

IgE Sensitization to *Aspergillus fumigatus* Is Associated with Reduced Lung Function in Asthma

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Rationale: The importance of *Aspergillus fumigatus* sensitization and colonization of the airways in patients with asthma is unclear.

Objectives: To define the relationship between the clinical and laboratory features of *A. fumigatus*-associated asthma.

Methods: We studied 79 patients with asthma (89% classed as GINA 4 or 5) classified into 3 groups according to *A. fumigatus* sensitization: (1) IgE-sensitized (immediate cutaneous reactivity > 3 mm and/or IgE > 0.35 kU/L); (2) IgG-only-sensitized (IgG > 40 mg/L); and (3) nonsensitized. These were compared with 14 healthy control subjects. Sputum culture was focused toward detection of *A. fumigatus* and compared with clinical assessment data.

Measurements and Main Results: *A. fumigatus* was cultured from 63% of IgE-sensitized patients with asthma ($n = 40$), 39% of IgG-only-sensitized patients with asthma ($n = 13$), 31% of nonsensitized patients with asthma ($n = 26$) and 7% of healthy control subjects ($n = 14$). Patients sensitized to *A. fumigatus* compared with nonsensitized patients with asthma had lower lung function (postbronchodilator FEV₁ % predicted, mean [SEM]: 68 [±5]% versus 88 [±5]%; $P < 0.05$), more bronchiectasis (68% versus 35%; $P < 0.05$), and more sputum neutrophils (median [interquartile range]: 80.9 [50.1–94.1]% versus 49.5 [21.2–71.4]%; $P < 0.01$). In a multilinear regression model, *A. fumigatus*-IgE sensitization and sputum neutrophil differential cell count were important predictors of lung function ($P = 0.016$), supported by culture of *A. fumigatus* ($P = 0.046$) and eosinophil differential cell count ($P = 0.024$).

Conclusions: *A. fumigatus* detection in sputum is associated with *A. fumigatus*-IgE sensitization, neutrophilic airway inflammation, and reduced lung function. This supports the concept that development of fixed airflow obstruction in asthma is consequent upon the damaging effects of airway colonization with *A. fumigatus*.

Keywords: asthma; lung function; *Aspergillus fumigatus*; induced sputum; neutrophil

Airways colonization by fungi, predominantly by *Aspergillus fumigatus*, has been demonstrated in airways of both subjects with and those without asthma. Allergic bronchopulmonary aspergillosis (ABPA) is a florid hypersensitivity reaction to *A. fumigatus* colonization of the airways reported in up to 8% patients with asthma (1) and 13% of patients with cystic fibrosis (CF) (2); ABPA affects around 40,000 people in the United Kingdom (3).

Aspergillus spp. are ubiquitous within the indoor and outdoor environment, particularly in soil, decaying vegetation, and

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Aspergillus sensitization is linked with asthma severity. The relationship between *Aspergillus fumigatus* sensitization and sputum culture is not clear. The effect of colonization on airway inflammation and lung function in asthma is largely unknown.

What This Study Adds to the Field

This study demonstrates that a focused approach increases sensitivity of detection of *A. fumigatus* in sputum, and is associated with *A. fumigatus*-IgE sensitization. In patients with moderate to severe asthma, *A. fumigatus*-IgE-sensitized patients exhibit elevated neutrophilic airway inflammation and reduced lung function.

water-damaged building materials (4). Inhalation of *A. fumigatus* spores can lead to colonization and, in damaged airways with retained mucus, germination within the bronchial tree through the production of hyphae. In some individuals, this stimulates a T helper (Th) type 2-mediated inflammatory response involving CD4⁺ T cells, IgE, and IgG antibodies (5). Recurrent airway inflammation, bronchial obstruction, and mucoid impaction (2) lead to the development of bronchiectasis. ABPA is commonly diagnosed based on five primary criteria: (1) asthma; (2) elevated serum IgE (>1,000 ng/ml); (3) elevated *A. fumigatus*-IgE and/or *A. fumigatus*-IgG; (4) positive skin prick test (SPT) to *Aspergillus* spp.; and (5) presence of proximal bronchiectasis (4). Secondary diagnostic criteria include pulmonary and sputum eosinophilia and positive sputum culture for *A. fumigatus* (5, 6). Many patients with *A. fumigatus*-associated asthma (AFAA), however, do not fulfill all of the diagnostic criteria for ABPA.

Detection of *A. fumigatus* in respiratory samples is only used as a minor diagnostic criterion for ABPA, as isolation of *Aspergillus* from respiratory specimens is unusual. Studies of treatment of patients with ABPA with itraconazole, an antifungal agent, as a monotherapy or combined therapy with glucocorticosteroids, have shown promising results, with a significant overall response to 16 weeks of itraconazole in a multicenter study of 55 patients (7). In addition, reduced sputum eosinophils, fewer exacerbations requiring corticosteroid treatment, and reduced serum IgE were observed in a study of 29 patients (8, 9). In the absence of a definitive diagnostic tool for demonstrating airways colonization, determining the efficacy of these treatments in eliminating fungi from the airways is difficult. Moreover, the efficacy of these treatments in AFAA without a diagnosis of ABPA is unclear, although a trial of itraconazole in patients with fungal sensitization in severe asthma improved asthma-related

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quality of life (10). Through a focused approach toward detection of *A. fumigatus* in sputum, we aimed to define the relationship between the clinical and laboratory features of AFAA. Our most striking and unanticipated observation was an association between *A. fumigatus*-IgE sensitization and evidence of fixed airflow obstruction. Some of the results of these studies have been previously reported in the form of an abstract (11–13).

METHODS

Subjects

Patients with asthma were recruited consecutively by A.J.W from respiratory and allergy clinics at Glenfield Hospital (Leicester, UK) from August 2007 to April 2009. Inclusion required a clear clinical history of asthma, with either: airflow obstruction on prebronchodilator FEV₁ and historical evidence of greater than 12% variability in their FEV₁; or history of asthma with a greater than 12% improvement in FEV₁ 15 minutes after 200 µg inhaled albuterol and/or a provocative concentration of methacholine required to cause a 20% fall in FEV₁ of less than 8 mg/ml at the time of recruitment. Exclusion criteria included a main respiratory diagnosis other than asthma or inability to produce sputum. Healthy subjects were recruited from staff at Glenfield Hospital. Subject groups were: (1) *A. fumigatus*-IgE (>0.35 kU/L or positive SPT to *A. fumigatus* wheal, 3 mm in diameter); (2) *A. fumigatus*-IgG only (>40 mg/L); (3) nonsensitized asthma (negative *A. fumigatus*-IgE and *A. fumigatus*-IgG <40 mg/L); and (4) healthy subjects. Asthma severity was assessed using the Global Initiative for Asthma (GINA) 2009 criteria (www.ginasthma.com). Refractory asthma was defined according to the American Thoracic Society Workshop definition (14). All subjects gave written informed consent, and the study was approved by the Leicestershire and Rutland ethics committee.

Clinical Assessment

The following clinical data were collected: sex, age at asthma onset, asthma duration, physiological parameters of spirometry (Vitalograph Gold Standard, Vitalograph Ltd, Maids Moreton, UK), sputum eosinophil, neutrophil and macrophage differential cell counts, smoking history, radiological evidence of bronchiectasis, and prescribed inhaled and systemic corticosteroid therapy. Inhaled corticosteroid doses were standardized to fluticasone (0.5 µg fluticasone = 1.0 µg budesonide = 1.0 µg beclomethasone dipropionate).

CF genotype was requested from the local clinical genetics service.

Pulmonary Function Testing and Methacholine Challenge Testing

Spirometry was measured using standard methods (15). Airway hyper-responsiveness was assessed as previously described using less than 8 mg/ml as a cut off (16). Reversibility was measured as change in FEV₁ 15 minutes after 200 µg albuterol.

Cross-Sectional Imaging

High-resolution computed tomography of the thorax was performed using a Picker PQS (Picker International, Cleveland, OH) or Siemens sensation 16 scanner (Siemens Healthcare, Knoxville, TN). Cross-sectional images were obtained using settings of 1-mm collimation at 10-mm intervals in full inspiration. Bronchiectasis was deemed to be present where reported or if there was bronchial dilatation (internal bronchial diameter greater than the accompanying pulmonary artery). Diagnosis of bronchiectasis was based on the radiologist's clinical report.

Allergy Testing

Atopy was assessed using SPTs to common aeroallergens, including grass pollen, dog and cat fur, *Dermatophagoides pteronyssinus*, and a fungal panel of *A. fumigatus*, *Alternaria alternata*, *Botrytis cinerea*, *Cladosporium herbarum* and *Penicillium chrysogenum* (Alk-Abello, Denmark). Total IgE, *A. fumigatus*-IgE, and *A. fumigatus*-IgG levels were measured using the UniCAP 250 system (Pharmacia, Milton Keynes, UK). Sensitization to *A. fumigatus* was defined as

positive where *A. fumigatus*-IgE was greater than 0.35 kU/L and *A. fumigatus*-IgG greater than 40 mg/L, according to the manufacturer's instructions.

Sputum Induction, Processing, and Mycology

Sputum induction was performed as described previously (15, 17, 18). Sputum plugs were separated from saliva and divided into two parts. The first part was used for cytopins for a differential inflammatory cell count (15). The second part was used for mycological culture. Aliquots of undiluted sputum plug of approximately 170 mg (±80 mg) were inoculated on potato dextrose agar (PDA) containing 16 µg/ml chloramphenicol, 4 µg/ml gentamicin, and 5 µg/ml fluconazole. All plates were incubated at 37°C and inspected frequently for up to 7 days. Subcultures of filamentous fungi were produced and *A. fumigatus* colonies identified based on criteria for macroscopic morphology of colonies (19).

Repeatability of sputum culture was analyzed in a subset of patients, where sputum was obtained on more than one visit when the patient was clinically stable. Data from subsequent visits was only used to assess repeatability, and was not included in the main study results.

Data Handling

All data were entered electronically into a secure Access database (Microsoft, Redmond, WA) and analyzed using GraphPad (version 5; GraphPad Software Inc., La Jolla, CA) and SPSS for Windows (version 11.0; SPSS, Inc., Chicago, IL). Before data analysis, laboratory personnel were blinded to clinical characteristics of patients.

Parametric data was expressed as means (±SEM) and analyzed by Bonferroni-corrected one-way analysis of variance. Nonparametric data were expressed as medians with interquartile ranges and analyzed using Mann-Whitney, Chi-square, and Dunn-corrected Kruskal-Wallis tests.

Kappa statistics were performed on the data using MedCalc for Windows (version 9.5.4.0; MedCalc Software, Mariakerke, Belgium).

Clinical data were investigated *a priori* to create a multilinear regression model using the enter method for prediction of FEV₁ (% predicted, postbronchodilator). For seven subjects, only prebronchodilator data were used, as postbronchodilator FEV₁ were not available. For the comparison of FEV₁, including the regression model, only data from the asthma groups were used.

RESULTS

Patient Demographic Data

The numbers of patients recruited to each group and their clinical characteristics are shown in Table 1. Sex and age were well matched within the asthma groups, but healthy subjects were significantly younger ($P < 0.0001$). There was no significant difference in age, and no relationship between age and *A. fumigatus*-IgE sensitization in the asthma population (data not shown). The ratio of never-smokers to subjects with a smoking history was highest in the healthy subjects, but well matched among the asthma groups. The majority of participants were no longer smokers. Although no significant difference in age at onset was found between the asthma groups, asthma duration was significantly higher in *A. fumigatus*-IgE-sensitized patients with asthma ($P < 0.05$). There was no significant difference in prescription of oral or inhaled corticosteroids between the three asthma groups. A total of 43 (54%) of the patients were classed as having refractory asthma; 32 (42%) patients were GINA 5, 38 (48%) were GINA 4, 7 (9%) were GINA 3, and 2 (2%) were GINA 2.

A. fumigatus Sensitization and Culture

A. fumigatus culture rates were significantly different across groups ($P = 0.004$). Significantly higher rates of *A. fumigatus* were detected in sputum from *A. fumigatus*-IgE-sensitized patients with asthma (63%) in comparison to nonsensitized

TABLE 1. STUDY COHORT CHARACTERISTICS

Characteristics	Healthy	Asthma Af-IgE (\pm Af-IgG)	Asthma Af-IgG only	Nonsensitized Asthma
Subjects, <i>n</i>	14	40	13	26
Male, <i>n</i>	9	19	5	11
Age, mean (SEM)	33 (2.5)	58 (2.0)*	58 (4.9)*	53 (2.6)*
Never-smokers, <i>n</i>	11	19	7	13
Ex-smokers, <i>n</i>	3	17	6	13
Current Smokers, <i>n</i>	0	4	0	0
Age at asthma onset, yr [†]	—	24 (3–44)	30 (11–63)	39 (23–50)
Duration of asthma, yr [†]	—	27 (15–45) [‡]	18 (7–38)	10 (5–28)
Requiring oral steroids, <i>n</i> (%)	—	12 (30)	6 (46)	11 (42)
Prednisolone oral dose, median, mg [§]	—	10	10	10
ICS dose, μ g day ^{-1†}	—	1,000 (800–1,000)	700 (400–950)	800 (800–1,000)

Definitions of abbreviations: Af = *Aspergillus fumigatus*; ICS = inhaled corticosteroid.

* $P < 0.0001$ versus healthy controls by analysis of variance.

[†] Median with interquartile range shown in parentheses.

[‡] $P < 0.05$ versus nonsensitized asthma by Kruskal-Wallis test.

[§] Median dose of those taking oral steroids.

patients with asthma (31%) ($P < 0.05$; Table 2). There was no significant difference between *A. fumigatus*-IgG-only-sensitized patients with asthma and *A. fumigatus*-IgE-sensitized patients with asthma or nonsensitized patients with asthma.

Repeatability of the sputum culture was analyzed in 17 patients (8 negative on the first visit) where sputum was obtained on 2 occasions within 6 months when the patient was clinically stable. Of the nine patients with *A. fumigatus* culture-positive sputum, seven remained positive, and seven of the eight culture-negative patients remained negative. The kappa value was 0.648 (0.184), which is regarded as showing substantial agreement (20).

Lung Function According to *A. fumigatus* Sensitization and Culture

Reduced FEV₁ (% predicted, postbronchodilator) was observed in *A. fumigatus*-IgE-sensitized patients in comparison to nonsensitized patients with asthma, independent of *A. fumigatus* sputum culture ($P < 0.05$; Table 3, Figure 1). *A. fumigatus*-IgE-sensitized patients also showed significantly lower airway reversibility ($P < 0.05$), reduced FEV₁ in the presence of *A. fumigatus* sputum culture ($P < 0.05$), and significantly higher rates of bronchiectasis ($P < 0.05$) in comparison to nonsensitized patients with asthma (Table 3).

Airway Inflammation according to *A. fumigatus* Sensitization

Differential cell counts of sputum neutrophils were significantly higher in patients with *A. fumigatus*-IgE sensitization ($P < 0.01$;

Table 2) in comparison to nonsensitized patients with asthma (Table 2). Elevated neutrophils were also shown in nonsensitized asthma in comparison to healthy subjects ($P < 0.01$; data not shown). Sputum eosinophils were significantly higher in *A. fumigatus*-IgE-sensitized ($P < 0.01$) and nonsensitized patients with asthma ($P < 0.05$) in comparison to healthy subjects, but did not differ within the asthma groups, concordant with peripheral blood eosinophils, which did not differ significantly between the groups.

A total of 95% of *A. fumigatus*-IgE-sensitized patients with asthma had elevated *A. fumigatus*-IgE greater than 0.35kU/L, with 65% having a positive SPT to *A. fumigatus* (Table 2). *A. fumigatus*-IgE-sensitized patients with asthma demonstrated significantly higher total serum IgE (IU/ml) than nonsensitized patients with asthma and *A. fumigatus*-IgG-only-sensitized patients with asthma ($P < 0.001$). A total of 48% of *A. fumigatus*-IgE-sensitized patients also had *A. fumigatus*-IgG sensitization, and 24.4% of *A. fumigatus*-IgE-sensitized patients had positive SPT to other fungi. Mutations in the CF gene (Δ f508) were found in four patients, all of whom were heterozygous (Table 2). Four patients with elevated *A. fumigatus*-IgE fulfilled all the major criteria for ABPA.

Regression Analysis of Predictor Variables with Measurements of Lung Function

A. fumigatus-IgE sensitization and sputum neutrophil differential cell count were the most important predictors of lung

TABLE 2. AIRWAY INFLAMMATION AND FUNGAL CULTURE ACCORDING TO ASPERGILLUS FUMIGATUS SENSITIZATION

	Healthy	Asthma + Af-IgE (\pm IgG)	Asthma Af-IgG Only	Nonsensitized Asthma
Sputum culture of Af, <i>n</i> (%)	1 (7.1)	25 (62.5)*	5 (38.5)	8 (30.8)
Sputum eosinophils, % [†]	0 (0–0.8)	2.1 (0.5–6.1)	1.2 (0.5–6.3)	8.7 (0.6–16.6)
Sputum neutrophils, % [†]	50.7 (29.5–62.8)	80.9 (50.1–94.1) [‡]	79.5 (50.1–87.5)	49.5 (21.2–71.4)
Blood eosinophil $\times 10^{9†}$	0.1 (0.1–0.4)	1.0 (0.7–1.7)	0.8 (0.6–1.2)	1.0 (0.5–1.5)
Total IgE IU/ml [†]	36.9 (7.2–52.0)	791 (359.3–2,415.0) [§]		150.0 (82.08–320.0)
Af-IgE > 0.35 kU/L, <i>n</i> (%)	0	39 (95)	0	0
Af-IgG > 40 mg/L, <i>n</i> (%)	2 (14)	20 (48)	13 (100)	0
Atopy, %	21	55	38	54
Positive SPT Af, <i>n</i> (%)	0	26 (65)	0	0
Sensitization to other fungi, %	7.1	24.4	0.0	0.0
CF genotype mutations not detected, <i>n</i>	—	37	13	23
Heterozygous, <i>n</i>	—	3	0	1

Definitions of abbreviations: Af = *Aspergillus fumigatus*; GM = geometric mean; SPT = skin prick test.

* $P < 0.05$ versus nonsensitized patients with asthma by Chi-square analysis.

[†] Median (interquartile range).

[‡] $P < 0.01$ versus nonsensitized asthma by Kruskal-Wallis test.

[§] $P < 0.001$ versus IgG-only and nonsensitized patients with asthma by Kruskal-Wallis test.

^{||} Positive SPT test with a wheal diameter greater than 3 mm on exposure to common aeroallergens, excluding *A. fumigatus*.

TABLE 3. SPIROMETRY AND BRONCHIECTASIS ACCORDING TO *ASPERGILLUS FUMIGATUS* SENSITIZATION

	Healthy	Asthma Af-IgE (±IgG)	Asthma Af-IgG (only)	Nonsensitized Asthma
FEV ₁ after BD*	111 (3)	68 (5) [†]	77 (7)	88 (5)
FEV ₁ :FVC after BD*	85 (1)	64 (2) [‡]	64 (5) [†]	75 (2)
FEV ₁ after BD with positive Af culture*	—	69.30 (6)	68.60 (10)	86.50 (6)
Bronchiectasis, n (%)	—	27 (68) [§]	5 (38)	9 (35)

Definition of abbreviations: Af = *Aspergillus fumigatus*; BD = bronchodilator.
 * Mean (SEM).
[†] P < 0.05 versus nonsensitized patients with asthma by analysis of variance (ANOVA).
[‡] P < 0.01 versus nonsensitized patients with asthma by ANOVA.
[§] P < 0.05 by Chi-square analysis.

function in the model (P = 0.016; Table 4), further supported by positive sputum culture of *A. fumigatus* (P = 0.046) and eosinophil differential cell count (P = 0.024; Table 4). Although asthma duration and *A. fumigatus*-IgE sensitization were shown to be closely correlated (r_s = 0.307; P = 0.011), duration of asthma did not significantly affect the clinical outcome when adjusted for other clinical explanatory variables in the model (Table 4). Removal of the four patients with ABPA strengthened the model (from r² = 0.493 [Table 4] to r² = 0.636). *A. fumigatus*-IgE sensitization, sputum neutrophil differential cell count, positive sputum culture, and eosinophil differential cell count remain predictors of lung function; however, duration of asthma became a supporting factor (from P = 0.105 [Table 4] to P = 0.04), with bronchiectasis approaching significance (P = 0.05).

TABLE 4. MULTILINEAR REGRESSION ANALYSIS PREDICTING POSTBRONCHODILATOR FEV₁ % PREDICTED IN SUBJECTS WITH ASTHMA

	Unstandardized Coefficient	Standardized Coefficients	
	B (SEM)	β	P Value
Af primary culture, positive	-14.577 (7.037)	-0.278	0.046
Sputum eosinophils, %*	-13.945 (5.891)	-0.340	0.024
Af sensitization (SPT or Af-IgE), kU/L	-18.570 (7.327)	-0.356	0.016
Sputum neutrophils, %	-0.382 (0.151)	-0.393	0.016
Af-IgG, mg/L	-0.036 (0.102)	-0.048	0.724
Bronchiectasis, presence/absence	9.849 (7.066)	0.188	0.172
Duration of asthma, yr	-0.280 (0.169)	-0.209	0.105

Definitions of abbreviations: Af = *Aspergillus fumigatus*; SPT = skin prick test.
 Model summary: r² = 0.493; P = 0.001.
 * Normalized by log transformation.

DISCUSSION

To our knowledge, this is the first study to show an association specifically between *A. fumigatus*-IgE sensitization and reduced lung function in asthma. A number of studies have associated fungal sensitization with asthma severity (21–23) and, specifically, *A. fumigatus* sensitization and ABPA have been associated with progressive lung function decline in CF, likely due, in part, to coinciding *Pseudomonas aeruginosa* infection (2, 24, 25). A more severe reduction in lung function has been shown in children with CF with *A. fumigatus* sensitization in combination with elevated total IgE (24). However, the relationship between sensitization, colonization, and lung function is poorly understood in the absence of CF or ABPA.

Our data from patients without CF with asthma support these studies, showing, specifically, that *A. fumigatus*-IgE sensitization, regardless of other ABPA diagnostic criteria, is an important indicator of reduced lung function.

The patients with asthma were recruited consecutively, rather than in a formally randomized fashion, raising the possibility of unconscious bias in recruitment toward poor lung function in *A. fumigatus*-IgE-sensitized patients with asthma and good lung function in nonsensitized patients with asthma. However, this is unlikely. The majority of patients attending the asthma clinic at Glenfield Hospital over the recruitment period that were willing to take part were included. All three asthma groups were matched for severity. The starting point of the study was to investigate the relationship between sputum culture and sensitization, and an effect on FEV₁ only became apparent during the analyses. The level of FEV₁ was, therefore, not part of the recruitment criteria.

Although there was no formal steroid trial, patients were stable and optimally treated, suggesting that the postbronchodilator FEV₁ reflects fixed airflow obstruction; a common finding in asthma, particularly in moderate to severe phenotypes where it affects around 23% of patients (26). The cause of fixed airflow obstruction in asthma remains unknown, although it has been related to neutrophilic airways inflammation (27, 28). It is therefore of interest that we found airway neutrophils to be increased in the *A. fumigatus*-IgE-sensitized group.

It is not possible to say from this study whether the association between *A. fumigatus*-IgE sensitization and reduced lung function is causal; it is possible that sensitization is related to long-term colonization of the airways in asthma, which may occur preferentially in damaged airways. This would be consistent with the observation that *A. fumigatus*-IgE-sensitized patients with asthma had a longer duration of asthma, as also shown previously (29). We showed a clear association between

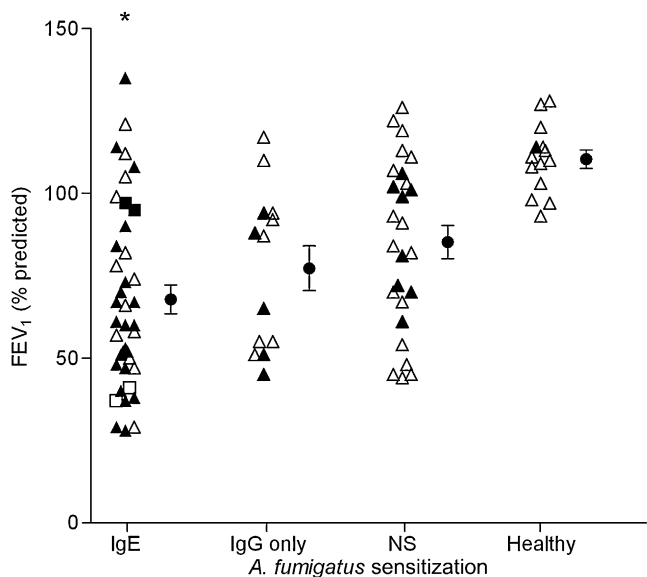


Figure 1. Geometric mean (SEM) FEV₁ (% predicted, postbronchodilator) according to *Aspergillus fumigatus* sensitization in the three asthma groups: *A. fumigatus*-IgE-sensitized ± *A. fumigatus*-IgG sensitization (IgE), *A. fumigatus*-IgG-only-sensitized (IgG only), and non-sensitized patients with asthma (NS), in comparison to healthy control subjects, in sputum culture-positive (closed symbols) and -negative (open symbols) subjects. Patients with classical allergic bronchopulmonary aspergillosis are represented by squares; all other subjects are represented by triangles. *P < 0.05 versus nonsensitized subjects with asthma by analysis of variance.

asthma duration and *A. fumigatus*-IgE sensitization, suggesting that the longer the duration of asthma, the more likely *A. fumigatus* sensitization will occur. Bronchiectasis, which again could be a consequence of colonization, did not explain the association between *A. fumigatus*-IgE and reduced FEV₁. It is therefore plausible that *A. fumigatus* is, at least in part, responsible for the development of fixed airflow obstruction in asthma, either as a result of the effects of its many toxins on the bronchial mucosa, or through stimulating a vigorous and persistent inflammatory reaction (30). Only longitudinal studies using antifungal therapy would resolve this question.

ABPA is a well established syndrome; however, the criteria for its diagnosis are restrictive. Only 4 of the 40 *A. fumigatus*-IgE-sensitized patients in our study fulfilled all of the major criteria for ABPA. Excluding these patients made no difference to the primary message of the article, although it made a minor difference to the outcome of the multilinear regression. We would hypothesize that *A. fumigatus* is important in many more patients with asthma than those who fulfill the ABPA criteria, and that a term such as AFAA should be used instead. ABPA is thought to be caused by chronic colonization of the airways with *A. fumigatus*; however, a positive sputum culture is not one of the major diagnostic criteria for ABPA. A key aim of this study was to determine the relationship between colonization, as defined by a positive sputum culture, and *A. fumigatus*-IgE sensitization. One of our most striking findings was the high rate of positive sputum culture in a specialist mycology laboratory (where more than 60% of *A. fumigatus*-IgE-sensitized patients with asthma were culture positive), compared with our routine National Health Service (NHS) clinical laboratory where less than 10% of patients historically had a positive culture for *A. fumigatus*. The research and clinical samples were not taken at the same time, so the results are not a direct comparison, but it does suggest that sensitivity of detection is very dependent on the method of culture.

There were a number of differences in technique between our culture method and that used by the routine laboratory that could explain this difference in detection rate, with quantity of inoculating material being the main difference. Routine clinical mycology laboratories process respiratory specimens according to a standard protocol issued by the Health Protection Agency, BSOP 57 (3), designed for the identification of both bacteria and fungi from a single sample. Briefly, samples comprising both saliva and sputum plug are digested in equal volumes of 0.1% dithiothreitol or equivalent solution, then further diluted 1:500 with sterile water; 1 μ l is then inoculated onto Sabouraud agar (SA) plates and incubated at 37°C for at least 2 days. For mycological investigation, our local clinical laboratory uses a modification of the standard protocol, inoculating 10 μ l of undiluted homogenized sputum/dithiothreitol mix onto the plates and observing for up to 5 days. In contrast, our method uses a far higher quantity of starting material, which consists of the sputum plugs removed from the saliva. The addition of undiluted sputum to culture plates has been used previously to investigate the prevalence of *A. fumigatus* in patients with CF (31). The other difference that is less likely to have affected the culture rate is choice of media. PDA and SA are both commonly used media for mycological investigations. We selected PDA over SA based on a comparison of sporulation from pure cultures of seven allergenic species representing different fungal genera, specifically *A. alternata*, *A. fumigatus*, *B. cinerea*, *C. herbarum*, *Epicoccum nigrum*, *Leptosphaeria coniothyrium*, and *P. chrysogenum* (unpublished data). Antibiotics were added to the media at optimum concentrations for isolation of pathogenic fungi (32), and fluconazole to enhance recovery of *A. fumigatus* through suppression of *Candida* growth (33).

The repeatability of our sputum method, which showed substantial agreement, was based on 17 patients where we had 2 samples within 6 months; an interval relevant to retesting after an intervention such as antifungal treatment. This suggests that our method is reasonably robust, considering the inherent variability in the amount and quality of sputum obtained by induction.

Although a positive sputum culture of *A. fumigatus* was strongly linked to *A. fumigatus*-IgE sensitization, a high rate of positive culture was also found in the *A. fumigatus*-IgG-only and nonsensitized patients with asthma. We had noted, anecdotally, that a number of patients with asthma had high levels of *A. fumigatus*-IgG, without evidence of *A. fumigatus*-IgE or high levels of total IgE. Speculating that these patients had an atypical form of AFAA, we investigated whether *A. fumigatus*-IgG-only-sensitized patients had similar rates of colonization to the *A. fumigatus*-IgE group. In fact, *A. fumigatus*-IgG-only-sensitized patients with asthma had closer colonization rates to the nonsensitized patients with asthma. However, both the *A. fumigatus*-IgG-only-sensitized and nonsensitized patients with asthma showed relatively high rates of *A. fumigatus* colonization, at over 30%. This was specific in the sense that healthy subjects had a low rate of culture, although one caveat is the younger age of our control subjects. Most of the patients in this study had refractory asthma, and it would be interesting to know the *A. fumigatus* culture rates in mild patients with asthma. We found no significant difference between *A. fumigatus*-IgG-only-sensitized patients with asthma and the other asthma groups, which may partly be due to low numbers within this group. However, raised *A. fumigatus*-IgG alone does not appear to be a particularly good marker of either colonization or reduced lung function, and may simply reflect high levels of exposure to *A. fumigatus* spores. Indeed, it would be interesting to know if there was a relationship between culture status and exposure in all the asthmatic groups. It is possible that *A. fumigatus* is simply a commensal in culture-positive, nonsensitized patients with asthma, but it is equally possible that *A. fumigatus* is responsible for driving the inflammatory response. It is possible that these culture-positive *A. fumigatus*-IgE-negative patients with asthma are those who, over the longer term, develop fixed airflow obstruction and *A. fumigatus*-IgE sensitization, especially as fungal culture was independently associated with reduced FEV₁ in the multivariate model. Why some patients are susceptible to colonization with *A. fumigatus* is not known (4); however, the potential involvement of immunogenetic factors has been suggested (6, 34).

The relationship between *A. fumigatus* airways colonization, sensitization, and inflammation is poorly understood. Isolation of *Aspergillus* spp. from respiratory samples has recently been shown to indicate colonization, as opposed to infection (35). We found that a focused approach toward culture of *A. fumigatus* in sputum was highly predictive of *A. fumigatus*-IgE sensitization, and, as such, should be considered in parallel with immunological measures of lung function in AFAA. Sputum eosinophilia is a response normally regarded as a hypersensitivity reaction to environmental antigens, whereas a neutrophilic profile usually suggests an infection (36). ABPA is characterized by eosinophilia, which is used as a diagnostic criterion for the disease; however, ABPA has also been correlated with increased sputum neutrophils, airway obstruction, and increased levels of IL-8 (37). Our data show elevated levels of neutrophils in *A. fumigatus*-IgE-sensitized patients in comparison to nonsensitized patients with asthma, suggesting a Th1- or Th17-mediated immune response. The multivariate analysis also brought out a relationship between sputum eosinophils and FEV₁, showing, specifically, that airway inflammation, determined through sputum differential cell counts, should be taken into consideration in the clinical characterization of AFAA.

In summary, we found a strong relationship between detection of *A. fumigatus* in sputum and *A. fumigatus*-IgE sensitization, in addition to a strong inverse relationship between *A. fumigatus*-IgE sensitization and lung function. Moreover, *A. fumigatus*-IgE sensitization, airway inflammation, and *A. fumigatus* culture from sputum can be used collectively to model lung function in AFAA. Future studies are necessary to assess the benefit of antifungal agents in these patients, particularly with regard to sputum culture indicating airways colonization.

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