

Fungal Sensitisation in Severe Asthma is Associated with the Identification of *Aspergillus fumigatus* in Sputum

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Abstract

Background: Fungal sensitisation is an important factor in severe asthma, not only for allergic bronchopulmonary aspergillosis (ABPA) but also the more recently described severe asthma with fungal sensitisation (SAFS). It is not known whether these diseases are driven by the presence of airway fungal colonisation. We aimed to determine if both SAFS and ABPA were associated with airway isolation of *Aspergillus fumigatus* and whether the frequency of isolation changed following anti-fungal treatment.

Methods: Sputum samples were collected from patients with SAFS, ABPA and a control group without fungal sensitisation. We recorded details of antifungal treatment, serum IgE and *A. fumigatus* specific IgE levels. In a subgroup (n = 9) we recorded serial sputum PCR measurements before and during itraconazole therapy.

Results: 244 sputum samples were provided by 135 patients, 41(17%) ABPA, 168(69%) SAFS, and 35(14%) controls. Sputum *Aspergillus fumigatus* PCR was positive in 61 SAFS patients (70%) and 6 ABPA patients (50%) not on anti-fungal treatment at the time of the test, compared to 3 (9%) in controls ($\chi^2 = 37.90, p < 0.001$). 19 patients with SAFS who were taking antifungal treatment (23%) were significantly less likely to be PCR positive than the 61 patients not on treatment (70%) ($\chi^2 = 36.66, p < 0.001$). All nine patients assessed serially during therapy had positive sputum PCR pre-treatment and all became negative during itraconazole treatment.

Conclusion: We have shown that isolation of fungus from the airway of severe asthma patients with fungal sensitisation is very common, supporting the hypothesis of a mechanistic link between fungal colonisation and sensitisation.

Introduction

Fungal exposure has long been recognised as a factor in asthma and other respiratory conditions, implicated in thunderstorm asthma and seasonal epidemics of high risk asthma events (1,2). The possible importance of airway colonisation with fungi and influence with regard to sensitisation remains controversial (3).

Phenotypes of fungal sensitised asthma have been defined: allergic bronchopulmonary aspergillosis (ABPA) requires a level of IgE above 1000 IU/mL and has been associated with evidence of airway colonisation identifiable in most patients; patients with severe asthma and fungal sensitisation (SAFS) have a lower total

IgE, and the role of airway colonisation is not clear. The low sensitivity of traditional culture techniques has hampered investigation of this, although recent improved techniques have been developed (4–8).

This controversy surrounding the role of airway fungus in these diseases has led to subsequent debate surrounding optimum treatment, in particular for SAFS. Denning et al demonstrated an improvement in health related quality of life in patients with SAFS treated with itraconazole for six months (9). Itraconazole, as well as being an antifungal, has a strong interaction with inhaled steroids enhancing systemic bioavailability and so it has been hypothesised that it is this steroid enhancing effect of itraconazole that could have resulted in the clinical response seen (10,11). A more recent study investigating three months treatment with voriconazole (which does not have steroid-potentiating effects) in patients whom colonisation had been indicated by sputum culture did not find any clinically relevant and sustained benefit at 12 months (12).

We hypothesised that *Aspergillus fumigatus* is present in the airways of severe asthma patients with fungal sensitisation, and could be detected using a highly sensitive and specific polymerase chain reaction (PCR) test (13–15). Low numbers of airway fungi might explain the previous difficulty in identifying colonisation using standard culture techniques, in SAFS compared to ABPA, especially as cultured based methods are poorly sensitive (8). We aimed to address this question by investigating data already obtained through our standard clinical practice in our regional severe asthma clinic, and to describe the prevalence of PCR positive samples for patients on and off anti-fungal treatment, by retrospectively identifying all patients who had samples collected and analysed. Secondly we aimed to document serial results in patients starting a course of itraconazole, who had an *Aspergillus* PCR test performed before the onset of therapy, by prospectively observing the impact of treatment on subsequent PCR tests. Whilst we recognise that SAFS is associated with a variety of fungi (16), ABPA is *aspergillus* specific therefore for the purposes of this study we investigated specifically *Aspergillus fumigatus* alone.

2. Methods

Study participants

Patients were classified as having severe asthma using the guidelines jointly defined by the American Thoracic Society and European Respiratory Society (17). Patients are classed as having severe disease if they are uncontrolled despite step 4 treatment or require step 5 management. (18)

In order for a patient to be considered fungal allergic they must have had a historical positive *Aspergillus fumigatus* specific IgE test >0.4 IU/mL or skin prick testing positivity 3 mm greater than negative control. Patients are classified as SAFS or ABPA on pre-determined criteria (see below).

All patients had their last total IgE measure identified prior to the onset of any anti-fungal therapy from review of clinical notes and pathology results (as itraconazole lowers total IgE levels with successful treatment). ABPA was defined if total IgE was ≥ 1000 IU/ml and patients met two of the following three criteria: positive serum IgE antibodies to *Aspergillus fumigatus*; the presence of bronchiectasis on high resolution CT scanning; a documented blood eosinophil count of >0.5 cells/nL (19–21). For a patient to be classified with SAFS they would not have met the criteria for ABPA but must still be classed as fungal allergic, with a total serum IgE <1000 IU/ml (5).

Controls were identified as patients who attended the severe asthma service at the University Hospital of South Manchester, had a sputum *Aspergillus fumigatus* PCR test but had a negative specific IgE and/or skin test to *Aspergillus fumigatus*.

Study Design

The cross sectional (retrospective) study arm comprised data from any patients having an *Aspergillus fumigatus* PCR test as part of their clinical care. Any patient attending either the National Aspergillosis Centre or the regional severe asthma clinic, (both based at the University Hospital of South Manchester) were available for inclusion if a clinical sample for sputum *Aspergillus* PCR had been performed at any time as part of their clinical evaluation. There are no specific criteria within the department which trigger a sputum PCR request. Further tests are then sent at the clinician's discretion.

The therapy response arm of the study identified all patients within the study period who had a diagnosis of SAFS or ABPA and who had provided a sputum PCR sample prior to initiation of treatment with itraconazole as part of their planned therapy and subsequently provided at least one further PCR test after the commencement of treatment.

Measurements

All patients within the study had demographic data collected, as well as: *Aspergillus* specific IgE, total serum IgE, high resolution CT abnormalities, and *Aspergillus fumigatus* sputum PCR results. All blood samples taken were analysed in the University Hospital of South Manchester biochemistry laboratory except for the *Aspergillus* specific IgE which were performed in the Immunology laboratory at Manchester Royal Infirmary for Immuno-Assay analysis. Sputum samples were obtained spontaneously and stored in specimen pots for transportation to the on-site Mycology laboratory.

The Mycology laboratory method for performing PCR is a two-step process involving DNA extraction and then Realtime PCR. The DNA extraction is performed using the Myconostica MycXtra fungal DNA Extraction Kit. The Realtime PCR uses the Myconostica MyAssay *Aspergillus* Kit (15,22). The reproducibility of the *Aspergillus* PCR testing results were not routinely checked nor were the samples also tested using sputum culture for comparison. The MyAssay detects a number of different species of *Aspergillus* spores and hyphae. However only the patients who were found to have *Aspergillus fumigatus* were included in the study.

The Myconostica MyAssay *Aspergillus* kit is reported to have a sensitivity and specificity of 94% and 77% respectively when compared to *Aspergillus* culture data. It is also reported to have a sensitivity and specificity of 94% and 91% respectively when compared to clinical diagnoses (22). Given the strength of the assay and for the purpose of the this study we assumed that presence of identifiable *Aspergillus fumigatus* through culture or *Aspergillus* PCR positivity, equated to colonisation, in the absence of direct signs/suggestion of invasion. It was not possible to assess if there were false positive results from sample contamination.

Data Analysis

Between-group comparisons for categorical data were performed using Chi squared tests. The statistical analysis was performed using Microsoft Excel.

Results

For the retrospective data analysis 135 patients had provided 244 sputum samples. The average patient age was 58 years and 61% were females, 60% of patients had SAFS, 20% ABPA and 20% were controls (See Table 1).

Table 1. Demographic data by disease classification. SAFS = Severe Asthma with Fungal Sensitisation; ABPA = Allergic Bronchopulmonary Aspergillosis; IQR = Interquartile range; FEV1 L = Forced Expiratory Volume in One Second; SD = Standard Deviation

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PCR positive samples were found in 28 (68%) and 80 (48%) samples from patients with ABPA and SAFS (both on and off treatment) respectively, compared to 3 (9%) samples from non-sensitised controls ($\chi^2 = 27.90$, 18.27 respectively $p < 0.001$).

In patients with SAFS tested only whilst not receiving active anti-fungal therapy the positive PCR rate was 70% ($n = 61$) in, compared to 23% ($n = 19$) in those on treatment. The off treatment PCR positivity was 50% ($n = 6$) in the ABPA population, lower than the rate of 76% ($n = 22$) seen in the on treatment population ($\chi^2 = 33.7$ $P < 0.001$) (See Table 2).

Table 2 Prevalence of PCR positivity by diagnosis (ABPA/SAFS) and in relation to whether patient was on or off with anti-fungal therapy at the time of the sputum test.

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We explored whether there was any association between bronchiectasis as reported on the most recent CT scan and Aspergillus PCR rate. 101 of the 135 patients had a CT scan within the study period (24 ABPA, 57 SAFS and 20 control patients). In the ABPA population 79% ($n = 19$) of patients had bronchiectasis, 61% ($n = 35$) in SAFS and 35% ($n = 7$) in the severe asthma controls. In ABPA, the sputum PCR positive patients had similar levels of bronchiectasis (84%) compared to those who were PCR sputum negative (73%). In the SAFS population 72% of those with an “ever positive” sputum sample had bronchiectasis compared to just 47% in those who had no positive samples ($\chi^2 = 4.68$, $P = 0.03$).

In the therapy response sub-study, all six SAFS and three ABPA patients provided PCR positive sputum samples prior to initiation of itraconazole. Samples from all nine became PCR negative after a period of treatment with itraconazole (treatment time prior to second sample varied from 3 - 36 months). Each patient's total serum IgE also declined (see table 3). A further patient who did not have sputum analysed by PCR pre-treatment, but who was PCR negative post-treatment, subsequently became PCR positive and had a rising serum IgE titre after treatment was discontinued.

Table 3 presents the Prospective Study Results

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4. Discussion

We believe this is the first study to explore the possibility of airway colonisation of severe asthma patients with fungi, using sputum PCR analysis in a clinical setting. The finding of a high colonisation rate has potential clinical, therapeutic and research implications.

The study has several limitations, largely due to its observational and “real life” nature. Firstly we acknowledge the use of total serum IgE's as the only criteria to differentiate between patients with SAFS and ABPA merits debate. The differentiation between ABPA and SAFS based on a potentially arbitrary threshold of a total serum IgE >1000 IU/ml, could clearly have led to misclassification. This figure has however been widely used in previous studies (19,23). Secondly the small study population and its single centre nature will need to be supported by larger multi-centre studies of prospectively characterised patients in order to prove the hypothesis theorised in this study. This should also be reinforced with careful assessment of contemporaneous clinical data including lung physiology, markers of airway inflammation and testing to distinguish between *Aspergillus* spores, hyphae and alternate fungi in the same patient cohort.

The high prevalence of 70% PCR positivity witnessed in patients in the absence of anti-fungal therapy supports the primary hypothesis of airway colonisation in the majority of patients with severe asthma and fungal sensitisation. The 70% PCR positive rate (off itraconazole treatment) compares well with the study by Fairs, which used extended culture technique and identified a prevalence rate of 63% positivity to culture in their *Aspergillus fumigatus* IgE sensitised cohort (24).

We have also identified that the presence of bronchiectasis is more likely to be associated with a positive *Aspergillus* PCR sample, compared to those patients without bronchiectasis, but only in the SAFS sub-population. This may be a consequence of definition, or could be due to higher fungal load in patients with areas of bronchiectasis. The small patient population in the secondary “therapy response” arm, supports the concept of relevant airway colonisation, with negative tests following therapy initiation in all nine patients followed prospectively. Further consistency is seen within the sub-analysis of the retrospective study based on analysis of whether the patients were on or off treatment with itraconazole when PCR tests were performed. The reduction in cross sectional rate of SAFS patient's PCR positive tests from 70% to 23% when on treatment reinforces the findings of the “therapy response” arm of the study. This is in contrast to the ABPA population, where fewer patients were PCR negative off treatment than on treatment. This may reflect lower levels of fungal burden and a greater ease of suppression/eradication of *Aspergillus fumigatus* in SAFS compared to those patients with ABPA.

One clinical question which has resulted from the study findings is the selection of patients to consider for itraconazole therapy. It could be argued that only patients who are both sensitised to *Aspergillus fumigatus* and have evidence of colonisation should be considered for therapy, especially bearing in mind the complexity of itraconazole therapy, side effects and requirement for careful monitoring. Denning reported a 60% response rate when patients were treated unselected for evidence of colonisation and clearly further studies should explore whether response to treatment is dependent on identification of active colonisation (6).

Conclusion

This study is the first study to our knowledge to find evidence of *Aspergillus fumigatus* colonisation using PCR technology in SAFS. We report a high prevalence of *Aspergillus fumigatus* PCR positivity in both ABPA and SAFS patients. The high prevalence of positivity in SAFS patients not receiving anti-fungal therapy and the conversion of all patients who were initiating treatment from PCR positive to PCR negative, supports

the hypothesis of active colonisation of the airways in patients with severe asthma with fungal sensitisation and a colonisation suppression or possible eradication effect of itraconazole as at least one part of its mechanism of action.

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	SAFS	ABPA	Controls
Number of Patients	82	27	26
Number of Sputum Samples provided	167	41	36
Age mean (SD)	59.4 (11.8)	57.7 (12.0)	52.7 (12.6)
Sex (M/F)	31/51	16/11	6/20
Mean FEV ₁ L(SD)	1.90 (0.72)	1.79 (0.82)	2.30 (0.83)
Number of patients with Bronchiectasis on High Resolution CT Scan	35 (64%)	18 (79%)	7 (35%)
Total IgE median IU/mL (IQR)	225 (405)	2200 (1450)	2.1 (0.0)
<i>Aspergillus fumigatus</i> IgE median IU/mL (IQR)	2.1 (7.5)	28.2 (39.6)	0.04 (0.0)
Blood eosinophils median cells/nL (IQR)	0.31 (0.35)	0.51 (0.44)	0.10 (0.07)

Sample Group	On Treatment		Off Treatment	
PCR Positive	PCR Negative	PCR Positive	PCR Negative	ABPA (n = 41)
22 (76%)	7 (24%)	6 (50%)	6 (50%)	SAFS (n = 168)
19 (23%)	62 (77%)	61 (70%)	26 (30%)	Control (n = 35)

Patient Number	Diagnosis	Pre Itraconazole PCR result	Pre Itraconazole IgE (IU/ml)	On Itraconazole PCR result	On Itraconazole IgE (IU/ml)
1	SAFS	2 Positive	30	1 Negative	12
2	SAFS	1 Positive	43	1 Negative	34
3	SAFS	1 Positive	780	8 Negative	510
4	SAFS	1 Positive	380	1 Weak Positive then 2 negative	140
5	SAFS	1 Positive	490	1 Negative	160
6	SAFS	1 Positive	250	1 Negative	86
7	ABPA	1 Positive	2300	1 Negative	1300
8	ABPA	1 Positive	1200	5 Negative	630
9	ABPA	4 Positive	4000	4 Negative	810