



# Sputum Inflammatory Mediators Are Increased in *Aspergillus fumigatus* Culture-Positive Asthmatics

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*Aspergillus fumigatus* sensitization and culture in asthma are associated with disease severity and lung function impairment, but their relationship with airway inflammation is poorly understood. We investigated the profile of 24 sputum inflammatory mediators in *A. fumigatus* culture-positive or-negative moderate-to-severe asthmatics. Fifty-two subjects were recruited from a single center. *A. fumigatus* was cultured from 19 asthmatics. Asthma control, symptom score, lung function, and sputum cell count were not significantly different between the asthmatics with and without a positive *A. fumigatus* culture. All of the sputum mediators were numerically increased in subjects with a positive versus negative sputum *A. fumigatus* culture. Sputum TNF-R2 was significantly elevated ( $P=0.03$ ) and the mediator that best distinguished *A. fumigatus* culture-positive from culture-negative subjects (receiver-operator characteristic area under the curve 0.66 [95% CI: 0.51 to 0.82,  $P=0.045$ ]). *A. fumigatus*-positive culture in moderate-to-severe asthma is associated with increased inflammatory sputum mediators.

**Key Words:** *Aspergillus fumigatus*; asthma; cytokines profile; inflammatory mediators

## INTRODUCTION

Asthma is a heterogeneous condition with variability in the clinical expression of disease, disordered airway physiology, inflammation, and frequency of exacerbations.<sup>1-4</sup> It affects over 300 million people worldwide.<sup>1,2</sup> To develop effective therapies for asthma, its mechanisms, pathogenesis, and progression need to be better understood. Cytokine profiles have provided insights into potential underlying mechanisms and responses to therapy in asthma and chronic obstructive pulmonary disease (COPD).<sup>4-9</sup> Sensitization to *Aspergillus fumigatus* and culture in sputum samples are associated with increasing severity of disease and airflow limitation in asthma.<sup>10-14</sup> However, the sputum cytokine profile in *A. fumigatus* culture-positive asthmatics is unknown.

We hypothesized that the sputum cytokine profile is different in asthmatics with sputum-positive versus sputum-negative culture for *A. fumigatus*. To test this hypothesis, we studied moderate-to-severe asthmatics during stable disease in a single center to determine the relationship between the sputum inflammatory profile and *A. fumigatus* culture.

## MATERIALS AND METHODS

### Subjects

In this study, 52 adults with moderate-to-severe asthma, according to the Global Initiative for Asthma (GINA) treatment step,<sup>1</sup> were recruited from a single center University Hospitals of Leicester NHS Trust, Leicester, UK. Some had participated in a previous cross-sectional study.<sup>5</sup> The diagnosis of asthma was made by the subjects' physician consistent with the GINA guidelines and assessed at stable visits at least 8 weeks free from an exacerbation, which was defined as an increase in symptoms necessitating a course of oral corticosteroids and/or antibiotic therapy. All subjects provided written informed consent, and the studies were approved by the local Leicestershire, Northamptonshire and Rutland ethics committee.

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• There are no financial or other issues that might lead to conflict of interest.

### Clinical assessments

Demographic, clinical, and lung function data were recorded, which included pre- and post-bronchodilator forced expiratory volume in 1 second (FEV1), forced vital capacity (FVC), asthma control questionnaire (ACQ), and symptom scores using the visual analogue scale (VAS). Fractional exhaled nitric oxide was measured, and sputum samples were collected. Sputum plugs were processed to generate cytopins for the assessment of sputum total and differential cell counts.<sup>5-9</sup> Sputum cell-free supernatants were stored at -80°C for later mediator analysis. Blood total IgE levels were measured using the ImmunoCap 250 system (Phadia, Milton Keynes, UK). Atopy was assessed by either skin prick testing (Alk-Abello, Horsholn, Denmark) or by blood specific IgE antibody levels (Phadia) and included cat, dog, timothy grass, *Dermatophagoides pteronyssinus*, and *A. fumigatus*.

### Sputum and blood mediator measurements

A panel of inflammatory mediators was measured in sputum supernatants using the Meso Scale Discovery Platform (MSD<sup>®</sup>, Gaithersburg, MD, USA) and ELISA as previously described.<sup>5,8,9</sup> Samples with mediator concentrations below the limit of quantification (BLQ) were assigned half of the limit of quantification. In addition, mediators that were below limit of quantification in more than 60% of the subjects participated in this study were excluded from further analysis.

### Bacterial and fungal culture

Standard routine culture to derive the number of colony-forming units (CFU) was performed according to the Health Protection Agency standard operating procedures.<sup>15</sup> Quantitative real-time polymerase chain reaction (RT-qPCR) was used to estimate bacterial load based on the abundance of 16S ribosomal subunit encoding genes as previously described.<sup>8</sup> The lower limit of quantification was  $1 \times 10^4$  genome copies/mL.

Fungal culture was performed as previously described.<sup>12,14,16</sup> In brief, undiluted sputum plugs were inoculated onto potato dextrose agar plates containing 16 µg/mL chloramphenicol, 4 µg/mL gentamicin and 5 µg/mL fluconazole; incubated at 37°C and inspected daily for up to 7 days. *A. fumigatus* colonies were identified by colony formation and microscopy.

### Statistical analysis

Statistical analysis was performed using STATA/IC version 13.0 for Windows (Stata Corp, College Station, TX, USA). The demographic and clinical characteristics, and the patterns of the inflammatory mediators were compared across subjects with and without *A. fumigatus*. Parametric data are presented as mean with standard error of the mean (SEM), and log-transformed data as geometric mean with 95% confidence intervals (CI). Receiver operating characteristic (ROC) curves were used to determine which inflammatory mediators discriminated be-

tween asthmatics with positive versus negative *A. fumigatus* culture, both significantly and with a ROC area under the curve >0.65. A *P* value of less than 0.05 was considered significant.

## RESULTS

The demographic and clinical characteristics were compared across the subgroups with versus without *A. fumigatus* as shown (Table 1). There were no significant differences between the asthmatics with positive *A. fumigatus* culture versus without in terms of demographics, symptoms, asthma quality of life, or lung function. The bacterial load assessed by either CFU or total 16S was also not significantly different between the groups.

The sputum mediator concentrations in asthma subjects with and without *A. fumigatus*-positive culture are shown in Table 2. All the mediators were numerically increased in subjects with positive versus negative *A. fumigatus* culture (24 vs 0, *P*<0.001), with sputum TNF-R2 increased significantly (Figure). The best predictor of positive versus negative *A. fumigatus* culture was TNF-R2 (ROC area under the curve 0.66 [95% CI: 0.51 to 0.82, *P*=0.045]).

We further categorized the subjects into 4 groups according to the by presence or absence of *A. fumigatus* sensitization and culture. Comparing the 4 groups by ANOVA and then by pairwise comparisons, we found no significant differences in the sputum cytokines between the subjects that were culture-positive and sensitization-positive versus those that were both culture-and sensitization-negative (data not shown).

## DISCUSSION

We report here from a single center the sputum cytokine profile for moderate-to-severe asthmatics with or without positive culture for *A. fumigatus*. We found that although there were no differences in the clinical characteristics between the groups, sputum TNF-R2 concentrations were elevated in those with positive sputum *A. fumigatus* culture.

In keeping with earlier reports in asthma-positive culture for *A. fumigatus* was common and detected in 36% of moderate-to-severe asthmatics.<sup>12</sup> Positive culture for *A. fumigatus* and moreover sensitization in asthma and COPD are associated with airflow limitation.<sup>11,12,14</sup> Here, we observed no significant difference in spirometry between subjects with or without positive culture for *A. fumigatus*, although our sample size was small and may have reduced our ability to detect a significant difference. Consistent with other reports, we did not demonstrate differences in demographics or symptoms. Reports of an association between positive culture for *A. fumigatus* with sputum neutrophilic inflammation in asthma are inconsistent,<sup>11,12</sup> with one further report of an association in COPD.<sup>14</sup> Here, we observed no significant differences between groups for differential or total neutrophil counts.

**Table 1.** Clinical Characteristics in asthmatics with and without positive sputum *A. fumigatus* culture

Variable	<i>A. fumigatus</i> negative (n=33)	<i>A. fumigatus</i> positive (n=19)	Pvalue
Female/Male (n)	16/17	6/13	0.23
Age (yr)	54.7 (2.24)	57.2 (2.45)	0.48
Current or ex-smokers [n (%)]	13 (39.4)	6 (31.6)	0.57
Pack year history*	2.25 (1.15 to 17.26)	10.01 (10.00 to 13.01)	0.64
BMI (kg/m <sup>2</sup> )	30.8 (1.3)	28.9 (1.5)	0.34
Atopy (n)	16	12	0.31
<i>Aspergillus fumigatus</i> sensitized (n)	8	11	0.02
IgE (IU/L) <sup>‡</sup>	121 (60 to 242)	341 (159 to 731)	0.06
Exacerbations (OCS courses in last year)*	2 (2 to 4)	3 (1 to 3)	0.38
GINA classification III/IV/V (n)	2/14/17	1/6/12	0.71
Daily ICS dose (BDP equivalent µg/day)*	1,600 (1,000 to 2,000)	2,000 (1,000 to 2,000)	0.61
Daily prednisolone dose (mg) <sup>†</sup>	10 (7.5 to 10)	10 (7.5 to 10)	0.67
Pre-bronchodilator FEV1 (L)	2.2 (0.13)	2.0 (0.18)	0.32
Post-bronchodilator FEV1 (L)	2.4 (0.14)	2.2 (0.19)	0.37
Pre-bronchodilator FEV1 predicted (%)	79.8 (3.8)	70.8 (6.0)	0.19
Post-bronchodilator FEV1 predicted (%)	85.4 (3.8)	77.5 (6.1)	0.25
Pre-bronchodilator FEV1/FVC (%)	69.9 (3.2)	64.4 (3.2)	0.26
VAS score-cough (mm)	34.9 (4.6)	39.3 (7.3)	0.59
VAS score- dyspnea (mm)	33.1 (4.5)	36.9 (6.9)	0.63
VAS score- wheeze (mm)	22.8 (4.5)	37.6 (8.9)	0.11
Asthma Quality of life Questionnaire (AQLQ)	4.7 (0.21)	4.9 (0.32)	0.96
Asthma Control Questionnaire (ACQ6)	2.1 (0.22)	2.1 (0.32)	0.86
Blood neutrophils × 10 <sup>9</sup> /L	5.7 (0.32)	5.3 (0.54)	0.46
Sputum neutrophil count (%)	61.8 (4.4)	65.1 (4.9)	0.64
Blood eosinophil × 10 <sup>9</sup> /L <sup>‡</sup>	0.24 (0.18 to 0.32)	0.28 (0.14 to 0.57)	0.63
Sputum eosinophils count (%) <sup>‡</sup>	1.96 (1.03 to 3.76)	3.1 (1.58 to 6.07)	0.36
Total sputum eosinophils count × 10 <sup>4</sup> /g sputum <sup>‡</sup>	4.12 (1.84 to 9.23)	5.84 (2.66 to 12.81)	0.56
Total sputum neutrophil count × 10 <sup>4</sup> /g sputum <sup>‡</sup>	110.35 (59.54 to 204.54)	115.55 (65.5 to 203.84)	0.92
Fraction of exhaled Nitric Oxide (FeNO) (ppb) <sup>‡</sup>	24.45 (16.17 to 36.97)	23.78 (17.29 to 32.72)	0.93
16s Bacteria load <sup>  </sup>	9.5 (0.12)	9.2 (0.13)	0.14
CFU <sup>  </sup>	6.5 (0.15)	6.4 (0.26)	0.75

Data are presented as mean (standard error of mean [SEM]) unless stated; pack year history for current and ex-smokers.

OCS, oral corticosteroid; ICS, inhaled corticosteroid; BDP, beclomethasone dipropionate; VAS, Visual analogue score; BMI, Body Mass Index; FEV1, Forced Expiratory Volume in the First Second; FVC, Forced Vital Capacity.

\*Median (first and 3rd quartiles); <sup>†</sup>dose of prednisolone in those receiving prednisolone; <sup>‡</sup>geometric mean (95% CI); <sup>||</sup>logarithmic scale (base 10).

We have previously described that sputum cytokine profiles in both asthma and COPD clustered into 3 groups consisting predominately of T1, T2, or pro-inflammatory cytokines.<sup>5,9</sup> We observed no significant differences in T1 or T2 cytokines between the groups. However, all of the sputum mediators were numerically increased in subjects with a positive culture for *A. fumigatus*. The soluble receptor TNF-R2 was significantly increased in the subgroup with a positive culture for *A. fumigatus*. The TNF- $\alpha$ -converting enzyme TACE can cleave membrane-bound TNFR1 and TNFR2 to yield soluble TNF receptors, which have higher affinity for TNF- $\alpha$ . Thus, in asthma ele-

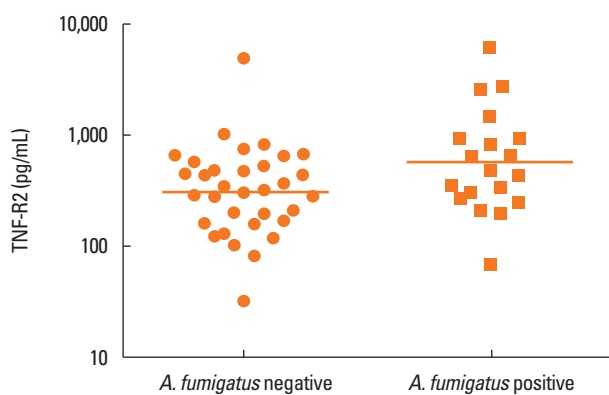
vated sputum TNF-R2 might attenuate the inflammatory response to *A. fumigatus*.<sup>17</sup>

Consistent with our report in COPD, positive culture for *A. fumigatus* was not associated with increased bacterial colonisation.<sup>14</sup> This suggests that the mechanisms increasing susceptibility to either bacterial or fungal colonisation are independent and that an underlying common impairment in the innate immune response is unlikely.

This report has some limitations. Importantly, this is a relatively small cross-sectional study in a single center and requires further validation in larger groups of subjects. Sputum media-

**Table 2.** Geometric mean (95% confidence intervals) sputum mediator concentration (pg/mL) in asthmatics with and without positive *A. fumigatus* culture

Variable	<i>A. fumigatus</i> negative	<i>A. fumigatus</i> positive	Pvalue
IL-1 $\alpha$	58.6 (40.7 to 84.5)	69.7 (42.2 to 115.2)	0.56
IL-1 $\beta$	81 (50.6 to 129.7)	113.5 (54.9 to 234.5)	0.41
IL-2	0.7 (0.5 to 1)	1.2 (0.6 to 2.5)	0.09
IL-5	4.4 (2.5 to 7.7)	6.3 (2.9 to 13.8)	0.43
IL-6	71.1 (45.8 to 110.3)	96.6 (46.2 to 202)	0.43
IL-6R	279.2 (216.9 to 359.3)	349.5 (232.3 to 525.8)	0.31
IL-8	4,350.8 (2,944 to 6,429.8)	7,770.9 (4,669.8 to 12,931.4)	0.07
IL-15	1.5 (1 to 2.3)	2 (0.9 to 4.3)	0.48
IL-18	31.2 (22.8 to 42.6)	46.3 (29.3 to 73.4)	0.14
CXCL-9	751.8 (422.6 to 1,337.3)	1,605.9 (656.7 to 3,926.8)	0.13
CXCL-10	864.6 (546.2 to 1,368.6)	1432.5 (705.6 to 2,908.3)	0.2
CXCL-11	59.4 (33.7 to 104.5)	98.3 (32.3 to 299.6)	0.36
CCL-2	336.5 (240.5 to 471)	461.6 (294 to 724.6)	0.25
CCL-3	40.2 (26.8 to 60.4)	67.6 (36.2 to 126.3)	0.14
CCL-4	589.7 (378.3 to 919.3)	1,097.2 (591.7 to 2,034.5)	0.09
CCL-5	8.2 (6.1 to 11)	14.6 (7.9 to 27.1)	0.05
CCL-11	78.4 (57.1 to 107.8)	81.9 (45 to 149)	0.89
CCL-13	26.4 (18.5 to 37.6)	29 (17.7 to 47.6)	0.75
CCL-17	36.2 (23.6 to 55.4)	52.4 (24.2 to 113.5)	0.35
CCL-26	14.0 (7.8 to 24.9)	14.2 (7 to 29)	0.97
TNF $\alpha$	4.2 (2.6 to 6.8)	6.8 (2.7 to 17.1)	0.28
TNF-R1	653.3 (488.3 to 874)	1,006.2 (646.3 to 1,566.5)	0.09
TNF-R2	320.7 (232.3 to 442.7)	592.1 (352.3 to 995.2)	0.03
VEGF	1,670.4 (1,364.8 to 2,044.3)	1,991.8 (1,335.8 to 2,969.9)	0.37

**Figure.** Sputum TNF-R2 concentration (plotted on a log scale) in subjects with or without positive *A. fumigatus* culture.

tor assessments are challenging; however, we have extensively validated these measurements and are therefore confident that the findings are robust. Although our group has carefully optimized fungal culture techniques in sputum samples, new molecular approaches to mycology are under development and future studies will need to include these new technologies. In

addition to considering the full mycobiome, the fungal load determined by spore count might be clinically important and should be included in future studies.<sup>18</sup>

In conclusion, we have found that sputum inflammatory cytokine concentrations were collectively increased in moderate-to-severe asthmatics with positive culture for *A. fumigatus*. Anti-fungal therapy has benefit in allergic bronchopulmonary aspergillosis (ABPA), although possibly due to its effects on corticosteroid pharmacokinetics.<sup>2,19</sup> Anti-fungal therapy in asthma beyond ABPA is contentious with inconsistent evidence from randomized controlled trials.<sup>2,20,21</sup> Whether anti-fungal therapy may be more effective in asthmatics with evidence of positive culture for *A. fumigatus* and increased airway inflammation warrants further investigation.

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## REFERENCES

- Global Initiative for Asthma. Global Initiative for Asthma guidelines [Internet]. [place unknown]: Global Initiative for Asthma; 2016 [2016 Jun 30]. Available from: <http://ginasthma.org/>.
- Chung KF, Wenzel SE, Brozek JL, Bush A, Castro M, Sterk PJ, et al. International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *Eur Respir J* 2014;43:343-73.
- Haldar P, Pavord ID, Shaw DE, Berry MA, Thomas M, Brightling CE, et al. Cluster analysis and clinical asthma phenotypes. *Am J Respir Crit Care Med* 2008;178:218-24.
- Wu W, Bleeker E, Moore W, Busse WW, Castro M, Chung KF, et al. Unsupervised phenotyping of Severe Asthma Research Program participants using expanded lung data. *J Allergy Clin Immunol* 2014;133:1280-8.
- Ghebre MA, Bafadhel M, Desai D, Cohen SE, Newbold P, Rapley L, et al. Biological clustering supports both "Dutch" and "British" hypotheses of asthma and chronic obstructive pulmonary disease. *J Allergy Clin Immunol* 2015;135:63-72.
- Desai D, Gupta S, Siddiqui S, Singapuri A, Monteiro W, Entwisle J, et al. Sputum mediator profiling and relationship to airway wall geometry imaging in severe asthma. *Respir Res* 2013;14:17.
- Piper E, Brightling C, Niven R, Oh C, Faggioni R, Poon K, et al. A phase II placebo-controlled study of tralokinumab in moderate-to-severe asthma. *Eur Respir J* 2013;41:330-8.
- Bafadhel M, McKenna S, Terry S, Mistry V, Reid C, Haldar P, et al. Acute exacerbations of chronic obstructive pulmonary disease: identification of biologic clusters and their biomarkers. *Am J Respir Crit Care Med* 2011;184:662-71.
- Bafadhel M, McCormick M, Saha S, McKenna S, Shelley M, Hargadon B, et al. Profiling of sputum inflammatory mediators in asthma and chronic obstructive pulmonary disease. *Respiration* 2012;83:36-44.
- Knutsen AP, Bush RK, Demain JG, Denning DW, Dixit A, Fairs A, et al. Fungi and allergic lower respiratory tract diseases. *J Allergy Clin Immunol* 2012;129:280-91.
- Fairs A, Agbetile J, Hargadon B, Bourne M, Monteiro WR, Brightling CE, et al. IgE sensitization to *Aspergillus fumigatus* is associated with reduced lung function in asthma. *Am J Respir Crit Care Med* 2010;182:1362-8.
- Agbetile J, Fairs A, Desai D, Hargadon B, Bourne M, Mutalithas K, et al. Isolation of filamentous fungi from sputum in asthma is associated with reduced post-bronchodilator FEV1. *Clin Exp Allergy* 2012;42:782-91.
- Wardlaw AJ, Woolnough K, Pashley CH. Lassoing a chimera: the semantics of allergic fungal airway disease. *Clin Exp Allergy* 2015;45:1746-9.
- Bafadhel M, McKenna S, Agbetile J, Fairs A, Desai D, Mistry V, et al. *Aspergillus fumigatus* during stable state and exacerbations of COPD. *Eur Respir J* 2014;43:64-71.
- Public Health England. Investigation of bronchoalveolar lavage, sputum and associated specimens: Bacteriology, B 57, Issue no: 3.1 [Internet]. London: Public Health England; 2015 [updated 2015 Oct 2; cited 2015 Dec 2]; Available from: [https://www.gov.uk/government/uploads/system/uploads/attachment\\_data/file/466668/B\\_57i3.pdf](https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/466668/B_57i3.pdf).
- Pashley CH, Fairs A, Morley JP, Tailor S, Agbetile J, Bafadhel M, et al. Routine processing procedures for isolating filamentous fungi from respiratory sputum samples may underestimate fungal prevalence. *Med Mycol* 2012;50:433-8.
- Brightling C, Berry M, Amrani Y. Targeting TNF- $\alpha$ : a novel therapeutic approach for asthma. *J Allergy Clin Immunol* 2008;121:5-10.
- Lin WR, Chen YH, Lee MF, Hsu LY, Tien CJ, Shih FM, et al. Does spore count matter in fungal allergy?: the role of allergenic fungal species. *Allergy Asthma Immunol Res* 2016;8:404-11.
- Shah A, Panjabi C. Allergic bronchopulmonary aspergillosis: a perplexing clinical entity. *Allergy Asthma Immunol Res* 2016;8:282-97.
- Agbetile J, Bourne M, Fairs A, Hargadon B, Desai D, Broad C, et al. Effectiveness of voriconazole in the treatment of *Aspergillus fumigatus*-associated asthma (EVITA3 study). *J Allergy Clin Immunol* 2014;134:33-9.
- Denning DW, O'Driscoll BR, Powell G, Chew F, Atherton GT, Vyas A, et al. Randomized controlled trial of oral antifungal treatment for severe asthma with fungal sensitization: the Fungal Asthma Sensitization Trial (FAST) study. *Am J Respir Crit Care Med* 2009;179:11-8.