) (blinded to any of the immunological data) for the presence or absence of bronchiectasis, bronchial wall

thickness, air trapping, tree-in-bud, mucoid impaction, collapse/consolidation or fibrosis. Fleeting shadows were recognised by their presence on serial chest x-rays.

Data handling

Data was analysed using SPSS for Windows (version 22; SPSS, Inc., Chicago IL), GraphPad (version 6; GraphPad software Inc., La Jolla, CA) and SAS version 9.4 (www.sas.com). Descriptive statistics were expressed as mean (SD, 95% CI) or median (interquartile range). Two group analyses were analysed via an independent t-test and a Bonferroni-corrected one-way analysis of variance for multiple groups. Two group analyses were performed using the Mann-Whitney test and the Dunn-corrected Kruskall-Wallis test was used for multiple group comparisons for non-parametric data. Cluster analysis was performed in order to map clinical characteristics to the immunological variables and to see how these immunological biomarkers clustered together. A correlation matrix of these ten immunological variables is presented in supplementary table 1. The clinical importance of these immunological variables and factor loading scores from a principle component analysis (supplementary table 2) was used to reduce the number of these immunological variables prior to performing cluster analysis. This would enable us to see whether these immunological variables could be used to identify distinct clinical groups that would be at risk of lung damage.

Data underwent logarithmic and z-transformation in order to perform univariate analysis and to remove problems with unequal variance during cluster analysis. Factor analysis was used to identify the highest loading immunological variables. A varimax rotation was used in the generation of the factor analysis and a cut off value of >0.75 was applied to determine the factors which would represent each domain. The SPSS two-step algorithm was used to estimate the number of clusters within the studied population(20). This estimate was used

to specify the number of clusters present in a K-means cluster analysis, which was used as the principle clustering technique.

RESULTS

Descriptive Clinical Data

431 sets of clinical data were collected. 329 (76.3%) subjects were sensitised to one or more fungi and 102 (23.7%) were not sensitised to any fungi. These two groups were well matched in terms of age, gender, smoking status, pack year history and GINA score (see table 1). Those sensitised to one or more fungi were seen to have early onset, atopic asthma (52.9%) or late onset eosinophilic asthma (37.1%); whilst those that were not sensitised to any fungi had a slightly greater BMI ($p<0.05$) and had predominantly late onset eosinophilic disease (67.6%). 31.3% (n=103) of the group sensitised to one or more fungi and 49% (n=50) of the group not sensitised to fungi were taking maintenance oral prednisolone. 69.4% (n=299) of the cohort had a thoracic CT scan, of these 222 were sensitised to fungi (67% of those with fungal sensitisation) and 77 (75% of those without fungal sensitisation) were not sensitised to fungi.

Effect of fungal sensitisation and atopic status on lung function

A reduction in post-bronchodilator FEV_1 was seen in those sensitised to the thermotolerant filamentous fungi (group 1) (73.1% predicted (23.2; 70.2-76)) compared to (i) those that were sensitised to thermotolerant yeasts (group 2: 77% predicted (20.28; 71.2-82.9), $p=0.263$), (ii) subjects sensitised to non-thermotolerant fungi (group3: 85.2% predicted (19.68; 76.7-93.7), $p<0.05$), and (iv) those that were not sensitised to any fungi (group 4: 81.8% predicted (21.45; 78.5-87.2), $p<0.001$) (see figure 1a). In addition, IgE sensitisation to

A. fumigatus, independent of atopic status, was associated with a significantly lower post-bronchodilator FEV_1 compared to those that were just atopic (71.9 (23.98; 67-77) vs 83.7 (19.76; 79.1-88.2), p=0.005) (figure 1b).

Measured quantitatively there were weak correlations between the post-bronchodilator FEV_1 and sIgE to *A. fumigatus* (r -0.254, p<0.001), sIgE to *P. chrysogenum* (r -0.198, p<0.001), sIgE to *C. albicans* (r -0.112, p<0.05) and total IgE (r -0.117, p<0.05). Post-bronchodilator FEV_1 was also weakly correlated with sputum neutrophil count (r -0.203, p<0.001) and duration of asthma (r -0.249, p<0.001), which may suggest that the nature of this relationship maybe non-linear. No significant correlation was seen with sIgG to *A. fumigatus*, sputum/blood eosinophil count or any of the other immunological variables.

Radiological abnormalities & serum biomarkers of fungal sensitisation

Bronchiectasis, tree-in-bud appearances and the presence of collapse /consolidation were significantly increased in frequency in the group sensitised to fungi compared to those that were not (p<0.05; figure 2a). Group 1 (subjects sensitised to thermotolerant filamentous fungi) had an increased prevalence of bronchiectasis (p<0.001), tree-in-bud appearances (p<0.05) and collapse/consolidation (p<0.05) compared to any of the other groups (figure 2b). In addition, an increase in level of the immunological biomarkers of sensitisation to *A. fumigatus* was seen in association with several of the radiological abnormalities (table 2). A raised total IgE was observed in the presence of tree-in-bud appearances (p=0.007) and fleeting shadows (p<0.001). *A. fumigutus* IgE was associated with the development of bronchiectasis (p<0.001), tree-in-bud appearances (p<0.001), fleeting shadows (p<0.001), collapse / consolidation (p=0.002) and fibrosis (p=0.023). sIgG to *A. fumigatus* was increased in bronchiectasis (p=0.003), tree-in-bud abnormalities (p=0.024) and fleeting shadows (p=0.014). A higher prevalence of radiological abnormalities was seen in those sensitised to

the thermotolerant filamentous fungi (group 1). However, sensitisation to the thermotolerant yeasts (group 2) or the non-thermotolerant fungi (group 3) was not associated with any of the radiological abnormalities (figure 2b).

Blood eosinophilia was the only inflammatory biomarker to be related to any of the radiological markers of lung damage that were studied. A raised blood eosinophil count was associated with the presence of tree-in-bud abnormalities (1.85(2.95; 0.93-2.79) versus 0.99(1.06; 0.86-1.12) $x10^9$/L; p<0.001) and fibrosis (1.7 (3.38; 0.41-2.99) versus 1.04 (1.11; 0.91-1.18) $x10^9$/L; p=0.024). Sputum eosinophilia and neutrophilia were not associated with any radiological abnormalities.

Receiver operating characteristic curves, which were obtained on all of the radiological abnormalities, demonstrated that only two of the fungal immunological biomarkers were associated with statistically significant area under the curve (AUC) values. *A. fumigatus* IgE had an AUC value of 0.77 (p=0.001), giving a sensitivity of 81.5% and specificity of 63.3%, when a cut off value of 1.88kUA/L was used for the development of bronchiectasis. *A. fumigatus* IgG produced an AUC value of 0.79 (p<0.001), a sensitivity of 77.8% and a specificity of 60% when a cut off value of 26.39mg/L was used for the development of bronchiectasis. None of the other immunological variables had statistically significant AUC values >0.75 for any of the remaining radiological abnormalities.

Cluster membership of asthmatics sensitised to fungi as a predictor of outcomes

Factor analysis showed that the highest loading immunological variables in the data set were: Total IgE, *A. fumigatus* IgE, *A. fumigatus* IgG and sputum eosinophil count. These variables were used in a K-means cluster analysis to reveal three cluster populations. The baseline characteristics of these clusters are shown in table 3. These cluster population

were matched in terms of age, gender, BMI, GINA and ACQ6 scores. Asthma endotype among these clusters were very similar, with the majority in each cluster having either early-onset atopic disease (cluster 1: 47.1%, cluster 2: 42.2%, cluster 3: 43.6%) or late onset inflammatory asthma (cluster 1: 47.1%, cluster 2: 45.1%, cluster 3: 43.9%). Cluster 1 was slightly younger in asthma onset with a longer duration of disease compared to the other clusters. Cluster 1 was also more neutrophilic, whilst cluster 2 was more eosinophilic than any of the other clusters. A similar proportion of atopy was seen between all clusters. Cluster 1 had higher values for each of the fungal immunological variables compared to the other two clusters with a greater loss of lung function and a higher rate of radiological abnormalities. In addition, the numbers of cases of ABPA defined according to recent criteria were similar in clusters 1 and 3 (table 3).

DISCUSSION

This large cross sectional study has demonstrated that the association between fungal sensitisation and fixed airflow obstruction is limited to the thermotolerant filamentous fungi *A. fumigatus* and *P. chrysogenum* and is not associated with the thermotolerant yeast *C. albicans* or any of the non-thermotolerant fungi. Similarly, the relationship between airflow obstruction and sensitisation to *A. fumigatus* was not simply a function of atopy as patients who were atopic without fungal sensitisation had well maintained lung function. We also observed from the receiver operating curve that this link with lung damage was quantitative as well as a qualitative, although with limited specificity. This study provides further support of an association between IgE sensitisation to colonising filamentous fungi and the development of lung damage; as opposed to IgE sensitisation being simply an incidental

biomarker or a secondary phenomenon. In addition to previous studies, this study has confirmed the association between IgE sensitisation to *A. fumigatus*, fixed airflow obstruction and bronchiectasis (11).

Patients with IgE sensitisation to *A. fumigatus* exhibited a number of radiological abnormalities including fleeting lung shadows, collapse and consolidation, lung fibrosis and tree-in-bud changes. The first three are recognised features of fungal allergy, but the link to tree-in-bud abnormalities is less well recognised although noted in other studies (13).

Fleeting shadows, although now unusual, are a classical hallmark of ABPA. There was a strikingly high total IgE in patients with this abnormality. A possible link between all the radiological abnormalities is chronic obstruction of the airway with viscid mucus. Consistent with this others have reported that high attenuation mucus is an important feature of ABPA, although this was not specifically reported by the radiologists in our cohort (14).

The importance of the total IgE is emphasised in the criteria for the diagnosis of ABPA. However our data would suggest this is misplaced. High levels of IgE were seen with (the unusual) fleeting shadows and to a lesser extent tree-in-bud shadowing, but total IgE was not associated with fixed airflow obstruction or any of the other radiological abnormalities, either qualitatively as reported in table 2 or quantitatively in the receiver operating curves. A high total IgE can be a feature of sensitisation to skin commensals such as *Malassezia* spp. in atopic dermatitis which may confound interpretation of the total IgE. Total IgE does not therefore appear to be a particularly useful biomarker for the risk of lung damage due to fungal allergy. In contrast we did find an association between radiological evidence of raised *A. fumigatus* specific IgG and bronchiectasis, tree-in-bud changes and fleeting shadows, although the difference in concentrations between those with abnormalities and those without was not marked and the mean value fell below the normal cut off of 40mg/L. An

association with specific IgG to *A. fumigatus* and lung damage was also demonstrated in the ROC curve for bronchiectasis. However at optimum values the specificity of both *A. fumigatus* IgE and IgG for the presence of bronchiectasis was not particularly strong.

ABPA has had a dominant place in the conceptual understanding of fungal allergy in asthma since its characterisation in several case series in the 1960s and 1970s. This suggests that an identifiable syndrome must exist even if the robustness of the criteria for its diagnosis are overstated. To determine if this endotype of asthma could be objectively identified in our cohort we used cluster analysis with immunological criteria as the input variables. Three clusters were revealed, which apart from a slight difference in length of time since asthma was diagnosed, did not show any significant demographic differences. The largest cluster (cluster 3) had no striking immunological signature. Cluster two was identified by a very high sputum eosinophilia and may equate to the 'hypereosinophilic' asthmatics identified in our previous cluster analysis (17). Cluster 1 which represented about 10% of the fungal-sensitised asthmatics had a florid immunological signature with a very high total IgE, specific IgG and IgE to *A. fumigatus*, and polysensitisation to fungal allergens. They also had significantly higher rates of radiological abnormalities than the other two clusters. This group therefore equate to the patients that would be identified by the ABPA criteria, although it is important to stress that there is overlap with the other two groups with no clear distinction between them in terms of total IgE or *A. fumigatus* specific IgG or IgE. There are however clearly a small number of asthmatics who develop a florid immunological response to fungi and are at high risk of developing lung damage. As the immunological response is seen across several fungal genera it is likely to be related to the vigour of the host immune response to fungal allergens rather than fungal specific or environmental factors.

Asthma is heterogeneous and can be subdivided into endotypes with distinct clinical and molecular signatures although whether these represent genuinely distinct conditions or just reflect the variable way in which asthma presents remains unclear (21). Two major types of asthma are childhood onset atopic asthma and adult onset eosinophilic asthma, which is often non-atopic. There was no difference in the rate of fungal allergy in these two groups suggesting on this measure at least that they reflect different aspects of the same disease. Indeed it is striking that although hypereosinophilia, both in the blood or sputum, is a feature of allergic fungal airway disease it is not at all specific for fungal involvement in asthma, at least as measured by specific IgE.

In summary we have made two clinically important observations. Firstly we have demonstrated a significant association between IgE sensitisation to colonising thermotolerant filamentous fungi and the presence of lung damage as demonstrated by fixed airflow obstruction and a number of radiological abnormalities on HRCT scanning in moderate to severe asthma. We have found that the total IgE is neither a sensitive or specific biomarker of the likelihood of lung damage in asthma. Secondly, we have identified a small subset of patients with fungal allergy and asthma who have a florid immunological response to fungal allergens with polysensitisation, high levels of total IgE and *A. fumigatus*-specific IgG and IgE, poor lung function and high rates of lung damage. These patients equate to those described as having ABPA, although it is important to emphasise that no specific criteria can be usefully used to identify them. Taken together these two observations suggests that IgE sensitisation to thermotolerant filamentous fungi is a risk factor for the development of lung damage in asthma irrespective of whether the criteria for ABPA are present.

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FIGURE 1. **1a.** Post-bronchodilator FEV_1 (% predicted (means; SD, 95%CI)) compared between different sensitisation patterns. Those sensitised to *A. fumigatus* and *P.chrysogenum* (group 1; mean FEV_1 73.04% (23.17; 70.2-76)) were found to have a lower FEV_1 compared to asthmatics not sensitised to fungi (group 4; mean FEV_1 81.8% (21.45; 78.5-87.2), $p<0.001$). Sensitisation to *C. albicans* (group 2; mean FEV_1 77.03% (20.28; 71.2-82.9)) and sensitisation *A. alternaria* and *C. herbarum* (group 3; mean FEV_1 85.2% (19.68; 76.7-93.7)) were not linked to a reduced FEV_1 compared to group 4 ($p>0.05$).

1b. Post-bronchodilator FEV_1 (% predicted (means; SD, 95% CI) in subjects grouped as being (i) atopic and sensitised to *A. fumigatus* (n=147), (ii) atopic but not sensitised to *A. fumigatus* (n=80), (iii) sensitised to *A. fumigatus* but not atopic (n=95), (iv) not sensitised to *A. fumigatus* nor atopic (n=104). Sensitisation to *A.fumigatus* (AF), but not atopy was associated with a significantly lower FEV1 compared to those that were just atopic (71.9% (23.98; 67-77) vs 83.7% (19.76; 79.1-88.2)), $p<0.05$).

FIGURE 2. (a) An increased frequency of radiological abnormalities was seen in asthmatics sensitised to fungi compared to non-sensitised asthmatics. (b) The increase in HRCT scan abnormalities was dependent on the sensitisation status with the major increase seen in patients sensitised to thermotolerant filamentous fungi (group 1 = sensitised to *A. fumigatus* and/or *P. chrysogenum*; group 2 = sensitised to *C. albicans*; group 3 = sensitised to *Alternaria alternata* and/or *Cladosporium herbarum*; group 4 = not sensitised to fungi). The significance levels are shown on the x-axis.

TABLE 1. Baseline clinical characteristics of asthmatics sensitised to fungi and those not sensitised to fungi. *mean (SD, 95%CI), #median (interquartile range)

Characteristic	Not sensitised to fungi (n=102, 23.7%)	Sensitised to fungi (n=329, 76.3%)	Significance level (p value)
Age (years)*	52.8 (16.42; 43.7-58.7)	50.9 (18.35; 43.2-57.1)	0.346
Male, *n* (%)	47 (48.1%)	168 (39%)	0.379
BMI (kg/m^2)*	29.9 (6.6; 26.2-33.5)	28.4 (6.5; 26.8-32.7)	<0.05
Ex/current smokers, *n* (%)	35 (34.3)	88 (26.7)	0.18
Smoking pack years (years)*	8.2 (5.6; 5.1-10.1)	8.5 (6.19; 6-10.6)	0.9
Age of asthma diagnosis (years)#	39 (21-50)	7 (2-36)	<0.001
Duration of asthma (years)*	16.3 (15.59; 9.5-22.6)	31.9 (20.54; 21.8-38.9)	<0.001
GINA≥3, *n* (%)	94 (92.2%)	299 (90.9%)	0.545
ACQ 6 score*	2.21 (1.39; 1.7-3.1)	2.29 (1.29; 2.1-3.1)	0.721
Atopic status, *n* (%)	31 (30.4)	198 (60.2)	<0.001

<u>TABLE 2.</u> Serum biomarkers associated with the presence of radiological abnormalities.

[#]median (interquartile range)

		Total IgE (kU/L)[#]	*A. fumigatus* IgE (kUA/L)[#]	*A. fumigatus* IgG (mg/L)[#]
Bronchiectasis	Present	578 (152-1582)	3.3 (0.23-26.5)	35.00 (19.25-62.00)
	Not present	406 (116-1535)	0.21 (0.04-1.34)	23.05 (12.80-47.50)
	P value	0.257	**<0.001**	**0.003**
Tree in bud	Present	987 (260-2680)	8.53 (0.58-24.10)	39.75 (18.90-79.50)
	Not present	406 (132-1336)	0.48 (0.05-5.36)	27.20 (14.50-52.85)
	P value	**0.007**	**<0.001**	**0.024**
Fleeting shadows	Present	2324 (1395-5000)	32.15 (16.3-75.6)	74.10 (27.7-181.0)
	Not present	440 (134-1336)	0.55 (0.06-6.51)	27.60 (14.65-54.65)
	P value	**<0.001**	**<0.001**	**0.014**
Collapse / consolidation	Present	574 (154-1787)	1.34 (0.12-16.6)	30.60 (15.40-57.60)
	Not present	442 (132-1453)	0.42 (0.05-5.53)	28.00 (14.60-53.50)
	P value	0.450	**0.002**	0.173
Fibrosis	Present	872 (147-1787)	1.68 (0.49-36.50)	40.60 (21.30-71.70)
	Not present	427 (136-1535)	0.53 (0.06-6.59)	27.20 (14.70-53.50)
	P value	0.205	**0.023**	0.065

TABLE 3. Cluster populations and their relationship to immunological biomarkers, lung function and radiological abnormalities (ABPA = allergic bronchopulmonary aspergillosis; ISHAM = international society for human and animal mycology; SAFS = severe asthma with fungal sensitisation). *mean (SD; 95%CI); #median (interquartile range).

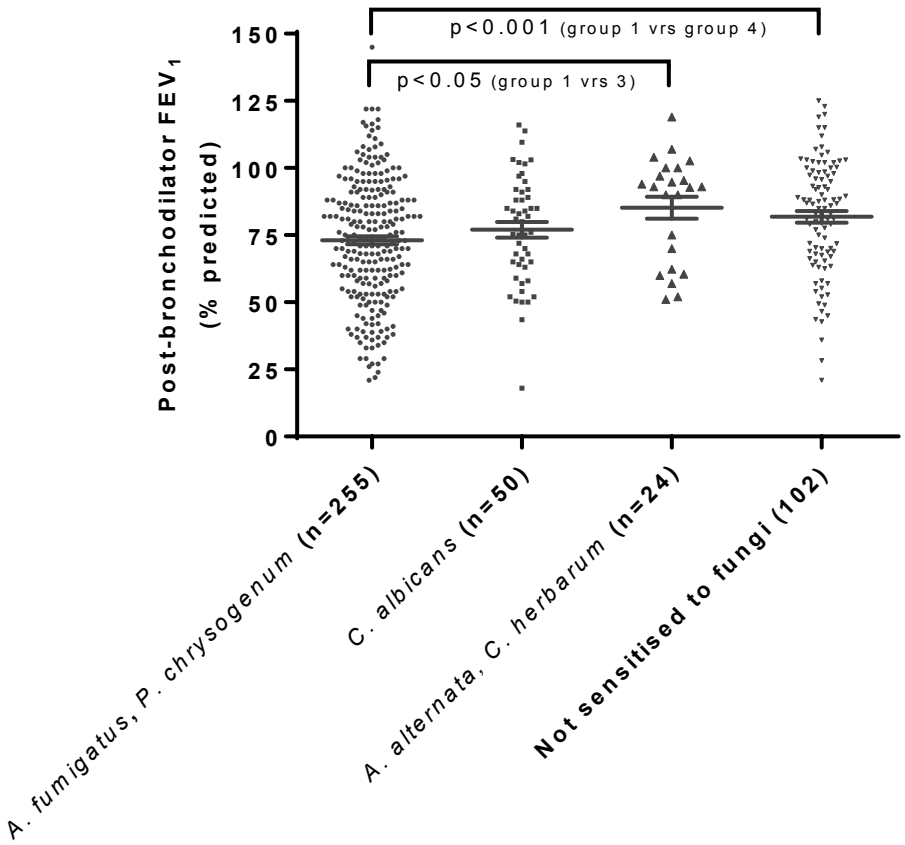
	Cluster 1 (n=34)	Cluster 2 (n=71)	Cluster 3 (n=326)	P value
Demographics				
Age (years)*	57.77 (15.02; 54.2-73.9)	51.78 (19.89; 41.6-58.6)	50.62 (17.65; 48.6-54.5)	0.084
gender (n, % male)	15 (44.1%)	36 (50.7%)	164 (50.3%)	0.781
BMI (kg/m^2)*	27.1 (7.3; 24.6-29)	29.2 (7; 25.4-30.2)	28.8 (6.4; 29.1-31.5)	0.264
Age of asthma diagnosis (years)#	6.0 (1-60)	18 (1-69)	19 (1-62)	0.528
Asthma duration (years)*	37.84 (23.39; 12.6-58.5)	28.68 (20.92; 13.6-28.1)	26.9 (19.93; 20.5-27.8)	0.014
GINA ≥3 (n, %)	34 (100%)	65 (91.5%)	294 (90.2%)	0.586
Maintenance steroids (n, %)	9 (26.5%)	25 (35.2%)	109 (33.4%)	0.586
ACQ6 score*	1.87 (1.13; 0.7-2.7)	2.15 (1.39; 1.3-2.5)	2.31 (1.33; 2-2.5)	0.512
Atopy (n, %)	19 (55.9%)	35 (49.3%)	175 (52.8%)	0.755
Immunological biomarkers				
Blood eosinophil count (x10^9/L)*	1.28 (0.94; 0.7-2)	0.98 (0.97; 0.77-1.69)	2.31 (1.38; 0.83-1.05)	0.419
Sputum neutrophils (%)*	80.37 (21.52; 52.68-99.36)	53.58 (31.31; 41.17-68.11)	75.73 (22.11; 74.63-82.05)	<0.001
Sputum eosinophils (%)#	8.25 (1.25-40.5)	58.25 (26.5-93.75)	4.25 (0-31)	<0.001
Fungal biomarkers				
Total IgE (kU/L)#	4669 (2023-5000)	279 (42-1791)	436 (32-5000)	<0.001

sIgE *A. fumigatus* (kUA/L)#	66.05 (27.8-100)	0.22 (0.01-24.9)	0.49 (0.02-21.6)	<0.001
sIgG *A. fumigatus* (mg/L)#	63.7 (12.3-200)	55.5 (5.4-200)	21.4 (4.1-87.4)	<0.001
sIgE *P. chrysogenum* (kUA/L)#	9.47 (1.6-30.7)	0.1 (0-4.7)	0.17 (0-6.09)	<0.001
sIgE *C. albicans* (kUA/L)#	5.44 (0.72-43.6)	0.24 (0.01-3.82)	0.45 (0-9.57)	<0.001
sIgE *A. alternata* (kUA/L)#	3.85 (0.22-23.75)	0.3 (0.03-8.64)	0.29 (0.02-8.2)	<0.001
sIgE *C. herbarum* (kUA/L)#	3.1 (0.31-13.74)	0.14 (0.01-4.98)	0.24 (0-16.2)	<0.001
Sensitisation group				
Sensitised to fungi (n, %)	33 (97.1%)	48 (67.6%)	248 (76.1%)	0.004
Group 1 (*A. fumigatus, P. chrysogenum*)	33 (97.1%)	34 (47.9%)	188 (57.7%)	
Group 2 *(C. albicans)*	0	10 (14.1%)	40 (12.3%)	
Group 3 (other fungi)	0	4 (5.6%)	20 (6.1%)	
Group 4 (not sensitised to fungi)	1 (2.9%)	23 (32.4%)	78 (23.9%)	
Overall group difference				<0.001
Lung function				
PBFEV$_1$ (% predicted)*	67.04 (23.13; 40.5-88.2)	76.14 (23.04; 64.3-85.8)	77.25 (22.3; 69.7-77.1)	0.043
Radiological abnormalities				
Bronchiectasis (n, %)	23 (79.3%)	17 (35.1%)	89 (40.1%)	<0.001
Tree-in-bud (n, %)	10 (34.5%)	7 (14.6%)	24 (10.8%)	0.002
Bronchial wall thickening (n, %)	16 (55.2%)	18 (37.5%)	106 (47.7%)	0.278
Mucoid impaction (n, %)	9 (31%)	12 (25%)	36 (16.2%)	0.084
Collapse/consolidation (n, %)	14 (48.3%)	19 (39.6%)	60 (27%)	0.026
Air trapping (n, %)	11 (37.9%)	15 (31.2%)	56 (25.2%)	0.286
Fleeting shadows (n, %)	6 (20.7%)	1 (2.1%)	3 (1.3%)	<0.001
Fibrosis (n, %)	7 (24.1%)	5 (10.4%)	17 (7.7%)	0.018

Cases of ABPA & SAFS				
ABPA ((ISHAM criteria) n, %)	27 (79.4%)	7 (9.9%)	29 (8.9%)	<0.001
SAFS (n, %)	0	13 (18.3%)	82 (25.2%)	<0.001

FIGURE 1.

1a.


p<0.001 (group 1 vrs group 4)
p<0.05 (group 1 vrs 3)
Post-bronchodilator FEV1 (% predicted)
150
125
100
75
50
25
0
A. fumigatus, P. chrysogenum (n=255)
C. albicans (n=50)
A. alternata, C. herbarum (n=24)
Not sensitised to fungi (102)

1b.

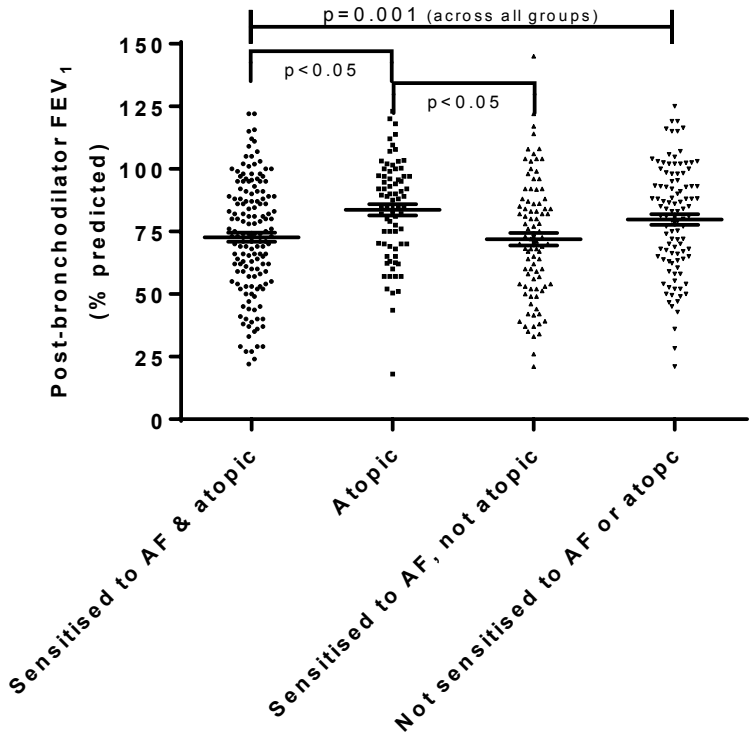
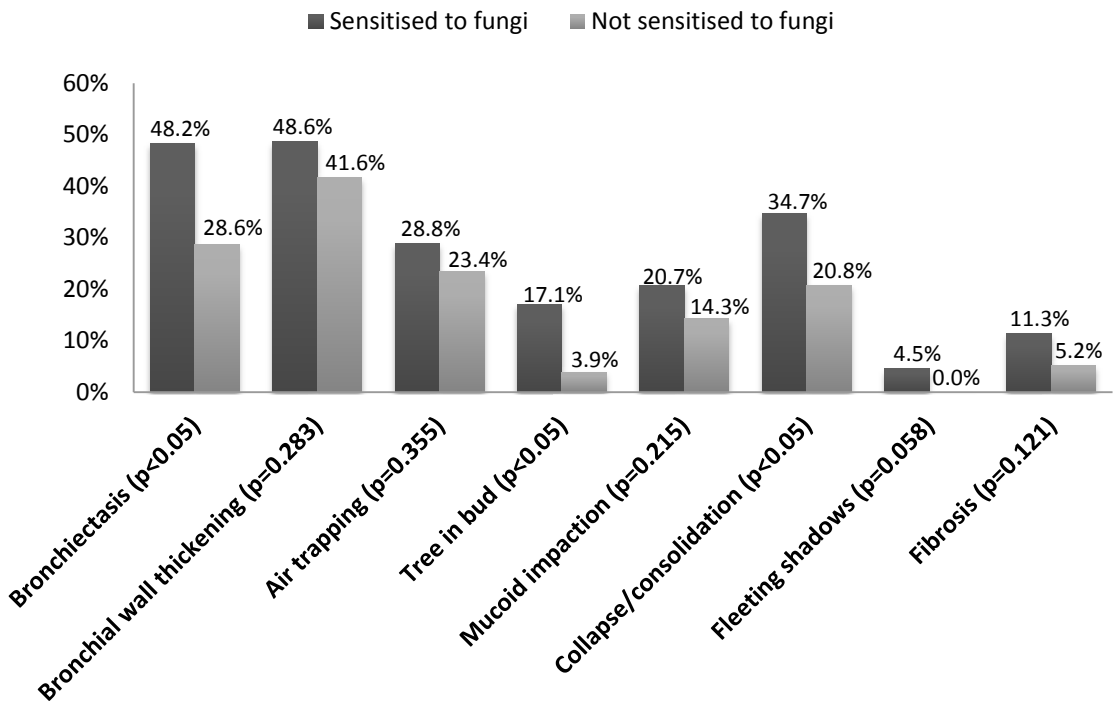

p=0.001 (across all groups)
p<0.05
p<0.05
Post-bronchodilator FEV1 (% predicted)
150
125
100
75
50
25
0
Sensitised to AF & atopic
Atopic
Sensitised to AF, not atopic
Not sensitised to AF or atopc

FIGURE 2.

2a.



2b.