Blood basophil activation is a reliable biomarker of allergic bronchopulmonary aspergillosis in cystic fibrosis

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ABSTRACT The diagnosis of cystic fibrosis (CF) patients with allergic bronchopulmonary aspergillosis (ABPA) is clinically challenging, due to the absence of an objective biological test. Since blood basophils play a major role in allergic responses, we hypothesised that changes in their surface activation pattern discriminate between CF patients with and without ABPA.

We conducted a prospective longitudinal study (Stanford cohort) comparing basophil activation test CD203c levels by flow cytometry before and after activation with *Aspergillus fumigatus* allergen extract or recombinant *Asp* f1 in 20 CF patients with ABPA (CF-ABPA) and in two comparison groups: CF patients with *A. fumigatus* colonisation (AC) but without ABPA (CF-AC; n=13) and CF patients without either AC or ABPA (CF; n=12). Patients were tested every 6 months and when ill with pulmonary exacerbation. We also conducted cross-sectional validation in a separate patient set (Dublin cohort).

Basophil CD203c surface expression reliably discriminated CF-ABPA from CF-AC and CF over time. *Ex vivo* stimulation with *A. fumigatus* extract or recombinant *Asp* f1 produced similar results within the Stanford ($p<0.0001$) and the Dublin cohorts. CF-ABPA patients were likelier to have elevated specific IgE to *A. fumigatus* and were less frequently co-infected with *Staphylococcus aureus*.

Basophil CD203c upregulation is a suitable diagnostic and stable monitoring biomarker of ABPA in CF.

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Introduction

In patients with cystic fibrosis (CF), proper treatment requires effective tools to distinguish patients with bacterial from those with fungus-associated disease, as their management is different. This distinction is further complicated by a paucity of distinguishing pathogen-specific symptoms and signs [1]. Allergic sensitisation to the filamentous fungus *Aspergillus fumigatus* significantly impacts the development and severity of CF lung disease [2–9]. Allergic bronchopulmonary aspergillosis (ABPA) is the most common form of allergic bronchopulmonary mycosis [1, 10]. ABPA is a complex pulmonary disorder caused by immunological reactions to antigens released by *A. fumigatus* colonising the tracheobronchial tree of some CF and asthma patients. ABPA is clinically characterised by wheeze and compromised lung function with pulmonary infiltrates, airway mucus plugging and bronchiectasis. In literature meta-analyses, the prevalence of ABPA, thought to be 1–3.5% in unselected asthma populations, is 13% in referred/specialty clinic asthma populations and 8% in patients with CF with an estimated adult global ABPA burden of close to 5 million, including >6500 CF adults [11–14].

Underdiagnosis of ABPA is present in the CF community [13, 15]. This observation may be partially explained by the lack of agreement on diagnostic criteria for ABPA in asthma and CF, despite attempts at harmonisation and updating [1, 16, 17]. They also could be explained by the fact that clinical onset of ABPA is a temporally dynamic process: only some patients colonised with *A. fumigatus* will develop sensitisation or ABPA. Thus, a reclassification of patients, as already suggested in CF, may be needed in order to better define patient subgroups at risk. In order to clarify this dynamic process, prospective longitudinal studies of such at-risk patient groups are required. In CF, delayed recognition and treatment of ABPA has been shown to lead to accelerated lung function decline and worsening of bronchiectasis [18, 19].

Recent studies have focused on better diagnostic tools to classify *Aspergillus* pulmonary diseases. In particular, Baxter *et al.* [8] reclassified pulmonary aspergillosis in an adult CF centre cohort by combining two newer biomarker methods for detecting *A. fumigatus* colonisation (AC) and infection (sputum galactomannan and real-time PCR) with conventional fungal culture, and total IgE, and *A. fumigatus*-specific IgE and IgG serology. As a crucial component of the underlying pathophysiology of ABPA involves type I hypersensitivity [20], we hypothesised that blood basophil activation surface markers such as CD203c could help in the diagnosis of CF patients with ABPA (CF-ABPA) and might help define ABPA exacerbations.

In a previous cross-sectional study using a flow cytometric basophil activation test (BAT), we showed that blood basophils are primed and hyper-responsive to *A. fumigatus* allergen stimulation in patients with CF-ABPA compared to CF patients with *A. fumigatus* colonisation (AC) without ABPA (CF-AC) and CF patients without AC or ABPA (CF) [21]. Here, in a prospective 2-year longitudinal cohort study (the first of its kind in ABPA, to our knowledge), we show that blood basophil CD203c surface expression can be used to reliably and robustly discriminate CF-ABPA from CF-AC and CF over time. This discriminative ability was confirmed with another CF centre in an independent cross-sectional cohort. Additionally, *ex vivo* stimulation with *A. fumigatus* extract or recombinant (r)*Asp* f1 allergen produced similar results. However, clinical pulmonary exacerbations of CF that might be due to ABPA were not identifiable by this method. Other potential diagnostic tools such as *A. fumigatus*-specific IgE or IgG to *A. fumigatus* extract or r*Asp* f3 and r*Asp* f4 did not reliably discriminate these groups. *A. fumigatus*-specific IgE was more commonly elevated in ABPA patients and, elsewhere, we show that in combination with the basophil CD203c activation test, CF-ABPA patients can also be distinguished from *A. fumigatus*-sensitised patients without ABPA [22]. Finally, analysis of bacterial co-infections revealed a surprising negative association between colonisation with a major bacterial CF pathogen, *Staphylococcus aureus*, and ABPA.

Methods

Study design and subjects

A prospective 2-year longitudinal cohort design was employed at Stanford University Medical Center (Stanford, CA, USA) while a cross-sectional validation cohort was enrolled at the Royal College of Surgeons Beaumont Hospital Cystic Fibrosis Center (Dublin, Ireland). In the longitudinal study at Stanford, patients were seen and tested every 6 months and when presenting as ill with a pulmonary exacerbation. The Stanford Administrative Panel of Human Subjects in Medical Research and the Beaumont Hospital Institutional Review Board approved the study. All subjects (or parents, for minors) signed informed consent forms before they underwent study procedures.

At entry, CF patients were categorised into one of four groups: 1) CF without AC or ABPA/serological ABPA (ABPA-S) (CF), 2) CF with *A. fumigatus* colonisation without ABPA/ABPA-S (CF-AC), 3) CF with ABPA-S (CF-ABPA-S) and 4) CF-ABPA. Two non-CF asthma patients with ABPA (one at Stanford and one in Dublin), as well as healthy volunteer controls, were also studied (table 1). The diagnosis of CF-ABPA was based on CF Foundation Consensus Conference criteria [1]. CF-ABPA-S was defined as CF with

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TABLE 1 Baseline summary clinical characteristics of the Stanford and Dublin cystic fibrosis cohorts

Category	Centre	Subjects n	Age years	IgE kU·L^{-1}	FEV_1 % pred	FVC % pred	Phe508del homozygotes %	Subjects on ITRA %	Subjects on OS %	Subjects on IS %
CF	Stanford	12	17.1±2.8	108.9±82.2	84.8±4.6	96.0±4.2	41.7	0.0	0.0	66.7
	Dublin	11	27.3±1.6	46.2±13.1	49.3±5.8	69.6±5.3	27.2	9.1	0.0	36.4
CF-AC	Stanford	13	24.8±3.4	66.6±25.0	80.4±5.1	92.8±4.5	69.2	23.1	7.7	76.9
	Dublin	8	31.8±7.0	64.1±21.1	47.2±9.3	66.2±7.7	37.5	0.0	12.5	37.5
CF-ABPA	Stanford	20	25.3±3.7	759.0±126.0	77.2±5.1	91.0±4.8	45.0	40.0	35.0	80.0
	Dublin	6	26.8±2.0	609.7±93.8	63.8±6.2	91.0±7.5	33.3	33.3	16.7	66.7
CF-ABPA-S	Stanford	3	18.0±2.3	225.7±90.5	93.7±2.3	99.0±2.3	33.3	0.0	0.0	100.0
	Dublin	1	28.0		74.0	99.0	0.0	0.0	0.0	0.0
ABPA-asthma	Stanford	1	8.0	2839.0	78.0	95.0		0.0	100.0	0.0
	Dublin	1	29.0	615.0	59.0	103.0		0.0	0.0	100.0

Data are presented as mean±SE unless otherwise stated. FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; ITRA: itraconazole; OS: oral corticosteroids; IS: inhaled steroids; CF: cystic fibrosis; AC: *Aspergillus fumigatus* colonisation; ABPA: allergic bronchopulmonary aspergillosis; S: serological.

serological criteria for ABPA but without bronchiectasis on high-resolution computed tomography (HRCT) of the chest [23]. CF-AC was defined by the presence of *A. fumigatus* in at least two different sputum cultures within the previous 2 years [24]. For more details, please see the online supplementary material.

Sample collection and processing

Blood was collected by venipuncture in EDTA Vacutainer tubes (BD Biosciences, San Jose, CA, USA) and immediately placed on ice.

Incubation with saline or antigens

We assessed blood basophil, neutrophil and eosinophil responses under four conditions: 1) on ice, at baseline; 2) incubation with PBS (referred to as "without stimulation"); 3) incubation with offending *A. fumigatus* allergen (*A. fumigatus* clinical grade skin test extract; Greer, Lenoir, NC, USA) or r*Asp* f1 (Indoor Biotechnologies Inc., Charlottesville, VA, USA); and 4) incubation with heterologous nonoffending antigen (peanut allergen extract; Greer). All subjects were screened and negative for history of peanut allergy. The *A. fumigatus* extract in Dublin was obtained from Mediwiss Analytic Gmb (Moers, Germany).

Cell staining

Granulocytes in blood and each condition for the blood basophil assay (without stimulation or with stimulation by *A. fumigatus* extract/r*Asp* f1/peanut extract) were characterised with surface staining combinations. Surface staining for CD203c and CD63 was performed prior to fixation, as described previously [21, 25].

Flow cytometry data acquisition and analysis

We used a LSRII digital flow cytometer (BD Biosciences) equipped with four lasers (535, 488, 633 and 405 nm), two light-scatter detectors (yielding forward and side scatter data) and 18 fluorescent detectors. Analytical gates for basophils included ⩾300 cells [26].

Statistics

We used the JMP12 and SAS 9.4 software (SAS Institute, Cary, NC, USA) for all statistical analyses presented here. Differences were considered significant if p-values were <0.05. Discriminant analysis was used to select the outcomes that were the most associated with the category assignment at baseline. Two basophil activation marker outcomes (CD203c and CD63) and traditional measures (*A. fumigatus*-specific IgE/IgG) were included in this discriminant analysis. CD203c, the marker that showed the best discriminatory performance in a previous ABPA study [21], was evaluated longitudinally over the four visits and in individual and pooled receiver operating curve (ROC) analyses. These marker outcomes were log-transformed. Longitudinal analysis was conducted for both continuous and binary data using repeated measurements analysis. Specifically, the six correlation coefficients that describe the correlations among the four visits were estimated from the data. Models included testing for visit and category. For more details, please see the online supplementary material.

Results

BAT CD203c is consistently elevated in CF-ABPA over time

In our previous CF-ABPA BAT study, we evaluated several basophil activation markers (including CD123 and CD63 as well as CD203c) and found that the best discriminatory performance was obtained with CD203c [21]. Here, we wanted to see whether this upregulation of CD203c was sustained over time, which could suggest monitoring, as well as diagnostic, utility. In addition, we wanted to determine if CD203c levels over time were influenced by key clinical variables such as bacterial co-infection and pulmonary exacerbations.

In discriminant analysis, CD203c showed better discriminatory performance than CD63 and the best results were obtained at 10 min following *A. fumigatus* stimulation. Figure 1 shows box plots of CD203c at 10 min (figure 1a) and 30 min (figure 1b) following *A. fumigatus* stimulation, as well as at 30 min following stimulation with r*Asp* f1 (figure 1c), at baseline. CD203c levels were highest in CF-ABPA and CF-ABPA-S. CD203c levels were lower and similar in CF, CF-AC and healthy controls (figure 1). Similar results were obtained in the cross-sectional study performed in the Dublin cohort (table 2). CD203c BAT values were significantly higher in CF-ABPA than in the CF and CF-AC groups ($p<0.0001$) when tested at either 10 or 30 min incubation with *A. fumigatus* extract, and were also at or approaching significance after incubation with r*Asp* f1 ($p=0.04$ for CF *versus* CF-ABPA, $p=0.08$ for CF-AC *versus* CF-ABPA) (figure 1). This suggests that r*Asp* f1 may be a suitable standardised molecular substitute for the complex and variable *A. fumigatus* extracts as an antigen source in the BAT assay.

CD203c BAT results also varied by visit after stimulation with heterologous allergen (peanut extract), perhaps indicating variations in laboratory conditions. Because of this element of variability, CD203c BAT measures after stimulation with peanut extract were included in final multivariate longitudinal models. Results for multivariate models are shown in figure 2, which include the adjusted log mean and standard error. In these adjusted models, CD203c levels after peanut stimulation are a predictor of the main outcome (CD203c levels 10 min after *A. fumigatus* stimulation), even with category and visit included in the model; and the effect of visit on CD203c levels after *A. fumigatus* stimulation was markedly reduced from the univariate analysis (results not shown).

For all three experimental outcomes (*A. fumigatus* extract incubation at 10 min, *A. fumigatus* at 30 min and r*Asp* f1 at 30 min), BAT values were significantly higher in CF-ABPA than in CF and CF-AC. At 10 min post-stimulation with *A. fumigatus* extract (figure 2a), the category effect had a p-value of <0.0001 and the effect of visit was not statistically significant ($p=0.1$). At 30 min post-stimulation with *A. fumigatus* extract (figure 2b), the category effect had a p-value of 0.002 and the effect of visit was also statistically significant ($p=0.03$). After stimulation with r*Asp* f1 for 30 min (figure 2c), the category effect had a p-value of <0.0001 and the visit effect had a p-value of 0.06. BAT CD203c was also increased following *ex vivo* stimulation with *A. fumigatus* extract in the two patients with asthma–ABPA as compared to healthy controls (data not shown).

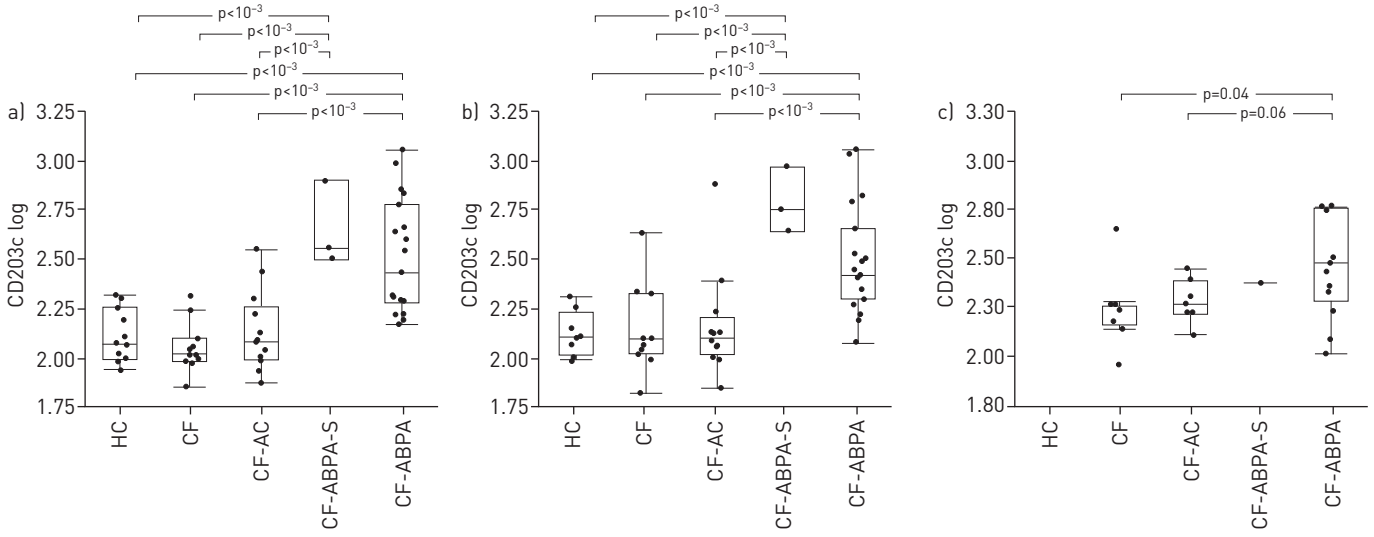

FIGURE 1 *Ex vivo* stimulation with *Aspergillus fumigatus* extract for a) 10 or b) 30 min, or c) recombinant *Asp* f1 allergen for 30 min, differentially increased the level of blood basophil surface CD203c expression in patients with cystic fibrosis (CF) with allergic bronchopulmonary aspergillosis (ABPA) or CF with serological ABPA (ABPA-S), as compared to CF with *A. fumigatus* colonisation (AC) or CF, as well as healthy controls (HC).

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TABLE 2 Statistical comparison of basophil CD203c levels upon 10-min *ex vivo* stimulation with *Aspergillus fumigatus* extract in the Stanford and Dublin cohorts

Centre	CF-ABPA *versus* HC	CF-ABPA *versus* CF	CF-ABPA *versus* CF-AC
Stanford	<0.001	<0.001	<0.001
Dublin	<0.001	<0.001	0.042

CF: cystic fibrosis; ABPA: allergic bronchopulmonary aspergillosis; HC: healthy control; AC: *A. fumigatus* colonisation.

ROC data are shown in figure 3 and table 3. At 10 min post-stimulation with *A. fumigatus* extract, the area under the ROC curve for CF-ABPA *versus* CF was 0.98 at visit 1 and 0.87 pooled across all visits (figure 3). Areas under the ROC curve for 10 min post-stimulation with *A. fumigatus* and 30 min post-stimulation with r*Asp* f1 at all four visits (table 1) indicate that both outcomes are useful in diagnosing CF-ABPA. Finally, we also conducted cross-sectional validation in a separate patient set (Dublin cohort). BAT values were significantly higher in CF-ABPA than in CF and CF-AC at 10 min post-stimulation with *A. fumigatus* extract (table 2).

Among conventional measures, total IgE best discriminates CF-ABPA

As expected, *A. fumigatus*-specific IgE antibodies varied significantly between categories. In the CF group, 81.8% of the patients had a level of specific IgE <0.35 kUA·L^{-1} (the clinical detection threshold) compared to 61.5% in the CF-AC group and 10.5% in the CF-ABPA group. Differences between the three groups were statistically significant (p<0.0001), with pairwise comparisons indicating differences in CF *versus* CF-ABPA (p=0.0003) and CF *versus* CF-AC (p=0.0022) (figure 4). One *A. fumigatus*-specific IgE study-negative patient in the CF-ABPA group had previously tested positive at time of ABPA diagnosis.

IgG antibodies to *A. fumigatus* antigens have generally been found to be increased in ABPA and contribute to the conventional serodiagnosis of ABPA in asthma [18]. In our study, specific IgG to *A. fumigatus* did not vary significantly between the CF-ABPA and CF-AC categories (numbers in the CF group were too small to make comparisons), suggesting potentially common IgG responses to frequent respiratory tract colonisation and localised infection with *A. fumigatus* in CF (supplementary figure 1).

In discriminant analysis at baseline, total IgE was found to the best predictor of category, which was expected because, according to current ABPA diagnostic criteria recommendations, we used total IgE level as an important *a priori* category differentiator to classify our CF patients into three groups. In longitudinal analysis, total IgE values maintained a difference between categories but did not differ significantly between visits. At baseline, the CF group had mean±SE total IgE levels of 108.9±82.2 IU·mL^{-1}, compared to 66.6±25.0 IU·mL^{-1} in the CF-AC group and 759.0±126.0 IU·mL^{-1} in the CF-ABPA group (table 1).

Among clinical correlates, S. aureus *infection is negatively associated with ABPA*

The mean age for the three groups was 17.1±2.8 years for CF, 24.8±3.4 years for CF-AC and 25.3±3.7 years for CF-ABPA (table 1); these differences were not statistically significant (p=0.2). At baseline, treatment with oral corticosteroids (n=8) was significantly associated with category (p=0.03), with the highest rates

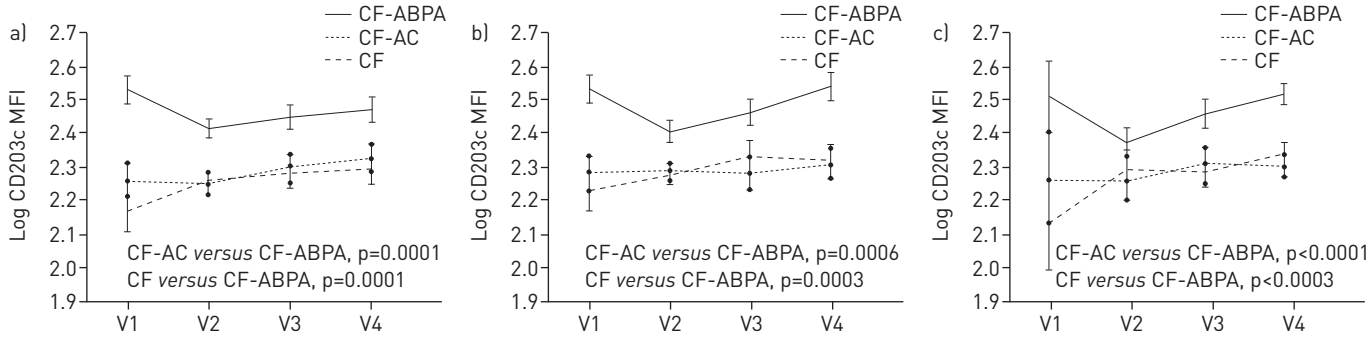

FIGURE 2 Multivariate longitudinal models showing effect of CD203c basophil activation test (BAT) measures. a) *Aspergillus fumigatus* extract stimulation for 10 min, b) *A. fumigatus* extract stimulation for 30 min and c) recombinant *Asp* f1 allergen stimulation for 30 min, by visit and category, controlling for peanut stimulation results at 10, 30 and 30 min, respectively. All BAT measures are log-transformed and shown with error bars (note error bars for cystic fibrosis (CF) with *A. fumigatus* colonisation (AC) are capped with a circle, while the other two category error bars are capped with lines). MFI: mean fluorescence intensity; ABPA: allergic bronchopulmonary aspergillosis; V: visit.

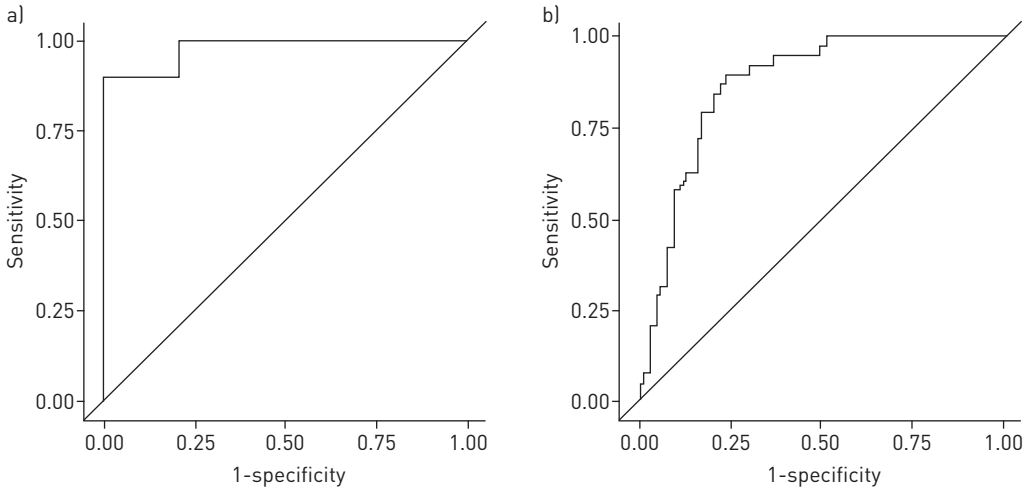

FIGURE 3 a) Receiver operating characteristic (ROC) curve for CD203c following 10 min of stimulation with *Aspergillus fumigatus* extract in cystic fibrosis (CF) with allergic bronchopulmonary aspergillosis (ABPA) *versus* CF category patients. Area under the curve: 0.97. b) Pooled ROC curve for CD203c following 10 min of stimulation with *A. fumigatus* extract at all visits combined, in CF-ABPA *versus* CF category patients. Area under the curve: 0.86.

of use in the CF-ABPA group (35%), compared to none of the CF group and 7.7% of the CF-AC group (table 1). In the multivariate model, treatment with itraconazole (n=11) or oral corticosteroids did not independently affect CD203c BAT outcomes (p=96 and p=022, respectively).

In assessing correlates of bacterial co-infection with *A. fumigatus* status, co-infection with *S. aureus* was significantly associated with category, with lower rates of infection in the CF-ABPA and CF-AC groups compared with the CF group. Evaluated longitudinally, *S. aureus* prevalence over the four visits was significantly higher for CF *versus* CF-AC (p=0.005) and for CF *versus* CF-ABPA (p=0.006) (figure 5). Co-infection with mucoid or nonmucoid *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* were not associated with CD203c BAT outcomes, either at baseline or in longitudinal analysis (data not shown).

Discussion

As basophils are considered an integral feature of allergic responses exhibiting functional aspects of both innate and adaptive immunity, and BATs have been found useful in several other allergic conditions including food, inhalant, drug and stinging insect allergies, we investigated the utility of BAT in CF patients [27]. In a prior cross-sectional single-centre study, we found that basophil CD203c upregulation distinguished CF-ABPA from CF-AC, as determined by respiratory culture (15–60% of CF patients), and from CF patients without either of these two clinical conditions [23]. The current study was conducted to examine the temporal behaviour and variability of basophil responses in these defined CF patient subgroups; to assess what, if any, clinical correlations might exist between basophil activation and other clinical features; and to further examine BAT utility as a novel diagnostic tool for ABPA in a prospective longitudinal manner. Additionally, we examined the validity of the CD203c BAT in a second external cross-sectional cohort, testing the portability of fluorescence-activated cell sorting (FACS)-based BAT and the generalisability of our prior findings. We were also interested in determining whether a standardised recombinant *A. fumigatus* allergen could substitute for crude *A. fumigatus* extracts, which are not

TABLE 3 Receiver operating characteristic (ROC) areas under the curve for blood basophil CD203c values to discriminate categories following 10 min *ex vivo* stimulation with *Aspergillus fumigatus* extract or 30 min *ex vivo* stimulation with recombinant (r)*Asp* f1 allergen

Condition	Outcomes	ROC area under curve			
		Visit 1	Visit 2	Visit 3	Visit 4
CF *versus* CF-ABPA	*A. fumigatus* extract (10 min)	0.98	0.89	0.74	0.76
CF *versus* CF-ABPA	r*Asp* f1 (30 min)	0.82	0.67	0.69	0.82
CF-AC *versus* CF-ABPA	*A. fumigatus* extract (10 min)	0.87	0.96	0.70	0.78
CF-AC *versus* CF-ABPA	r*Asp* f1 (30 min)	0.75	0.83	0.75	0.83

CF: cystic fibrosis; ABPA: allergic bronchopulmonary aspergillosis; AC: *A. fumigatus* colonisation.

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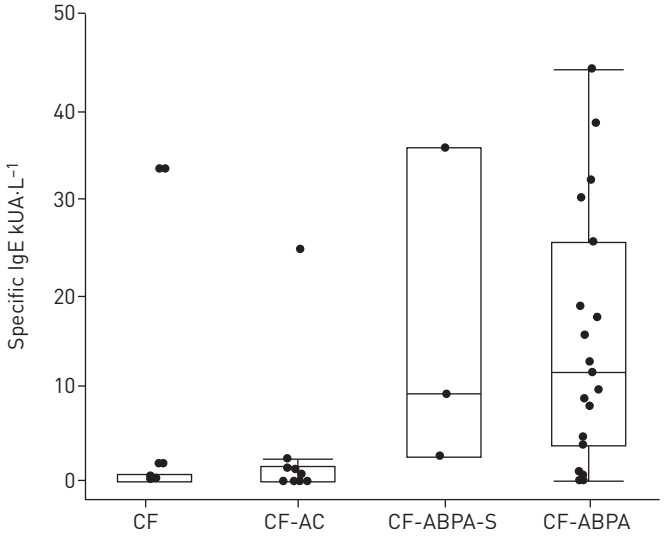

FIGURE 4 Blood IgE antibodies to *Aspergillus fumigatus* at baseline by category. Box plot represents 50% of the data. CF: cystic fibrosis; AC: *A. fumigatus* colonisation; ABPA: allergic bronchopulmonary aspergillosis; S: serological.

standardised and therefore might vary in diagnostic utility. We therefore employed r*Asp* f1, as this 18-kDa molecule, a member of the mitogillin family of cytotoxins, is a major species-specific *A. fumigatus* allergen not expressed by conidia and produced only after germination in the respiratory tract [28–31].

CD203c is an ectonucleotide pyrophosphatase/phosphodiesterase, which is rapidly increased on the surface of the basophils and mast cells upon stimulation with a sensitising allergen [27]. The physiological role of CD203c has been recently described by Tsai *et al.* [32], who have shown that CD203c, rapidly induced by FcεRI cross-linking, negatively regulates chronic allergic inflammation by decreasing ATP concentration and suppressing basophil and mast cell activity. The role of basophils in many clinical conditions including ABPA, however, remains unclear and needs to be explored by further studies. In addition to being a distinguishing feature of ABPA, the association of BAT upregulation of CD203c in combination with specific IgE against *A. fumigatus* may offer a method to further distinguish CF patients sensitised by *A. fumigatus* from those with ABPA [22]. While both *A. fumigatus* sensitisation and ABPA appear to accelerate lung function decline in CF, ABPA is a more serious complication associated with bronchiectasis and other structural lung pathology [2–9, 19, 20].

An area of uncertain clinical importance is the category of patients with ABPA-S, as it is currently unclear whether such patients represent a less aggressive form of ABPA or an earlier subset of patients detected

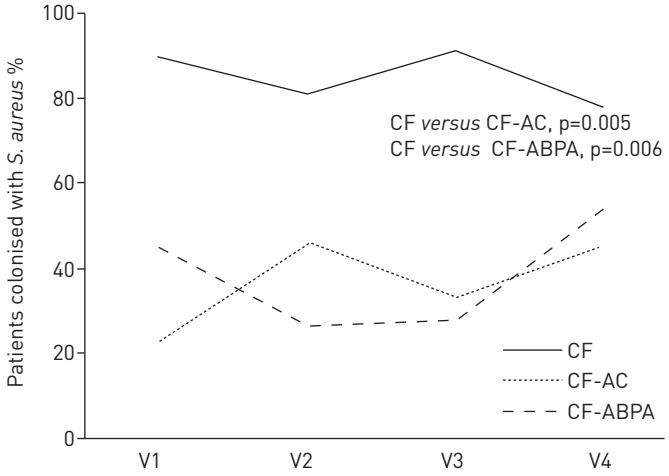

FIGURE 5 Prevalence of co-infection with *Staphylococcus aureus* by visit (V) and category. CF: cystic fibrosis; AC: *Aspergillus fumigatus* colonisation; ABPA: allergic bronchopulmonary aspergillosis.

before progression to overt structural lung damage [24, 33]. No prior longitudinal data on untreated ABPA-S patients has, to our knowledge, been published. BATs were in the CF-ABPA range in three such patients in our study cohort. Importantly, we observed that one of these three CF-ABPA-S patients (supplementary table 1) progressed to ABPA with the onset clinical signs and symptoms, HRCT findings of bronchiectasis and response to conventional ABPA treatment with systemic glucocorticosteroids. Evolution of ABPA-S into ABPA (*i.e.* progression of immunological response to irreversible lung damage) thus seems a potential pathogenetic clinical pathway over time. This supports the notion that ABPA-S represents a high-risk group for progression who should be monitored closely and treated early [33].

In assessing the relationship between our clinical categories (CF, CF-AC, CF-ABPA-S and CF-ABPA) and other features of CF disease, we observed an unanticipated, significant and persistent negative correlation between colonisation with *S. aureus* and ABPA, a previously unreported association that requires further studies. It seems possible that the presence of *S. aureus* as a prominent taxon in the CF lung microbiome, which contains a significant fungal representation [34, 35], could adversely affect the ability of *A. fumigatus* to colonise and/or infect sensitive patients. Although there is increasing investigational focus on potential interactions between another major bacterial CF pathogen, *P. aeruginosa,* and *A. fumigatus*, we did not find a strong persistent positive or negative correlation between *P. aeruginosa* colonisation and *A. fumigatus* colonisation or ABPA in this study. We were also interested in determining whether BAT levels might fluctuate in association with pulmonary exacerbations of CF but found no such association, implying that host responses associated with or responsible for pulmonary exacerbations probably do not include large changes in basophil priming or activation by *A. fumigatus*. As expected, CF-ABPA was associated with oral glucocorticoid use in our cohort.

ABPA in asthma is also likely to be underdiagnosed [15]. A delay in treatment can lead to worsening lung function and bronchiectasis as well as more severe asthma symptoms. Recent studies have tried to improve the diagnosis of ABPA in asthma by defining optimal cut-offs for total and *A. fumigatus*-specific IgE and *A. fumigatus*-specific IgG levels [28, 36], or by refinement of chest HRCT imaging findings, such as the occurrence of high attenuation mucus impaction [16]. Although we were only able to study two non-CF asthma ABPA patients (one at Stanford and one in Dublin), basophil CD203c surface expression was increased in both. This finding clearly needs to be confirmed by further studies in many more patients with asthma both without and with ABPA, but does suggest the possibility that the *A. fumigatus* BAT test may also have utility in asthma similar to what we found in CF for diagnosis.

A limitation of our study is that we used the level of total IgE as an important *a priori* category differentiator (according to current ABPA diagnostic criteria recommendations) to classify our CF patients into three groups. In the future, it may be advantageous to classify CF or asthma patients by CD203c rather than total IgE level and then compare utility to conventional IgE-based classification. Another limitation of the CD203c BAT is that it may be difficult to discriminate patients sensitised to *A. fumigatus* from patients with CF-ABPA, but we believe additional analysis of *A. fumigatus*-specific IgE levels may be able to resolve this issue in most cases [22]. Finally, it has been shown that HRCT can detect pulmonary exacerbations in CF patients [37]. As we did not design our study to compare BAT CD203c levels in patients with CF and ABPA to chest HRCT bronchiectasis scores, future long-term studies will be necessary to assess the potential correlation between the persistence of basophil CD203c activation in patients with CF-ABPA and progression of bronchiectasis on chest HRCT.

In conclusion, basophil surface CD203c expression, particularly after *ex vivo* stimulation with *A. fumigatus* allergen, offers a new FACS-based method for diagnosing ABPA. Similar results at Stanford and Dublin with different commercial *A. fumigatus* extracts indicate they may have similar diagnostic utility, while r*Asp* f1 allergen offers potential universal availability of a standardised allergen for the test. Importantly, *A. fumigatus*-specific basophil responses are sustained over time in CF patients with ABPA, are not affected by occurrence of pulmonary exacerbation or treatment with systemic steroids, and may also distinguish early ABPA (*i.e.* ABPA-S) as a high-risk subpopulation for early intervention.

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