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Title: Serum IgE and IgG Reactivity to Aspergillus Recombinant Antigens in Patients with Cystic Fibrosis

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Abstract:

Purpose: The diagnosis of aspergillosis in cystic fibrosis (CF) remains a challenge due to overlapping features of both diseases. This is further complicated by inconsistent antibody reactivity to the currently used crude antigen which has led a more focused evaluation of the efficacy of IgE response to a number of pure *Aspergillus fumigatus* recombinant proteins in patients with CF and asthma. In this study, we dissected the IgE and IgG responses to multiple *Aspergillus fumigatus* recombinant antigens in CF patients with different *Aspergillus* diseases.

Methodology: Serum IgE and IgG antibodies were measured in 12 CF patients with Allergic Bronchopulmonary Aspergillosis (ABPA), 12 with *Aspergillus* sensitization (AS), and 12 with *Aspergillus* bronchitis (AB) against recombinant antigens Asp f1, f2, f3, f4, and f6.

Results: The ABPA group showed significantly greater IgE reactivity to Asp f1, f2, f3 and f4 compared to patients with AS. Patients with AB expressed higher IgG positivity to Asp f1 and Asp f2 compared with ABPA. Very low IgE antibodies levels against all recombinant antigens in patients with *Aspergillus* sensitization. Aspf1 IgG reactivity in ABPA correlated with positive culture.

Conclusion: the use of multiple recombinant antigens may improve the diagnostic accuracy in CF complicated with ABPA or *Aspergillus* bronchitis. Asp f1 reactivity may relate to the presence of actively growing *Aspergillus spp*. which might be a useful marker for guiding antifungal therapy in ABPA.

Introduction:

Allergic bronchopulmonary aspergillosis (ABPA) is the one of the most serious fungal diseases in Cystic Fibrosis (CF). It is characterized by a hypersensitivity reaction to *Aspergillus spp.* with diverse clinical and radiological manifestations. The precise prevalence of ABPA in CF lungs is unclear and reports vary from 3% to 25% [1]. Due to the fact that ABPA has similar clinical features to poorly controlled CF lung disease, the diagnosis of ABPA in the context of CF can be extremely difficult compared with ABPA without CF.

Several diagnostic definitions for ABPA have been proposed, but in 2013 new criteria were developed by the International Society for Human and Animal Mycology (ISHAM) working group to assist physicians and clinical researchers [2]. The criteria are primarily serological. Although the isolation of *Aspergillus fumigatus* from sputum sample can sometimes be helpful, it was not included in new criteria due to its low sensitivity and specificity. In addition, consolidation seen in chest radiography of most ABPA patients with both CF and asthma is not very specific and can mimic other pulmonary infections including pulmonary tuberculosis as reported earlier [3]. Also, central bronchiectasis occurs at very late stage of ABPA and its demonstration indicates irreversible lung damage [4]. Consequently, many experts suggest that bronchiectasis is a complication rather than a criterion for ABPA diagnosis, and earlier stage diagnosis, before the development of permanent lung damage, is recommended [5].

Serological manifestations contribute strongly to the confirmation or exclusion of clinically suspected ABPA. Elevated total serum IgE >1000 IU/ml often suggests ABPA and is one of two required features [2]. The other required immunological parameter is the presence of raised serum IgE antibodies specific for *A. fumigatus*, although a specific level has not been defined. The presence of serum precipitins or raised specific IgG against *A. fumigatus*, eosinophilia and radiological signs are used as minor signs in these guidelines [2].

IgE and IgG antibodies to *Aspergillus* spp. antigens are usually evaluated with a crude antigen, which lack reproducibility and cross-react with other fungal antigens [6, 7]. Therefore, several attempts have been made to enable cloning of the genes encoding a number of *A. fumigatus* proteins [8], and the use of purified antigens to enhance the reliability of diagnosis have been reported in literature [9-15].

An additional *Aspergillus*-related disease entity was proposed in 2006, *Aspergillus* bronchitis, in a group of six CF patients who presented with respiratory deterioration despite standard antibiotic therapy, positive culture for *Aspergillus* sp. and an absence of evidence for an allergic reaction or atopy [16]. Later Baxter et al proposed an

immunological classification of *Aspergillus* diseases in CF, and Aspergillus Bronchitis (AB) in CF patients was characterized by markers of fungal infection (positive culture, high levels of *Aspergillus* DNA and galactomannan in sputum, and positive IgG to *A. fumigatus*) without any sign of a hypersensitivity response [17]. However, almost no serological analysis on the response to recombinant antigens had been carried out in CF patients with *Aspergillus* bronchitis, apart from analysis of IgE to Asp f4 and f6 in two patients with *Aspergillus* bronchitis [10]. Also, there has been no reported analysis of IgG responses to recombinant antigens in this patient group, which is more relevant.

The aim of this study was to evaluate the differential IgE and IgG responses to a set of recombinant antigens (Asp f1, Asp f2, Asp f3, Asp f4 and Asp f6) in CF patients with ABPA in comparison with *Aspergillus* bronchitis (AB), and a group of *Aspergillus*-sensitized (AS) patients in order to determine the diagnostic value of these antigens.

Methods:

Patient population:

This was a retrospective study utilizing sera from CF patients. The study was approved by Leeds East Research Ethics Committee in 2008 (08/H1306/103). Serum samples from three different groups of *Aspergillus* diseases were selected from samples collected between November 2016 and August 2017. Each serum was collected from different patients. Serum samples were stored at -20°C before use. In these patients, sputum samples were cultured on Sabourauds agar at 35°C and 45°C for 7 days. Once patient samples were identified, all were anonymised. Sera were analysed for reactivity to crude *A. fumigatus* antigens and recombinant antigens Asp f1, f2, f3, f4, and f6. The criteria used to select samples in the three groups were as follows:

Allergic Bronchopulmonary Aspergillosis (ABPA): criteria recommended by Agarwal et al [2] were used. These were elevated IgE levels against *Aspergillus fumigatus* (> 0.35 kUA/l), raised total IgE levels (> 1000 IU/ml), and at least two of the following: presence of *Aspergillus* IgG antibodies (> 90 mg/l), radiological findings compatible with ABPA, and eosinophilia (> 0.5 x 10^9 /l).

***Aspergillus* Bronchitis (AB):** elevated *Aspergillus* IgG (> 90 mg/l), and positive sputum culture for *Aspergillus* species (within a period of 3 months before or after the positive *Aspergillus* IgG result).

***Aspergillus* sensitization (AS):** elevated IgE levels against *Aspergillus fumigatus* (> 0.35 kUA/l) and IgG antibodies against *A. fumigatus* in serum < 90 mg/l.

A total of 36 serum samples were collected from the three groups: ABPA (n=12), AB (n=12), and AS (n=12). Both IgE and IgG antibodies were measured in ABPA samples and compared with the levels of IgE in AS and IgG in AB, respectively.

ImmunoCAP studies:

Serum antibodies testing was performed utilizing Phadia ImmunoCAP (Thermo Fisher scientific, Uppsala, Sweden). All sera were tested for the presence of specific antibodies against crude *A. fumigatus* antigen and recombinant antigens Asp f1, f2, f3, f4, and f6 (Thermo Fisher Diagnostics, UK). The procedures followed were exactly according to the manufacturer's protocol. Percent positivity to antigens was calculated where for IgG the response was > 2mg/L, and for IgE > 0.35 kUA/L

Statistical analysis:

The collected data were analysed using SPSS version 24.0. Since the data followed a non-normal distribution, non-parametric tests were used for analysis. The levels of IgE and IgG antibodies in sera from CF patients with ABPA were compared with those in the AS group and AB group, respectively. The statistical significance of differences between the groups were analysed by the Mann–Whitney *U*-test. A P value of < 0.05 was considered statistically significant.

Results:

Demographic data:

The demographic characteristics of the patients are shown in Table 1. The median IgG level to crude *A. fumigatus* antigen for ABPA and AB was almost identical. IgE to crude *A. fumigatus* antigen in ABPA was much higher than AS group and almost certainly relates to the fact that ABPA patients were also selected on the basis of total IgE levels of 1000 IU/l or higher.

Levels of IgE to *A. fumigatus* recombinant antigens: Sera in patients with ABPA showed significantly greater binding to Asp f1, f2, f3, and f4 antigens than patients in the AS group ($P < 0.05$ by Mann–Whitney *U*-test). (Figure 1A). Antibodies against Asp f6 demonstrated very weak binding in both groups ($P > 0.05$ by Mann–Whitney *U*-test). In general, the levels of IgE antibodies against all recombinant antigens in patients with AS were very low (Fig. 1A).

When the patterns of reactivity were analysed among patients in the same disease group, some ABPA patients showed strong binding to multiple recombinant markers, whereas a few reacted to only a single one (range 55%-100%); all ABPA subjects reacted to Asp f2 (Table 2). Lower frequencies of responses to recombinant antigens were noticed in patients with *Aspergillus* sensitization (range 0%-67%) (Table 2). No antibodies against Asp f4 were detected in the sensitized group. One AS patient showed no reaction to any recombinant markers. Another AS patient had barely detectable level of IgE antibodies against Asp f3 and no reactivity to other antigens.

Levels of IgG to *A. fumigatus* recombinant antigens: IgG antibodies against the Asp f1 antigen were high in both ABPA and AB groups compared with other markers (Figure 1B). Patients with *Aspergillus* bronchitis expressed significantly greater positivity to Asp f1 and Asp f2 compared with reactions in patients with ABPA ($P < 0.05$ by Mann–Whitney *U*-test) (Figure 1B). When Asp f3, Asp f4, Asp f6 antigens were tested, lower reactivity was detected in ABPA and AB groups and there were no significant differences in reactions to these antigens between ABPA and AB (Mann-Whitney *U*-test).
The differences in IgG response to each recombinant antigens among ABPA patients was noticeable among patients (range 75%-100%) (Table 2) As with IgE results, all patients were positive for Asp f2. Notably, of the 12 ABPA subjects studied, one showed IgG response to Asp f2 alone and failed to demonstrated reactivity with other antigens. Among *Aspergillus* bronchitis group, there was a strong binding of IgG antibodies to all the markers (range 95%-100%), which lead to higher frequency of IgG positivity to

recombinant antigens in *Aspergillus* bronchitis than in patients with ABPA (Table 2). None of the antigens showed consistent IgG binding in ABPA or AB, making it difficult to determine the validity of these markers for defining patient groups. Interestingly, we found that the median of all Asp f1 IgG levels is three times higher in ABPA patients with positive sputum *Aspergillus* culture (n=6), compared to those with negative culture (n=4) 27.15 mg/l and 10.19 mg/l respectively (for two out of the twelve ABPA patients, sputum culture results were not available). Although this was not significant, it suggests a correlation between positive culture and Asp f1.

Discussion:

ABPA is associated with high risk of irreversible lung damage, which requires early initiation of steroid treatment to control progressive lung destruction. It is widely believed that there is a fundamental role for serology in confirming or ruling out the disease. Several reports on serological markers for ABPA in CF as well as asthma have been conducted, and there is a substantial body of literature analysing their effectiveness for ABPA diagnosis [9-15]. In contrast, almost no serological analysis on the usefulness of recombinant antigens has been conducted on *Aspergillus* bronchitis patients.

Our study shows that the dissection of the IgE and IgG responses to crude antigens by using recombinant markers produces some interesting results in patients with different *Aspergillus* diseases. The pattern of reactivity of IgE to recombinant antigens demonstrated that Asp f1, f2, f3 and f4 are major antigens in individuals with ABPA and there was significantly higher levels of IgE compared to the AS group.

Antigens Asp f2 and f4 emerged as the strongest candidates to differentiate CF patients with ABPA from CF patients with asthma, although none of the recombinant antigens clearly differentiated between these groups [14]. According to Kurup et al, Asp f2, Asp f4, and Asp f6 showed significant binding to IgE in asthma patients with ABPA [13]. The reactivity of Asp f6 in this study was too low to be considered as a good marker. This has also been reported in several studies on CF patients with ABPA where Asp f4 sensitivity was described as superior to Asp f6 [10, 12, 18]. Moreover, the utility of Asp f6 for the diagnosis of Aspergillus diseases is still debatable as it has been reported to be a pan-allergen exhibiting cross-reactivity with *Alternaria* and *Malassezia* species [5, 7]. Other studies on asthma patients have also reported that IgE reactivity to Asp f2 [19, 20] and Asp f4 [21] distinguished ABPA from those with *Aspergillus* sensitization. Although previous works found Asp f1 and f3 antibodies in the sera of *Aspergillus* sensitized patients [9, 13, 21], this finding was not seen in the present study as all the patients with AS showed very low levels of IgE reactivity to recombinant markers. We noted a significant difference in the levels of total IgE and specific IgE to crude *Aspergillus* antigen between ABPA and AS groups. In our study, sensitized patients were defined by lower levels of total IgE and this is likely to be linked to lower levels of IgE to crude and recombinant *Aspergillus* antigens compared with patients with ABPA.

To the best of our knowledge, this is the first study to evaluate the performance of IgG reactivity to recombinant antigens in *Aspergillus* bronchitis. The high rate of seropositivity to Asp f1 and Asp f2 in patients with AB was significantly higher than in patients with ABPA, and might be attributed to the presence of actively

219 growing fungus in the airway of patients with AB. It is well known that Asp f1 is a species-specific major
220 allergen [22], and produced only during germination and growth of the fungus [23]. In this study, 60% of ABPA
221 patients were sputum culture positive whilst AB patients were defined by a positive *Aspergillus* sputum culture.
222 We also noticed that sera from ABPA patients with positive sputum culture in the current data showed a higher
223 median IgG level to Asp f1 compared with those with negative culture. Although this was not statistically
224 significant, it was an interesting finding that had not seen with other recombinant markers. This suggests that
225 Asp f1 might also play an important role in differentiating ABPA, where *Aspergillus* is actively growing
226 regardless of the culture result. If this observation validated in further studies, it may have implications for the
227 use of antifungals as an alternative or adjunct to steroid therapy in CF patient with *Aspergillus* disease.

228 The reactivity of the recombinant antigens varied considerably among patients in the same disease group
229 for IgE testing in ABPA and IgG testing in AB; this is also apparent from other studies without being
230 commented on by their authors [14, 20]. The reason for this variation between patients is unknown. However, it
231 may relate to variation in the interactions between the complex mixture of bacterial and fungal microbes
232 residing in the CF lung. *Peudomonas aeruginosa*, the most dominant and persistent pathogen found in CF, has
233 been shown to inhibit *A. fumigatus* filamentation mediated by both direct contact and indirect interaction via the
234 release of molecules responsible for intra-cellular communication [24, 25]. In this study, however, the analysis
235 was limited to *A. fumigatus* and the status of other microorganisms within individual patients was not known.
236 Another possible explanation is that different isolates of *A. fumigatus* might express varied concentrations of
237 recombinant antigens. Previous studies have confirmed that *A. fumigatus* antigens are heterogeneous, and
238 concentrations of individual proteins produced vary between and within different strains of the fungus [26, 27].
239 Furthermore, the stages of ABPA disease seem to play an important role in the immune recognition of the
240 recombinant antigens. Knusten et al, highlighted the usefulness of a number of recombinant antigens in
241 discriminating between ABPA stages of flares and remission. The group reported Asp f1, f2, f3, and f6 as major
242 antigens in flares while Asp f3 and Asp f4 remained raised even during period of remission [12]. It is also
243 believed that steroid therapy increases the growth rate of the *A. fumigatus* by 30-40% [28, 29], and this may lead
244 to a stronger immune response and higher antibody production against *Aspergillus* antigens, as seen with Aspf1.
245 Furthermore, genetic background may also leads to different immune response in individual patients, as reported
246 previously for IgG [30].

247 This study is limited by the fact that patient's numbers were modest, and their diseases were defined almost

entirely based on laboratory test results and not pulmonary function or the general clinical picture. Thus, larger comprehensive standardized studies are required to confirm the validity of these observations.

In conclusion, it seems likely that the response to different recombinant antigens are involved in the immunopathogenesis of different *Aspergillus* diseases. The IgG seropositivity to Asp f1 and Asp f2 might be of value in the diagnosis of *Aspergillus* bronchitis and possibly in a subset of ABPA patients. Whereas, in patients with ABPA, due to the wide pattern of markers reactivity, the use of multiple IgE antigens together with the other ABPA criteria could potentially provide greater diagnostic accuracy with the classic allergic form of the disease.

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Conflict of interest: none

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Tables:

Patients groups	Number of samples	Sex f/m	Age Y (median, range)	Asp IgG mg/l (median, range)	Asp IgE IU/l (median, range)	Total IgE IU/l (median, range)	Eosinophils $\times 10^9$/l (median)	Culture positive*
ABPA	12	4/8	16,3-23	132, 105-200	26, 14-57	2210, 1095-5000	0.60	6/10
AB	12	7/5	25,5-36	130, 98-200	<0.35	11, 2-63.7	0.20	12/12
AS	12	3/9	26,13-52	48, 18-64	5, 1-13	130, 21-392	0.28	3/12

Table 1: Characteristics of patients studied. (ABPA: Allergic Bronchopulmonary Aspergillosis, AS: *Aspergillus* Sensitization, AB: *Aspergillus* Bronchitis). * Positive culture for *Aspergillus fumigatus.*

	IgE		**IgG**	
	ABPA	**AS**	**ABPA**	**AB**
Antigen	% positive	% positive	% positive	% positive
Crude	100	100	100	100
Asp f1	83	67	83	100
Asp f2	100	33	100	100
Asp f3	92	58	92	100
Asp f4	75	0	92	92
Asp f6	55	25	75	92

375

376 Table 2: Proportion of patients with positive reactions (IgE >0.35 kUA/L, IgG >2mg/L) to *Aspergillus*
377 crude antigen and recombinant antigens according to patients group. (ABPA: Allergic
378 Bronchopulmonary Aspergillosis, AS: *Aspergillus* Sensitization, AB: *Aspergillus* Bronchitis).

379

380 Fig. 1: Box plots of the: (A) Serum IgE reactivity pattern to recombinant antigens Asp f1, f2, f3, f4,
381 and f6 in ABPA and AS patients. (B) Serum IgG reactivity pattern to recombinant antigens Asp f1, f2,
382 f3, f4, and f6 in ABPA and AB patients. *P* value indicates the statistical significance between the
383 groups by Mann–Whitney *U*-test.

384 ○, * outliers, ■ ABPA, ▨ AS, ▩ AB. (ABPA: Allergic Bronchopulmonary Aspergillosis, AS:
385 *Aspergillus* Sensitization, AB: *Aspergillus* Bronchitis).

386