

# Fungal sensitization and positive fungal culture from sputum in children with asthma are associated with reduced lung function and acute asthma attacks respectively

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## Abstract

**Background:** Sensitization to thermotolerant fungi, including filamentous fungi and *Candida albicans*, is associated with poor lung function in adults with severe asthma. Data in children are lacking. Environmental exposure to fungi is linked with acute severe asthma attacks, but there are few studies reporting the presence of fungi in the airways during asthma attacks.

**Methods:** We investigated the association between fungal sensitization and/or **positive** fungal **sputum** culture and markers of asthma severity in children with chronic and acute asthma. Sensitization was determined using serum-specific IgE and skin prick testing against a panel of five fungi. Fungal culture was focused towards detection of filamentous fungi from sputum samples.

**Results:** We obtained sensitization data and/or sputum from 175 children: 99 with chronic asthma, 39 with acute asthma and 37 controls. 34.1% of children with chronic asthma were sensitized to thermotolerant fungi compared with no children without asthma ( $p = < 0.001$ ). These children had worse pre-bronchodilator lung function compared with asthmatics without sensitization including a lower FEV<sub>1</sub>/FVC ratio ( $p < .05$ ). The isolation rate of filamentous fungi from sputum was higher in children with acute compared with chronic asthma.

**Conclusions:** Fungal sensitization is a feature of children with chronic asthma. Children sensitized to thermotolerant fungi have worse lung function, require more courses of systemic corticosteroids and have greater limitation of activities due to asthma. Asthma attacks in children were associated with the presence of filamentous fungi positive sputum culture. Mechanistic studies are required to establish whether fungi contribute directly to the development of acute asthma.

## KEYWORDS

airway obstruction, allergens and epitopes, *Aspergillus*, asthma, IgE, Paediatrics, sensitization

Kathryn G. Welsh and Karl A. Holden contributed equally to the manuscript preparation and share joint first authorship, Catherine H. Pashley and Erol A. Gaillard share senior authorship.

## 1 | INTRODUCTION

Childhood asthma is a global health problem and is the most common chronic respiratory disorder affecting children.<sup>1,2</sup> Between 1% and 5% of all school-age children are considered to have severe asthma that is not controlled with high doses of inhaled corticosteroids.<sup>3,4</sup> The healthcare burden of severe asthma is considerable.<sup>5</sup> In many individuals, severe adult asthma starts in childhood<sup>6</sup> and children with severe asthma are at high risk of progressive loss of lung function.<sup>7</sup>

Severe asthma is a heterogeneous condition.<sup>8-10</sup> Lotvall *et al*<sup>11</sup> and a European Academy of Allergy and Clinical Immunology (EAACI) task force<sup>12</sup> proposed the term endotypes to describe subtypes of asthma with characteristics linked across several disease dimensions from pathophysiology to clinical features and outcomes. The consensus statements suggest that fungal allergic disease is one such endotype, particularly associated with more severe disease and the progressive development of lung damage.<sup>11,12</sup>

There is considerable evidence linking fungi with severe asthma in adults, either through increased exposure in the environment or through IgE sensitization.<sup>13-15</sup> While mesophilic fungi such as *Alternaria* and *Cladosporium* spp., which are unable to germinate in the airways, can cause an allergenic stimulus in some circumstances, it is IgE sensitization to thermotolerant fungi such as *Aspergillus fumigatus* (AF) and *Candida* spp., which can colonize the lungs, that is associated with chronic severe disease and the development of bronchiectasis, fixed airflow obstruction and lung fibrosis.<sup>16,17</sup> In adults, both IgE sensitization to thermotolerant fungi and culture of these fungi from sputum are associated with lung damage with an apparent additive effect.<sup>18,19</sup> Adults with severe asthma and fungal sensitization frequently harbour fungi in the airways, supporting the hypothesis of a mechanistic link between fungal sensitization and airway colonization.<sup>20</sup>

The role of fungi in asthma is increasingly recognized as a spectrum, ranging from the somewhat rigid and arbitrary criteria for Allergic Bronchopulmonary Aspergillosis/Mycosis (ABPA/ABPM)<sup>21-23</sup> to more 'umbrella-like' terms such as severe asthma with fungal sensitization (SAFS) in adults,<sup>24</sup> fungal asthma in children<sup>25</sup> or Allergic Fungal Airway Disease (AFAD).<sup>16,26</sup> There are subtle differences between these labels, broadly separated by the level and relevance of total IgE cut-off values.

Compared with adults, there are little data on the effect of fungal sensitization on airway inflammation and lung function in children with asthma and none reporting airway fungal culture. The incidence of childhood asthma that meets the criteria for ABPA remains controversial. Several reports from the Indian subcontinent highlight high rates of ABPA in children with poorly controlled asthma.<sup>22,27,28</sup> These children are characterized by high values of total IgE, but findings with respect to lung function deficits and findings of bronchiectasis are inconsistent.<sup>23,28</sup> In Europe, this diagnosis is considered rare.<sup>25</sup> The diagnostic label 'severe asthma with fungal sensitization' (SAFS) is used for adults with severe asthma who do not fulfil the criteria for ABPA, in particular those with a total serum IgE below 1000 IU/L.<sup>24</sup> There are no defined and universally accepted criteria

for ABPA<sup>21</sup> and SAFS in children. A study in children with severe asthma and sensitization to fungi without ABPA found higher total IgE values in sensitized children, greater frequency of maintenance oral steroids and higher sputum IL-33 levels.<sup>29</sup>

There are almost no data in children with mild-moderately severe asthma and children with exacerbation-prone asthma and no paediatric studies reporting airway fungal culture data during asthma attacks in a real-life clinical setting.

The aims of this research were therefore to 1. study the association between sensitization to thermotolerant fungi and lung function in a large number of children with chronic asthma, 2. investigate the sputum of children with acute and stable asthma for the presence of fungi and 3. evaluate the association between sensitization to thermotolerant fungi and a positive fungal culture, and markers of asthma severity in children.

## 2 | MATERIALS AND METHODS

### 2.1 | Study participants

We conducted a single-centre, prospective observational cohort study in children aged 5 to 16 years with doctor-diagnosed asthma. Doctor-diagnosed asthma was determined by the clinical team caring for the patient using the clinical history and investigations such as spirometry, FeNO and others.<sup>30,31</sup> Two groups of children with asthma were recruited; children who attended our secondary and tertiary asthma or allergy clinics designated 'chronic asthma' and a second group of children who attended our emergency department with a doctor-diagnosed asthma attack designated 'acute asthma'. The research team completed a case report form for all the participants. History of clinical allergy was determined by asking participants whether they had a current history of hayfever, eczema or food allergy and recording this information in the case report form. A control group of children with no history of wheeze were recruited from surgical wards and general outpatient clinics. Recruitment took place over a three-year period from May 2013-2016. The East Midlands Research Ethics Committee (UK) approved all aspects of this study, reference: 12/WM/0413. We obtained written, informed consent from the legal guardian of all children prior to enrolment.

### 2.2 | Allergy testing

Sensitization to three thermotolerant fungi (the filamentous fungi; *A. fumigatus* and *Penicillium chrysogenum*, and the yeast *Candida albicans*), two non-thermotolerant filamentous fungi (*Alternaria alternata* and *Cladosporium herbarum*) and common aeroallergens including *Dermatophagoides pteronyssinus* (house dust mite), grass mix pollen, cat and dog dander were tested with either skin prick test (SPT) (Soluprick, ALK-Abello, Hørsholm, Denmark) or sandwich immunoassay. The SPT was deemed positive where the weal size was  $\geq 3$  mm greater than control.<sup>32</sup> Serum total IgE and specific IgE were quantified

using the ImmunoCap250 system (Pharmacia, Milton Keynes, UK). A positive specific IgE was designated as  $\geq 0.35$  kU/L. We considered patients to be sensitized to either fungi or aeroallergens if they had a positive result in either skin prick test or specific IgE to one of the five fungi or one of the four aeroallergens tested, respectively. Children who presented to hospital with an asthma attack frequently had routine blood tests arranged by the attending medical team. Where this was the case, specific IgE allergy testing was arranged from an aliquot of blood taken at the same time as the routine blood tests. In children where the medical team did not arrange for blood tests, children were offered the specific IgE blood test as an additional research procedure. Those children/families who declined the blood test were offered skin prick testing during follow-up. Children with stable asthma were given the choice between SPT and blood testing.

### 2.3 | Sputum collection, spirometry and exhaled nitric oxide

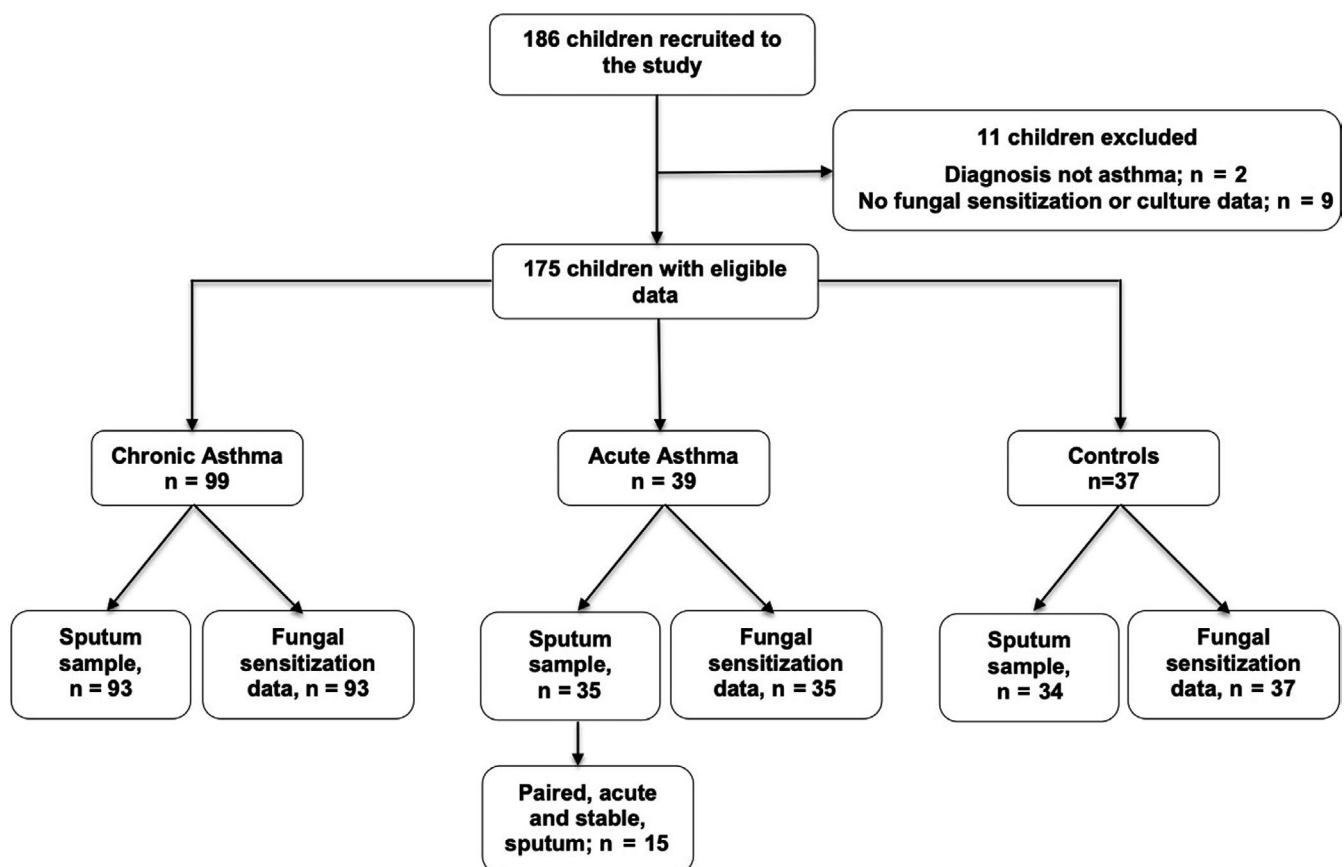
Children with chronic asthma and control children had one visit to the paediatric respiratory laboratory for spirometry, fraction of exhaled nitric oxide (FeNO; Niox Mino, Circassia Pharmaceuticals,

UK), allergy testing and sputum induction with hypertonic saline. Spirometry was undertaken using a MicroLab spirometer (CareFusion, USA) following American Thoracic Society (ATS) and European Respiratory Society (ERS) standards.<sup>33</sup> We calculated z-scores using global lung initiative reference values.<sup>34</sup>

Children attending the emergency department with acute asthma were invited to produce a sputum sample during their admission either spontaneously following bronchodilation or after 0.9% saline nebulization as previously described.<sup>35</sup> Children were invited to return after recovery from their asthma attack to repeat spirometry, FeNO testing, sputum induction and allergy testing if it had not been performed during the emergency department visit. For children recruited during an asthma attack who declined or did not return for the second, stable visit, recovery spirometry and FeNO results were taken not earlier than six weeks after the attack from their clinical outpatient records when they were reviewed and felt to be clinically stable.

### 2.4 | Sputum processing and fungal culture

Sputum plugs were separated from saliva. Aliquots of approximately 125 mg were inoculated directly onto potato dextrose agar (PDA)



**FIGURE 1** Consort diagram of recruitment Footer: Prior to data analysis, the research team reviewed the medical notes of all the children recruited into the study. At this point, one child recruited into the stable asthma group had also been diagnosed with primary cilia dyskinesia. Due to this significant co-morbidity, a decision was taken a priori to any data analysis, to exclude this participant from the data analysis. The second child, recruited as an acute asthmatic, was excluded when it became known that the child also had an innate and secondary immunodeficiency

TABLE 1 Demographics, clinical features, spirometry and FeNO in controls, chronic asthma and acute asthma groups

Demographic features	N	Control (n = 37)	N	Chronic asthma (n = 99)	N	Acute asthma (n = 39)	p-value (all 3)	p-value (Chronic vs. acute)
Age (years)	37	12 (5–16)	99	11 (5–17)	39	9 (5–15)	0.01*	0.01**
Male (n (%))		10 (27)		57 (58)		24 (62)	<0.003***	0.82***
BMI (kg/m <sup>2</sup> )		20.1 (14.6–34.6)		19.3 (13.9–31)		17 (13.9–36.7)	0.02*	0.03**
Low or very low BMI <sup>a</sup>	37	2 (5.4)	99	7 (7.1)	39	2 (5.1)	0.13***	0.57***
Normal BMI		18 (47.6)		54 (54.5)		26 (66.7)		
Overweight		10 (27.0)		10 (10.1)		2 (5.1)		
Obese or morbidly obese (n (%))		7 (18.9)		28 (28.3)		9 (23.1)		
History of atopic disease								
Age of wheeze onset (years)	–	–	35	2 (0–14)	79	2 (0–11)	–	0.34**
Any atopic disease <sup>b</sup> (n (%))	37	5 (13.5)	99	89 (89.9)	39	31 (79.5)	<0.001***	0.18***
Hayfever (n (%))	37	1 (2.7)	99	75 (75.8)	39	26 (66.7)	<0.001***	0.38***
Eczema (n (%))	37	3 (8.1)	99	69 (69.7)	39	22 (56.4)	<0.001***	0.20***
Food allergy (n (%))	37	2 (5.4)	99	29 (29.3)	39	10 (35.6)	0.004***	0.83***
Severity of asthma <sup>c</sup>								
Mild-moderate (n (%))	–	–	99	51 (52)	39	33 (85)	–	0.001***
Severe (n (%))	–	–	–	48 (48)	–	6 (15)	–	<0.001**
Dose of ICS								
Total daily dose of ICS (micrograms)	–	–	99	400 (0–2000)	39	400 (0–2000)	–	<0.001**
Stable pre-bronchodilator spirometry								
FEV <sub>1</sub> z-score (range)	23	-0.17 (-2.26–1.25)	90	-1.09 (-3.96–2.04)	24	-1.56 (-3.34–1.54)	0.02*	0.09**
FVC z-score (range)		-0.24 (-1.89–1.00)		-0.05 (-3.02–3.06)		-0.33 (-2.58–1.94)	0.46*	0.47**
FEV <sub>1</sub> /FVC		0.88 (0.76–0.98)		0.79 (0.54–0.99)		0.74 (0.57–0.89)	<0.001*	0.11**
FeNO								
Stable FeNO (ppb)	22	13 (5–55)	87	28 (5–300)	25	30 (6–145)	0.02*	0.72**
Total serum IgE (KU/L)	12	44 (2–411)	78	429 (7.6–5000)	22	550 (85–5000)	<0.001*	0.07**
Sensitization to aeroallergens <sup>d</sup>								
Sensitized to at least one aeroallergen (n (%))	37	10 (27)	92	75 (81.5)	33	29 (87.9)	<0.001***	0.59***
Grass pollen mix	37	5 (13.5)	96	61 (63.5)	33	18 (54.5)	<0.001***	0.36***
D pteronyssinus	37	7 (18.9)	97	60 (61.9)	33	24 (72.7)	<0.001***	0.26***
Dog dander	37	2 (5.4)	94	50 (53.2)	39	13 (33.3)	<0.001***	0.17***
Cat dander	37	5 (13.5)	95	50 (52.6)	39	12 (30.8)	<0.001***	0.11***

Note: All data are presented as median (range) unless otherwise stated (eg z-score).

BMI, Body Mass Index; FeNO, fraction of exhaled nitric oxide; FEV<sub>1</sub>, forced expiratory volume in one second; FEV<sub>1</sub>/FVC, forced expiratory volume in one second over forced vital capacity ratio; FVC, forced vital capacity; ICS, inhaled corticosteroids; ppb, parts per billion.

<sup>a</sup>Low or very low BMI = BMI 9th centile or less; Normal BMI = BMI centile 25–75th centile; Overweight = BMI 91st centile; Obese or morbidly obese = BMI > 98th centile.

<sup>b</sup>Atopy was defined as any history of either hayfever, eczema or food allergy.

<sup>c</sup>Asthma severity based on Global Initiative for Asthma (GINA).

<sup>d</sup>Evidence of sensitization to either: D pteronyssinus, grass mix pollen, cat or dog.

\*Dunn-corrected Kruskal-Wallis.

\*\*Mann-Whitney U.

\*\*\*Chi-square.

P values which are less than .05 are considered statistically significant and indicated in bold and italic.

containing 16 µg/ml chloramphenicol, 4 µg/ml gentamicin and 5 µg/ml fluconazole as previously described<sup>18</sup> and incubated at 37°C for up to 7 days. A minimum of 60 mg of sputum was required and samples were discarded if this was not available. Fungi were identified based on macroscopic and microscopic morphology.<sup>36</sup> Where sufficient sputum volume was available, a leucocyte differential cell count was performed as previously described.<sup>37</sup>

## 2.5 | Statistical analysis

All data were entered into REDCap (<https://www.project-redcap.org/>), a research electronic data capture tool, a secure web-based application designed to support data capture for research studies<sup>38</sup> hosted at University Hospitals of Leicester NHS Trust and analysed using SPSS, version 25 for Windows (SPSS, Inc, Chicago, IL). Data distribution was investigated using the Shapiro-Wilk test. Parametric data were expressed as means and standard deviation and analysed by one-way analysis of variance. Nonparametric data were expressed as medians with ranges or interquartile ranges and analysed using Mann-Whitney and Dunn-correct Kruskal-Wallis tests for continuous variables. Chi-square and Fisher's exact test were used to analyse categorical variables. The Wilcoxon matched-pair test was used to analyse paired data. A *p*-value of < 0.05 was considered significant.

## 3 | RESULTS

### 3.1 | Recruitment

We recruited 186 children and obtained sputum and/or sensitization data from 175 participants: 37 controls, 99 chronic asthma and 39 acute asthma. The consort diagram is shown in Figure 1. Of the 39 children recruited during an asthma attack, 15 returned to the respiratory laboratory for paired stable induced sputum sampling.

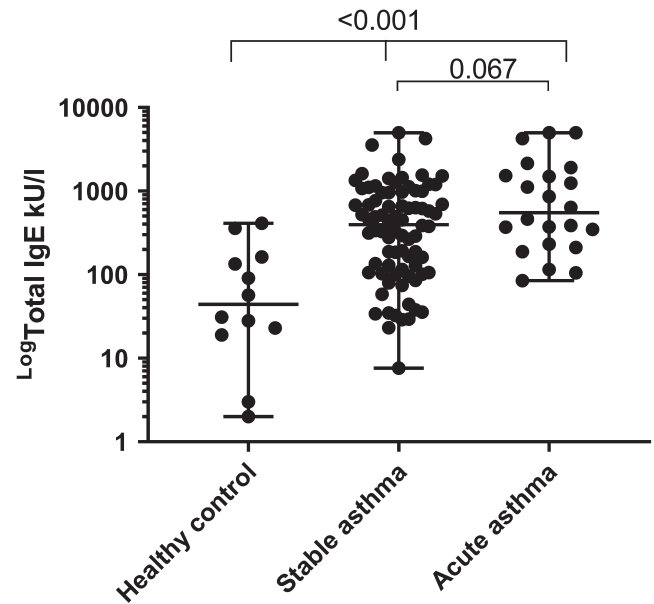
Data on demography, lung function, FeNO and atopic status are shown in Table 1.

### 3.2 | Total serum IgE

The distribution of total serum IgE values are presented in Figure 2. Serum total IgE was significantly different between healthy controls and patients with asthma but not between stable and acute asthma.

### 3.3 | Patterns of fungal IgE sensitization

No control child was sensitized to any fungi tested. In contrast, 47.8% of children with chronic and 43.3% with acute asthma were sensitized to at least one fungus (Table 2). There was no difference in the rate of sensitization between chronic stable and acute asthma for any fungi. The most common sensitizations were to *A alternata* (29.9%) and *C*



**FIGURE 2** Total serum IgE in healthy controls and children with stable and acute asthma. Footer: The bars represent the median and range of total serum IgE. Data have been log transformed due to not being normally distributed. The bars at the top of the image represent *p*-values between the groups shown

*herbarum* (29.1%), followed by *C albicans* (27.2%), *A fumigatus* (21.8%) and *P chrysogenum* (18.8%), respectively, for the chronic stable and acute asthma groups combined (total *n* = 121) where sensitization data were available. Forty-three (35.5%) children in this combined group were sensitized to a thermotolerant fungus (Table 2). Nearly two-thirds of fungal sensitized children with asthma were sensitized to more than one species; with 35% being sensitized to four or more (data not shown). Twenty-seven percent of controls were atopic to non-fungal aeroallergens compared with 81.5% of children with chronic and 87.9% with acute asthma (Table 1), and of the 43 asthmatic children sensitized to any fungi, 41/42 (97.6%) in whom data were available were sensitized to at least one non-fungal aeroallergen.

### 3.4 | Relationship between fungal sensitization and clinical outcomes

We combined the available stable asthma data from the two asthma groups. Asthmatic children sensitized to the thermotolerant fungi (*Aspergillus*, *Penicillium* or *Candida*) had a lower pre-bronchodilator FEV<sub>1</sub> z-score, FVC z-score and FEV<sub>1</sub>/FVC ratio (Table 3). The difference was not seen in the post-bronchodilator measurements (data not shown). In addition, children sensitized to thermotolerant fungi had a greater requirement for systemic corticosteroids in the previous 12 months, and a higher total serum IgE and FeNO compared to children with asthma who were not sensitized to thermotolerant fungi. They also were significantly more likely to be atopic and were more eosinophilic (Table 3).

Twenty-two children included in our study had a clinically directed computed tomography (CT) chest scan. Thirteen scans were

TABLE 2 Sensitization and fungal culture isolation in controls, chronic asthma and acute asthma

Sensitization to fungi n (%)	Control		Chronic asthma (CA)		Acute asthma (AA)		p-value* (all 3 groups)	p-value* (CA vs. AA)
	N	N (%)	N	N (%)	N	N (%)		
<i>Aspergillus fumigatus</i>	37	0 (0)	93	21 (22.6)	35	7 (20)	0.01	0.82
<i>Penicillium chrysogenum</i>	35	0 (0)	91	16 (17.6)	31	7 (22.6)	0.02	0.60
<i>Alternaria alternata</i>	37	0 (0)	92	30 (32.6)	35	8 (22.9)	<0.001	0.39
<i>Cladosporium herbarum</i>	37	0 (0)	92	28 (30.4)	35	9 (25.7)	0.001	0.67
<i>Candida albicans</i>	37	0 (0)	92	24 (26.1)	33	10 (30.3)	0.002	0.65
Sensitized to any fungi	35	0 (0)	90	43 (47.8)	30	13 (43.3)	<0.001	0.67
Sensitized to thermotolerant fungi <sup>a</sup>	35	0 (0)	91	31 (34.1)	30	12 (40)	<0.001	0.56
Fungal culture isolation n (%)								
Isolation of Filamentous fungi	34	8 (23.5)	93	19 (20.4)	35	15 (42.9)	0.03	0.01
Isolation of <i>Aspergillus fumigatus</i>	34	7 (20.6)	93	16 (17.2)	35	12 (34.3)	0.11	0.04
Isolation of yeast	34	6 (17.6)	93	36 (36.6)	35	10 (28.6)	0.12	0.40

<sup>a</sup>Thermotolerant fungi = *Aspergillus fumigatus*, *Penicillium chrysogenum* and *Candida albicans*.

\*Chi-square.

P values which are less than .05 are considered statistically significant and indicated in bold and italic.

reported to show either bronchial wall thickening, atelectasis, bronchiectasis or air trapping. There were no significant differences in these abnormalities between children sensitized to thermotolerant fungi or not (data not shown). No child with asthma in our study fulfilled the ISHAM diagnostic criteria for ABPA.

### 3.5 | Relationship between fungal culture and clinical outcomes

In the children with chronic asthma where data for fungal culture and fungal sensitization were available, we found no statistically significant differences in the rate of fungal sensitization between those with positive and negative fungal sputum cultures. Moreover, we found no significant differences in pre- or post-bronchodilator FEV<sub>1</sub>, FVC or FEV<sub>1</sub>/FVC, need for systemic corticosteroids in the previous 12 months or limitation of activities due to asthma between asthmatic children with positive and negative fungal sputum culture taken in stable disease. We also found no significant difference between FeNO, sputum eosinophils or serum total IgE in fungal culture positive and negative asthmatic children (data not shown).

We also report no difference in the number of control children who were fungal culture positive or negative in terms of sensitization to aeroallergens or difference in their pre- or post-bronchodilator FEV<sub>1</sub>, FVC or FEV<sub>1</sub>/FVC (data not shown).

### 3.6 | Fungal culture in acute asthma

Children with acute asthma had significantly higher rates of positive filamentous fungal sputum culture than children with chronic asthma and controls (Table 2). We found no seasonal differences in the rates of positive filamentous fungal cultures. There was no association between the presence of a positive sputum fungal culture and the severity of the asthma attack based on peak expiratory flow rates or the need for supplemental oxygen or intravenous bronchodilator/anti-inflammatory medication (data not shown). Median hospital admission was three days (range 0 to 12 days) for children with positive fungal culture and three days (range 1 to 5 days) for children with negative fungal culture. Children with acute asthma and positive sputum culture for filamentous fungi had higher blood eosinophils (median  $0.68 \times 10^9/L$  (n = 13) vs  $0.13 \times 10^9/L$  (n = 15);  $P = .05$ ) during the attack and higher sputum eosinophilia at recovery (median 12.5% (n = 5) vs 0.25% (n = 3);  $P < .05$ ) compared to children with negative sputum culture.

Paired acute and stable sputum samples were available from 15 children. Nine of these children (60.0%) were sputum culture positive for filamentous fungi during the asthma attack (seven for *A fumigatus*) of which only one (6.6%) remained culture positive (to *A fumigatus*) at the subsequent stable visit. One culture negative child during the acute attack became culture positive for a non-*A fumigatus* fungus at the stable visit ( $p = 0.04$ ; Wilcoxon matched-pair test). We found no significant relationship between positive sputum

**TABLE 3** Comparison between stable asthmatic children with sufficient sensitization data to be characterized as sensitized or non-sensitized to thermotolerant fungi

		Children with asthma sensitized to thermotolerant fungi <sup>a</sup> (n = 43)		Children with asthma not sensitized to thermotolerant fungi <sup>b</sup> (n = 78)		p-value
	N	Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)	
Gina treatment step <sup>c</sup> n(%)	1-3	43	21 (48.8)	78	49 (62.8)	0.18 <sup>*</sup>
	4-5		22 (51.2)		29 (37.2)	
Atopic status	Any atopic disease <sup>d</sup>	42	41 (97.6)	76	56 (73.7)	0.001 <sup>*</sup>
	Total serum IgE (KU/L)	38	961.5 (74.3-5000)	55	266 (7.60-2383)	<0.001 <sup>**</sup>
	FeNO (ppb)	36	49 (5-145)	66	19 (6-300)	<0.001 <sup>**</sup>
Stable pre-bronchodilator spirometry	FEV <sub>1</sub> z-score (range)	36	-1.76 (-3.96-1.87)	66	-0.965 (-3.62-2.04)	0.02 <sup>**</sup>
	FVC z-score (range)	36	-0.59 (-2.16-3.06)	66	0.19 (-3.02-2.45)	0.03 <sup>**</sup>
	FEV <sub>1</sub> /FVC (range)	36	0.76 (0.54-0.99)	66	0.81 (0.56-0.93)	0.04 <sup>**</sup>
Stable sputum inflammatory cells	% neutrophils	26	33 (10-97.8)	33	63.5 (6-99)	0.09 <sup>**</sup>
	% eosinophils	26	4.38 (0-29)	33	0.80 (0-30.75)	0.03 <sup>**</sup>
Courses of oral steroids in the preceding 12 months	0	31	6 (19.4)	58	22 (37.9)	0.04 <sup>*</sup>
	1-2		8 (25.8)		13 (22.4)	
	3-5		6 (19.4)		16 (27.6)	
Asthma symptoms limiting activities	>5		11 (35.5)		7 (12.1)	
	No (<twice a week)	37	21 (56.8)	69	52 (75.4)	0.08 <sup>*</sup>
	Yes (>twice a week)		16 (43.2)		17 (24.6)	

Note: All data are presented as median (range) unless otherwise stated (eg z-score).

FeNO, fraction of exhaled nitric oxide; FEV<sub>1</sub>, forced expiratory volume in one second; FEV<sub>1</sub>/FVC, forced expiratory volume in one second over forced vital capacity ratio; FVC, forced vital capacity; ppb, parts per billion.

<sup>a</sup>121 participants had complete stable asthma datasets for sensitization including thermotolerant fungi (*A fumigatus*, *P chrysogenum* and *C albicans*).

<sup>b</sup>Either sensitized to *Alternaria alternata* and/or *Cladosporium herbarum* only or not sensitized to fungi.

<sup>c</sup>Asthma severity based on Global Initiative for Asthma (GINA).

<sup>d</sup>History of atopy including hayfever, eczema or food allergy.

\*Chi-square.

\*\*Mann-Whitney U.

P values which are less than .05 are considered statistically significant and indicated in bold and italic.

filamentous fungal culture during the asthma attack and thermotolerant fungal sensitization.

## 4 | DISCUSSION

This study is to our knowledge the first comprehensive prospective investigation of the relationship between fungal isolation from sputum, sensitization and markers of disease severity in a large, real-world school-age asthma population. One of the key findings was that fungal sensitization is common in asthmatic children and that those with evidence of sensitization to thermotolerant fungi had worse lung function and were prescribed more courses of oral steroids in the preceding 12 months compared with children either not sensitized to fungi or to non-thermotolerant fungi only. Children with sensitization to thermotolerant fungi also had significantly greater sputum eosinophils, total serum IgE and higher FeNO.

Severe asthma with fungal sensitization (SAFS) in adults is characterized by the presence of severe asthma and fungal sensitization akin to ABPA, but without bronchiectasis and mucus plugging, and total IgE values < 1000 IU/mL.<sup>24,39</sup> ABPA is defined as total IgE values  $\geq$  1000 IU/mL, positive *A fumigatus*-specific IgE or SPT, and 2 of 4 criteria from the following: *A fumigatus* precipitans, raised blood eosinophils, positive *A fumigatus*-specific IgG and radiological changes on chest radiograph or computer tomography.<sup>12</sup> ABPM is similar to ABPA but the sensitising fungus is a different genus to *Aspergillus*.<sup>12</sup> The diagnostic criteria for both SAFS and ABPA are exclusive, have considerable limitations and are based on arbitrary cut-offs for total IgE values.<sup>17,40</sup> Statistics of the immunological measurements relevant to fungal lung disease only IgE sensitization to thermotolerant fungi, particularly *A fumigatus*, is consistently associated with evidence of lung damage in asthma which is the hallmark of fungal involvement.<sup>17</sup> We therefore prefer the inclusive term 'allergic fungal airway disease' (AFAD), defined as airway disease in the context of IgE sensitization to thermotolerant fungi, to describe this endotype of adult asthma, with a separate quantification of severity to accurately describe the patient's condition.<sup>26</sup> Children with asthma rarely have the fleeting lung shadows, fixed airflow obstruction or bronchiectasis<sup>41</sup> so it is unclear whether the above terms are relevant in childhood asthma. The use of the term 'fungal asthma' may be more appropriate at this stage in our understanding.<sup>25</sup>

Approximately half the children with asthma in our study were sensitized to fungal allergens. Similar rates were reported from Kuwait where > 60% of asthmatic children were found to be sensitized to fungi.<sup>42</sup> In contrast, in our study, no child without asthma was sensitized to fungi despite a third being atopic and 23.5% isolated filamentous fungi from their sputum. This is consistent with a study from Finland reporting < 1% fungal sensitization in an unselected cohort of children.<sup>43</sup>

In a retrospective case series in children with asthma, sensitization to fungi was more prevalent in those with severe disease and sensitized children had lower FEV<sub>1</sub> and FEV<sub>1</sub>/FVC ratios and more

eosinophils in bronchoalveolar lavage fluid (BALF).<sup>44</sup> Another retrospective study limited to children with severe asthma found no differences in lung function between children with and without fungal sensitization. Sensitized children had higher sputum IL-33 and greater levels of total serum IgE.<sup>29</sup> None of these studies reported whether the association with reduced lung function was limited to specific fungi, such as thermotolerant fungi and neither study reported sputum fungal culture results.

Fungi can be divided into mesophilic and thermophilic, the important difference being that the latter can colonize the airways causing a persistent allergenic and even infective stimulus which could result in greater tissue damage. In support of this concept, several recent studies in adults with asthma found that patients sensitized to *A fumigatus* and/or *Penicillium* spp. had a lower post-bronchodilator FEV<sub>1</sub> compared with non-sensitized patients with asthma or patients sensitized only to genera containing predominantly mesophilic fungi such as *Alternaria* and *Cladosporium*.<sup>18-20</sup> The average age of patients in the adult studies was > 50 years, and therefore, it is possible that airway remodelling in asthma patients with thermotolerant fungal sensitization is a slow process before clear differences emerge. Whether evidence of fungal sensitization early on in childhood asthma predisposes asthmatics to a more rapidly declining lung function trajectory would be an important research question to answer in the future.

Chronic asthma prone to acute attacks is considered a more severe form of asthma. Several mechanisms could explain the link between sensitization to thermotolerant fungi with asthma severity. In addition, airway damage as a result of severe asthma may create the environment for fungi to more readily colonize the damaged airways and lead to sensitization. Airway damage may already develop in school-age children in severe childhood asthma.<sup>41</sup> It is also possible that fungi directly contribute to the development of severe asthma by augmenting the immunological response in sensitized individuals. Data from this study to support this are higher FeNO, total IgE and a greater proportion of eosinophils in the sputum of asthmatics sensitized to thermotolerant fungi. Only well-designed longitudinal studies that include the evaluation of potential mechanistic pathways will be able to answer this important question.

To our knowledge, this is also the first study to report isolation of fungi from airway samples obtained during an acute asthma attack in either children or adults. We found that children with acute asthma had a significantly higher rate of filamentous fungal culture from sputum compared to children with stable asthma. In addition, of seven children with a positive *A fumigatus* sputum culture during the asthma attack only one child was still positive for this fungus after a full recovery from the acute episode.

Several new inhaled antifungal treatments are currently in various stages of clinical development. The long-acting inhaled azole PC945, a potent inhibitor of *A fumigatus*, may provide a targeted approach in the lung.<sup>45</sup> More recently, PC1244 has shown activity against azole-resistant *A fumigatus*.<sup>46</sup> In addition, an early phase randomized controlled trial (RCT) of PUR1900, a dry powder itraconazole preparation has shown good safety and tolerability, higher lung



and lower plasma concentration compared with oral treatment.<sup>47</sup> Identifying children who may benefit from these treatments for inclusion in clinical trials would be an important clinical advance.

Two recent studies in adults reported fungal culture positivity (*A fumigatus*) in approximately half of the severe chronic asthma patients studied.<sup>19,20</sup> A further recent study found a similar *A fumigatus* isolation rate (46%) from BALF in a mixed severity adult asthma population.<sup>48</sup> These rates in adults are higher than the just under 17% culture positivity we found in our study using the same culture method. Increased airway damage has been reported in adults with long standing asthma including bronchiectasis and tree-in-bud appearance on chest CT imaging.<sup>18</sup> These abnormal airways may be more prone to harbour fungi. The presence of bronchiectasis is associated with impaired mucociliary clearance<sup>49</sup> which creates an environment in the airways that makes fungal colonization more likely, for example in children with cystic fibrosis.<sup>50</sup> Steroid burden has been linked to the presence of an increased filamentous fungal burden in the airways,<sup>51</sup> but this has not been confirmed by all studies.<sup>48</sup>

#### 4.1 | Strength and limitations

We report fungal sensitization and culture from sputum in a large prospective cross-sectional observational study involving children with asthma. Data on pre-bronchodilator lung function, blood and sputum biomarkers were available in the vast majority of patients with asthma studied. A major strength of the current study is the inclusion of a control group of children without asthma. No controls were included in the two other large studies of fungal asthma in children.<sup>29,44</sup>

Further analysis of *A fumigatus* antigens, such as Asp F1, F2 and F4, would be beneficial, as described in a previous report.<sup>52</sup> Due to limitations on blood volumes imposed by the research ethics committee, we did not have enough serum to perform this on this occasion. These tests should be the focus of future studies.

A number of studies have shown that the traditional method used in routine microbiology laboratories in the UK to culture fungi is insensitive.<sup>41,42</sup> The method used in this study uses a high concentration of sputa and a media that inhibits the size of the yeast colonies that grow, thus increasing the likelihood of detecting co-culture of filamentous fungi.<sup>53</sup> It does not, however, prevent yeast colonies from growing or affect the number of yeast colonies observed when compared with alternative culture media (unpublished data). We isolated filamentous fungi from 23.5% of healthy control children with 20.6% having a positive culture result for *A fumigatus*. This rate is higher than that previously reported in healthy adults. Sputum samples were treated identically in controls and asthma patients and the laboratory personnel were blinded to sample origin. In addition, samples were processed in the same laboratory as the adult samples previously reported.<sup>19,20</sup> We have no reason to believe that these results are not robust. We cannot exclude a type 2 error due to issues with power but the number of controls in our study were greater than in either adult

study. Children with stable asthma and controls had their sputum sampled in a specially designed negative pressure room. The sputum of children with acute asthma was collected in the emergency department. There was no difference in fungal culture rates depending on where the sputum was obtained. We allowed for environmental exposure by inviting a selection of children controls/stable asthma to have sputum induction on the children's ward. Of the seven children having sputum induction on the children's ward, all had negative fungal culture. We also analysed nebulizer mist cultures from ward sputum inductions and separately from the ultrasonic nebulizer equipment used and none of them tested positive for fungi. It was not possible for logistic, clinical safety or ethical reasons to sample all the sputum in the same place. It is possible but unlikely that environmental contamination played a role in the high filamentous fungal isolation rate in acute asthma patients as new and sealed nebulization circuits and masks were used for every patient.

Chest CT scans are currently not routinely recommended for children with (severe) asthma, and bronchiectasis is found infrequently where this is performed.<sup>41</sup> Given the radiation exposure, this would not have been authorized by the research ethics committee. The decision to perform a CT scan was therefore a clinical one and most children in our cohort did not have a CT chest. Twenty-two children included in our study had a CT scan (decided clinically, not as part of their inclusion in the study). Thirteen had an abnormality on their CT scan, defined as either bronchial wall thickening, atelectasis, bronchiectasis or air trapping of which three had bronchiectasis (all in the sensitized group). None of these were significantly different between children sensitized to thermotolerant fungi or not.

All the data were obtained during a single visit to either the emergency department or the respiratory function laboratory limiting the conclusions that can be drawn on causality. We do not know whether a positive fungal culture preceded the asthma attack or whether the airway milieu during the acute asthma attack created a favourable environment for fungi to colonize the airway at least transiently. However, the link noted with eosinophilic inflammation suggests the observation is clinically relevant.

Unpicking the contribution of fungal sensitization and presence of fungi in sputum to attack-prone and severe asthma characterized by reduced lung function will require more research, in particular longitudinal studies. Many children with fungal sensitization also had concomitant aeroallergen sensitization and as previously reported, a significant number of our healthy cohort also had aeroallergen sensitization but none were sensitized to fungi. This suggests that fungal sensitization is associated with diseases including asthma whereas aeroallergen sensitization is not. In contrast, isolation of fungi from sputum occurs in people with asthma and healthy controls at similar rates in children. We can only speculate that unless there is co-existing fungal sensitization, harbouring fungi in the airways may not itself be harmful.

Studying the seasonal effect of fungal sensitization and presence of fungi in the airways and asthma attacks is challenging. We know

that asthma attacks are more frequent in the colder and damper seasons when fungi also thrive. Demonstrating causality is challenging but should be addressed in future studies.

*In summary*, there are three key questions in trying to understand the role of fungi in a fungal asthma endotype; 1. When do fungi become relevant in the pathogenesis of this endotype? 2. Which fungi are implicated? and 3. What is the pathological pathway(s) through which their affect is mediated?

We have addressed the first two questions and found that nearly half of school-age children with chronic asthma are sensitized to fungi compared with none in the controls, and that sensitization to thermotolerant fungi is associated with worse lung function, greater IgE and more airway inflammation. We also found significantly greater numbers with a positive sputum fungal culture during the asthma attack compared with periods of asthma stability.

### CONFLICT OF INTERESTS

None of the authors have a conflict of interest with respect to this manuscript. EAG has received grants and consultancy paid to his institution from Chiesi, Gilead, Circassia and Boehringer Ingelheim. AJW has received grants and consultancy from Pulmocide and GlaxoSmithKline and consultancy from Astra Zeneca and TEVA. CHP has received grants and consultancy from Pulmocide.

### AUTHOR CONTRIBUTIONS

C. H. P., A. J. W. and E. A. G. conceived and designed the study. K. G. W., J. S. and W. M. participated in the data acquisition. K. G. W. and K. A. H. analysed the data. K. G. W. and K. A. H. jointly wrote the first draft of the manuscript. All authors contributed to the interpretation of the work, contributed to the critical revision of the manuscript and approved the final version submitted for publication.

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### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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