The basophil surface marker CD203c identifies Aspergillus species sensitization in patients with cystic fibrosis.

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Citation
The Basophil Surface Marker CD203c Identifies *Aspergillus* Sensitization in Cystic Fibrosis

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Abstract

Background: Colonization by Aspergillus fumigatus (Af) in cystic fibrosis (CF) may cause Af sensitization and/or allergic bronchopulmonary aspergillosis (ABPA) which affects pulmonary function and clinical outcomes. Recent studies show that specific allergens upregulate the surface-expressed basophil marker CD203c in sensitized individuals, a response that can be readily measured by flow cytometry. 

Objective: To identify Af-sensitization in CF using the basophil activation test (BAT).

Methods: Patients with CF attending Beaumont Hospital were screened for study inclusion. BAT was used to identify Af sensitization. Serologic (total IgE and Af-specific IgE), pulmonary function and body mass index measurements were performed.

Results: The BAT discriminates Af-sensitized from non-sensitized patients with CF. Persistent isolation of Af in sputum is a significant risk factor for Af sensitization. Levels of the Af-stimulated basophil activation marker, CD203c, inversely correlated with pulmonary function and body mass index in Af-sensitized but not non-sensitized patients with CF. Total andAf-specific IgE, but not IgG, are elevated in Af-sensitized CF patients with ABPA when compared to Af-sensitized and non-sensitized CF patients without ABPA. Itraconazole treatment did not affect Af sensitization.

Conclusion: Combining the BAT with routine serological testing allows classification of patients with CF into three groups: non-sensitized, Af-sensitized and ABPA. Accurate and prompt identification of Af-associated clinical status may allow early and targeted therapeutic intervention potentially improving clinical outcomes.

Key Messages

- The basophil activation test can identify Aspergillus fumigatus-sensitized individuals in the cystic fibrosis population.
- CD203c levels inversely correlate with pulmonary function and body mass index in A. fumigatus-sensitized people with cystic fibrosis.
- Prompt identification of A. fumigatus sensitization may improve the management of A. fumigatus-associated disease in cystic fibrosis.
Capsule Summary
This study demonstrates a novel means of identifying *A. fumigatus* sensitization in cystic fibrosis using the basophil activation test. Simpler classification of *Aspergillus*–associated disease will likely improve both clinical management and outcomes.

**Keywords:** allergic bronchopulmonary aspergillosis; *Aspergillus fumigatus*; basophil activation test; body mass index; CD203c; cystic fibrosis; flow cytometry; forced expiratory volume in the first second; itraconazole; sensitization.

**Abbreviations:**
- ABPA: allergic bronchopulmonary aspergillosis
- *Af*: *Aspergillus fumigatus*
- BAT: basophil activation test
- BMI: body mass index
- CF: cystic fibrosis
- EDTA: ethylenediaminetetraacetic acid
- FEV<sub>1</sub>: forced expiratory volume in the first second
- GM: galactomannan
- MFI: mean fluorescence intensity
- PWCF: people with CF
- ROC: receiver-operating characteristic
- sIgE: specific IgE
- sIgG: specific IgG
INTRODUCTION

Cystic fibrosis (CF) is an inherited disorder characterized by recurrent polymicrobial pulmonary exacerbations and chronic neutrophil-dominated inflammation. Dysfunctional CFTR protein leads to impaired mucociliary clearance and colonization of the CF lung by bacteria and fungi. Much focus has been given to the role of bacteria in the CF airway; however, an increasing recognition of fungi has emerged.\textsuperscript{1–3} Aspergillus fumigatus (Af) is the most commonly isolated fungus in CF with a prevalence of up to 60%.\textsuperscript{4} The fungus is associated with a range of manifestations in CF, most commonly allergic bronchopulmonary aspergillosis (ABPA) and less commonly, aspergillomas and invasive pulmonary aspergillosis.\textsuperscript{5–7}

ABPA affects between 2%-15% of patients with CF (PWCF) and recurrent episodes impact pulmonary function.\textsuperscript{1,8–11} It manifests as an allergic, hypersensitive, Th2 CD4\textsuperscript{+} cell-driven response to Af and the diagnosis of ABPA in CF is particularly challenging due to overlapping clinical, immunological and radiological features that are similar to those of a pulmonary exacerbation. To address this, consensus conference criteria were published to aid clinicians in diagnosing CF-ABPA.\textsuperscript{12} Despite this, inherent weaknesses in these criteria exist. They can be difficult to employ in the CF setting and many CF patients with ABPA do not fulfill these criteria despite a good response to treatment. The criteria have not been updated for 12 years despite advances in both the understanding and the treatment of CF-ABPA. Hence, there is a growing need for a simplified, updated classification to robustly diagnose ABPA, facilitating earlier intervention to improve clinical outcome.

Sensitization is an immunological phenomenon, defined by the production of specific IgE (sIgE). It can arise from a combination of genetic predisposition and allergen exposure.\textsuperscript{13,14} Recent reports show that Af sensitization is associated with a greater decline in lung function and an increased rate of pulmonary exacerbations in CF.\textsuperscript{15–17} The basophil activation test (BAT) is a novel technique that measures upregulation of CD203c upon stimulation with the specific allergen to which an individual is sensitized.\textsuperscript{18} CD203c is an ectonucleotide pyrophosphatase/phosphodiesterase expressed on the surface of basophils, which are important effector cells in type II immune responses.\textsuperscript{18,19} CD203c can be rapidly measured by flow cytometry and has been proposed as a diagnostic tool in atopic disease, including peanut, drug and wasp venom allergy.\textsuperscript{20–22}
It has been shown that basophils are primed and hyper-responsive to Af allergen stimulation in CF-ABPA.\textsuperscript{18} In this current study, BAT to Af was employed to identify Af sensitization in a CF cohort and it was correlated with key CF clinical measurements. Furthermore, Af-stimulated CD203c, in conjunction with commonly available immunological parameters, was examined for use in the classification of patients with CF into non-sensitized, sensitized or ABPA groups.
METHODS

Patient recruitment
We prospectively recruited 48 PWCF to the study between October 2012 and October 2014. As controls, 11 healthy non-CF volunteers were also recruited. Ethical approval was obtained from our Institutional Review Board. CF was confirmed by sweat chloride levels (>60 mmol/L) and genotyping. Pulmonary function testing (Online Supplement) and serum sampling was performed on the day of the BAT. Total circulating IgE, sIgE and specific IgG (sIgG) levels to Af were determined using the ImmunoCap assay (Phadia, Uppsala, Sweden). Patient demographics are outlined in Supporting Table EI. Quarterly sputum samples were routinely collected for standard microbiological evaluation, including fungi. Exclusion criteria were pregnancy, lung transplantation, peanut allergy and those under 16 years of age.

Cohort characterization
The CF-ABPA cohort was diagnosed as per previously published consensus criteria. Specific IgE (sIgE) levels to Aspergillus were used as an alternative to skin prick testing that are interchangeable in consensus criteria. To differentiate between Af-sensitized and non-sensitized CF individuals, an arbitrary cut-off (1.36) was set at three standard deviations above the mean value of the stimulation index of the healthy control population, with the stimulation index defined as the ratio between Af- and PBS-stimulated CD203c values.

Basophil activation test
Samples were processed as previously described. Briefly, venous blood was collected in S-Monovette® ethylenediaminetetraacetic acid (EDTA) blood tubes (Sarstedt, Germany) and centrifuged at 400g for 10 min at 4 °C. The supernatant was further centrifuged at 3000g for 10 min at 4 °C and 97 µl of platelet-free plasma was added to 100 µl of erythrocyte/leukocyte pellet and incubated for 30 s at 37 °C. Samples were incubated with 3 µl Af extract (Mediwiss Analytic GmbH, Moers, Germany) for 10 min at 37 °C. PBS and peanut extract (Mediwiss) were used as controls. Untreated basophils were also evaluated. After washing, cells were stained with FITC mouse anti-human CD3, HLA-DR, CD41a, and CD66b, PerCP-Cy5.5 mouse anti-human CD123 (BD Biosciences, San Jose, CA) and PE mouse anti-
human CD203c (Biolegend, San Diego, CA) at saturating concentrations. LIVE/DEAD® FixableNear-IR Dead Cell stain kit (Invitrogen, Carlsbad, CA) was used to distinguish between live and dead cells. After washing, erythrocytes were lysed and leukocytes were fixed with Lyse/Fix buffer (BD Biosciences) for 30 min on ice. After centrifugation at 490g for 5 min, cells were resuspended in 2.5 mM EDTA containing 5 % FCS and analyzed by flow cytometry on a BD FACSCalibur (BD Biosciences) equipped with BD CellQuest Pro Software. Compensation was performed using CaliBRITE Beads (BD Biosciences) and at least 200 basophils per sample were analyzed. Data were analyzed using FlowJo software (Ashland, OR).

**Statistical Analysis**

Statistical analysis was performed using GraphPad PRISM 4.0 (San Diego, CA). Data were tested for normality using the Kolmogorov-Smirnoff test. Normal data were compared using a two-tailed independent Student’s t-test and for non-normal data, the Mann-Whitney U test was performed. Differences were considered significant at \( P < 0.05 \).
RESULTS

*Basophil activation test discriminates between non-sensitized and* Af-sensitized *patients with CF*

Flow cytometry was used to measure basophil activation in response to *Af*. Basophils were gated as the Live/Dead−/CD3−/HLA-DR−/CD41a−/CD66b−/CD123+ population and evaluated for CD203c expression following *Af* extract stimulation (Supporting Figure E1). PBS and peanut extract were used as non-offending and immunogenic controls, respectively. To differentiate between *Af*-sensitized and non-sensitized PWCF, an arbitrary cut-off of 1.36 was determined using the stimulation index of the non-CF controls (mean ± SD = 1.018 ± 0.114; n = 11). A positivity threshold of three standard deviations above the healthy mean of the stimulation index was determined as before.24 Using this cut-off, 23 (47.9%) of the 48 recruited PWCF were *Af*-sensitized. The ability of sIgE to distinguish between sensitized and non-sensitized individuals has been previously proposed using a cut-off of 0.35 kU/L.16 The diagnostic performance of the BAT was examined against sensitization status determined with sIgE (*Af* sensitization cut-off 0.35 kU/L; ImmunoCap assay)16 using receiver-operating characteristic (ROC)-curve analyses. The area under the ROC curve was 0.9134 (P < 0.0001; Supporting Figure E2) indicating the excellent discriminating ability of the BAT between non-sensitized and *Af*-sensitized PWCF and corroborating the strength of *Af*-stimulated CD203c levels as an indicator of *Af* sensitization in CF.

*Af*-sensitized PWCF have higher frequency of *Af*-positive sputum cultures compared to non-sensitized PWCF

Patients with CF were screened for the presence of *Aspergillus* spp. in their quarterly sputum samples as part of routine care, amounting to eight sputum samples from each patient in the two year period preceding the BAT. Fourteen out of 23 (60.9%) *Af*-sensitized patients, but only 6 out of 25 (24.0 %) non-sensitized PWCF, grew *Af* on at least one occasion in the 2-year period preceding the BAT. *Af*-sensitized PWCF displayed higher frequency of *Af* sputum isolation compared to non-sensitized PWCF (P = 0.0038; Figure 1). To verify the specificity of the BAT to *Af*, *Candida albicans* colonization was also assessed. In contrast to *Af*, 19 out of 23 (82.6%) *Af*-sensitized and 17 out of 25 (68.0 %) non-sensitized PWCF grew *C. albicans* at least once in the
2 years preceding the BAT. There was no significant difference in the frequency of *C. albicans* isolation between groups (Supporting Figure E3), illustrating the specificity of the BAT to *Af*. Similar results were observed when colonization with *Pseudomonas aeruginosa* was investigated (Supporting Figure E4). No significant difference in *Af*-stimulated CD203c levels between patients with non-mucoid (*P* = 0.3263) or mucoid *P. aeruginosa* (*P* = 0.2351) compared with their non-colonized counterparts was noted. In summary, BAT to *Af* is specific and PWCF with increased frequency of *Af* in sputum are more likely to develop sensitization to *Af*.

**Af-stimulated CD203c levels are increased in blood basophils from *Af*-sensitized compared to non-sensitized PWCF**

*Af*-sensitized PWCF had significantly higher *Af*-stimulated CD203c values compared to healthy controls (*P < 0.0001*) and non-sensitized PWCF (*P < 0.0001*; Figure 2A). Interestingly, *Af*-stimulated CD203c values were significantly higher in *Af*-sensitized PWCF with ABPA than *Af*-sensitized CF individuals without ABPA (*P = 0.0408*; Figure 2B). This demonstrates that the BAT to *Af* distinguishes *Af*-sensitization from non-sensitization along a quantitative spectrum and could also be potentially used in the diagnosis of individuals with ABPA within the *Af*-sensitized cohort.

**CD203c expression levels inversely correlate with FEV₁ and BMI of *Af*-sensitized PWCF**

*Af* sensitization has previously been shown to be associated with worse lung function in a variety of pulmonary diseases, including asthma, chronic obstructive pulmonary disease and CF.¹⁶,¹⁷,²⁷,²⁸ We therefore explored the relationship of *Af*-stimulated CD203c levels with lung function (forced expiratory volume in the first second, FEV₁) and body mass index (BMI), both established determinants of mortality in CF.²⁹ We observed a significant correlation of *Af*-stimulated CD203c levels with declining FEV₁ in *Af*-sensitized patients (*n = 16, r² = 0.6409, *P* = 0.0002; Figure 3A). Interestingly, no correlation between *Af*-stimulated CD203c values and FEV₁ in non-sensitized PWCF was detected (*n = 25, r² = 0.0198, *P* = 0.5027; Figure 3A). CF-ABPA patients receiving corticosteroid treatment were excluded from this analysis due to the potential interference of corticosteroid therapy with CD203c levels (see Supporting Figure E5 and E6). BMI was also shown to inversely correlate with *Af*-stimulated CD203c levels in *Af*-sensitized (*n = 16, r² = 0.3009, *P* = 0.0278) but not non-
sensitized PWCF (n = 25, \( r^2 = 0.0905, P = 0.1439 \); Figure 3B). Together, these data illustrate that \( Af \)-stimulated CD203c levels correlate with key clinical parameters in CF, including FEV\(_1\) and BMI, confirming the clinical impact of the \( Af \)-sensitized state.

**Total IgE and \( Af \)-specific IgE, but not \( Af \)-specific IgG levels, are elevated in serum of \( Af \)-sensitized patients with ABPA**

Total IgE, \( Af \) sIgE and sIgG levels were evaluated in serum samples from non-sensitized, \( Af \)-sensitized and CF patients with ABPA. As described above, the stimulation index threshold was used to distinguish sensitized (> 1.36) from non-sensitized (< 1.36) individuals. The CF-ABPA individuals were defined as those meeting classical consensus criteria for ABPA.\(^{12}\) Total IgE levels were significantly higher in CF-ABPA (n = 7) when compared to non-sensitized and \( Af \)-sensitized PWCF (n = 25, \( P < 0.0001 \) and n = 16, \( P < 0.0001 \), respectively; Figure 4A). Similarly, when sIgE was assessed, significantly higher levels were observed in CF-ABPA than in non-sensitized and \( Af \)-sensitized patients with CF (\( P = 0.0004 \) and \( P < 0.0001 \), respectively; Figure 4B). \( Af \)-sensitized PWCF without ABPA had higher sIgE levels than non-sensitized PWCF (\( P <0.0001 \)). No increase in sIgG levels were observed in the CF-ABPA group when compared to \( Af \)-sensitized and non-sensitized patients with CF; however, \( Af \)-sensitized PWCF without ABPA had higher sIgG levels than non-sensitized PWCF (\( P = 0.0302 \); Figure 4C). Therefore, total serum IgE and sIgE, but not sIgG, are useful in aiding the detection of CF-ABPA in CF and in differentiating it from \( Af \)-sensitization.

**Antifungal therapy does not alter \( Af \)-stimulated CD203c expression levels on basophils**

Recent work by our group has shown that elimination of \( Af \) bioburden with itraconazole (400 mg daily for 6 weeks) improves clinical outcome.\(^{23}\) To investigate the effect of fungal eradication on \( Af \) sensitization, BAT to \( Af \) was performed. Although we have previously shown that itraconazole reduces the fungal load in sputum,\(^{23}\) no change to CD203c levels pre- and post itraconazole treatment was observed (n = 8, \( P = 0.4028 \); Figure 5). Additionally, no difference in baseline CD203c expression was observed (n = 8, \( P = 0.3829 \); Supporting Figure E5A). This shows that decreasing the fungal burden in patients with CF undergoing anti-fungal therapy with itraconazole does not affect \( Af \) sensitization, once it is established.
Additionally, in a small number of patients (n = 4) receiving monthly pulses of high dose intravenous methylprednisolone for ABPA (10-15 mg/kg for three days), a trend towards efficacy of treatment was observed with \( Af \)-stimulated CD203c values halving (57.6%) one week after treatment initiation \( (P = 0.1604, \text{Supporting Figure E6}) \). This trend however decreased to 30% after one month, and prior to the next infusion of corticosteroids \( (n = 4, P = 0.2672; \text{Supporting Figure E6}) \), suggestive of the transient effect of corticosteroid therapy with time.
DISCUSSION

The role of bacteria in the CF lung has been extensively studied and potential roles for fungi are beginning to emerge. Recently, much focus has been given to the wide spectrum of Af-associated morbidities in CF, including Af sensitization and ABPA. Both of these clinical states are associated with poorer clinical outcomes.\textsuperscript{16,31} For this reason, identifying individuals with Af sensitization with or without ABPA is important to ensure appropriate and timely therapeutic intervention to minimize pulmonary impact.\textsuperscript{30} In this study we focused on the basophil activation marker CD203c as an indicator of Af sensitization and assessed its effect on important clinical measures of CF disease.

Sensitization to Af has previously been associated with poorer lung function,\textsuperscript{16,17} a finding further confirmed by this study. Gernez et al.\textsuperscript{18} recently reported that basophils from CF-ABPA patients are primed and hyper-responsive to stimulation with Af extract. In the current study, we employed the BAT to identify the Af sensitization status of our CF cohort. From a logistical viewpoint, the BAT to Af can be performed in under 4 hours using a maximum of 2 ml of whole blood. This can be taken in the same blood draw as the routinely measured immunological investigations minimizing discomfort for the patient. A flow cytometer and appropriately trained staff are required to perform the BAT, which would represent the only limitations to its applicability in routine CF centre practice.

Almost half of the patients studied were Af-sensitized, which is in accordance with previous studies (31-61\%) that employed serology and skin prick testing (SPT) alone.\textsuperscript{15–17} Elevated serum IgE to Af and SPTs are considered the gold standards for routine laboratory investigations and are part of the established consensus criteria for the diagnosis of ABPA in CF.\textsuperscript{12} Nevertheless, both of these tests harbour disadvantages. Specific IgE to Af is an easily accessible test for the clinician and is convenient for the patient. It has been shown however, that specific IgE levels tend to change over time and have to be interpreted along with the constellation of other clinical and laboratory variables and patient history to reach a diagnosis.\textsuperscript{32,33} SPTs are often laborious and time consuming as the full investigation may take up to 72 h to complete. Furthermore, subcutaneous injection of the antigen will induce swelling, itching and reddening at the site of injection in an allergic person and is as such a source of discomfort to the patient. The SPTs can be subjective for the attending clinician and open to interpretation as some suggest a wheal diameter of > 3 mm.
while others recommend a diameter of > 4 mm for a positive result.\textsuperscript{32} Accumulating reports have shown striking differences in individual patients between SPT and serology test results suggesting an important role for the effector cells of immediate hypersensitivity (mast cells and basophils) and their activation during an allergic response.\textsuperscript{32,34–37} Therefore, there is a great need for a reliable \textit{in vitro} method to complement the routine laboratory tests for sensitization/allergy when the latter show discrepancies or are not feasible.\textsuperscript{33} Of note, \textit{Af}-specific IgE levels correlated significantly with CD203c levels (Supporting Figure E7). The BAT is a specific and reliable measure of IgE-dependent response in sensitized individuals because (i) the mast cells are tissue-bound, (ii) the ease of basophil accessibility and (iii) the fact that the upregulation of basophil surface markers can be conveniently measured by flow cytometry. By comparing CD203c levels to the already established cut-off sIgE value of 0.35 kUa/L\textsuperscript{16} to distinguish between sensitized and non-sensitized individuals, ROC curve analysis further confirmed the usefulness of the BAT as an indicator of \textit{Af} sensitization in CF. Persistent and prolonged exposure to the fungus is a significant risk factor for \textit{Af}-sensitization as sensitized patients display a higher frequency of \textit{Af} isolation from sputum culture. Although a study by Baxter \textit{et al.}\textsuperscript{15} showed no correlation between \textit{Af} colonization and sensitization, it is conceivable that these different outcomes may be related to methodological differences in defining \textit{Af} sensitization, colonization and the microbiological techniques employed. Interestingly, the \textit{Af}-sensitized ABPA cohort had higher \textit{Af}-stimulated CD203c values than \textit{Af}-sensitized patients without ABPA illustrating that levels of measured CD203c may allow differentiation between \textit{Af}-sensitization and CF-ABPA. We and others have previously shown that persistent carriage of \textit{Af} in the CF airway is associated with radiological abnormalities, including more severe bronchiectasis and mosaic pattern perfusion and has an impact on pulmonary function.\textsuperscript{16,38} \textit{Af} sensitization is also associated with lung function decline and increased duration of intravenous antibiotic treatments.\textsuperscript{15,17} In line with this, our data illustrate a significant inverse correlation between \textit{Af}-stimulated CD203c values with FEV\textsubscript{1} and BMI. These findings are in concordance with previously published studies suggesting a negative impact of \textit{Af} sensitization in CF.\textsuperscript{15–17} \textit{Af} sensitization likely facilitates CF-ABPA and multiple factors, including degree of fungal exposure, mucus viscosity, immune status, atopy and age may also have interdependent roles.\textsuperscript{12,32,39} Other factors, such as HLA-DR/HLA-DQ subtypes and the presence of single nucleotide polymorphisms
in the IL-4 binding site of IL-4 receptor alpha have also been described to associate with development of ABPA.\textsuperscript{40–42} Longitudinal studies are needed to confirm that \textit{Af} sensitization routinely precedes ABPA. Baxter \textit{et al.}\textsuperscript{11} have recently proposed a novel classification of aspergillosis in adult CF. Using a combination of serologic (total IgE, \textit{Af} slgE and slgG), RT-PCR, and galactomannan (GM) data, they distinguish between non-diseased, CF-ABPA, \textit{Af} sensitization and \textit{Af} bronchitis. In accordance with their findings, we found that CF patients with ABPA had higher total IgE and \textit{Af} slgE levels compared to non-sensitized and \textit{Af}-sensitized patients without ABPA. Our study showed no difference in slgG levels between PWCF with or without ABPA. Additionally, when applied to our CF population, both the GM assay and RT-PCR for \textit{Af} detected a high number of \textit{Af}-positive sputum samples (data not shown), preventing adequate differentiation between the cohorts according to the aforementioned classification. This high positivity may be due to the fact that RT-PCR identifies both live and dead organisms,\textsuperscript{15} and that GM assays can yield false-positive results in the presence of penicillin-derived antibiotics, to which many of our patients with CF are exposed.\textsuperscript{43,44} This limits its utility in the current study.

For the purposes of the current study, we adopted a novel, simplified classification that allows differentiation between non-sensitized, \textit{Af}-sensitized and CF-ABPA. We employed a combination of \textit{Af}-stimulated CD203c, total IgE and \textit{Af} slgE levels (Table I) to identify ABPA, avoiding the need for RT-PCR, GM or \textit{Af} slgG testing that is not always accessible in CF centers. Elevated \textit{Af}-stimulated CD203c values (> 1.36) combined with elevated serum slgE (> 1.45 kUa/L) and total IgE (> 185 kU/L) correctly identified all cases of CF-ABPA by the consensus conference criteria. Of note, the slgE cut-off (1.45 kUa/L) used in the proposed classification system for identification of CF-ABPA is higher than the slgE cut-off (0.35 kUa/L) routinely used to identify sensitization alone. Furthermore, the total IgE cut-off herein has been considerably reduced compared to consensus values used (minimum 500 kU/L) in line with previously published ROC-curve analysis demonstrating that the optimum level for ABPA diagnosis is >185 kU/L, giving 91% sensitivity and 90% specificity.\textsuperscript{11} The inclusion of the reduced level in our proposed classification system was validated when one patient meeting our classification criteria for CF-ABPA, \textit{i.e.} elevated CD203c, total IgE and \textit{Af} slgE, but not consensus conference criteria (total IgE > 500 kU/L), developed ABPA as defined by consensus conference criteria.
shortly after the study conclusion. A graphical representation of our classification is depicted in Figure 6.

Our previous work has shown that itraconazole alleviates fungal burden in PWCF colonized with *Af* 

In this study, itraconazole administration did not influence *Af*-stimulated CD203c levels, suggesting that fungal eradication from the lung does not impact *Af* sensitization once established. Taking into consideration that persistent *Af* colonization is a significant risk factor for sensitization, early itraconazole intervention may be warranted to clear colonization, reduce exposure and thereby minimize the risk of sensitization to *Af*. Acute ABPA flares can be treated with intravenously administered pulsed methylprednisolone at 10-15 mg/kg/day for a three day period every month. 

Methylprednisolone was administered to four CF-ABPA patients in this study and a trend towards reduction in *Af*-stimulated CD203c values was observed after treatment. However, this trend was not significant (*P* = 0.1604) warranting future longitudinal study with increased numbers to examine the effect of corticosteroid treatment on *Af* sensitization and potentially the use of the BAT to monitor treatment responses.

In conclusion, we propose a novel and simplified means of identifying sensitization to *Af* using *Af*-stimulated CD203c values. Using the BAT we show that increased incidence of *Af* colonization is associated with *Af* sensitization and the latter state impacts lung function. The practical applicability of the BAT to *Af* in the clinical setting includes an evaluation of *Af*-stimulated CD203c values for patients positive for *Af* on at least two occasions within the preceding two years (Figure 7). If the BAT is negative, itraconazole treatment may be offered for fungal eradication however, a positive result indicates sensitization and a potential increased future risk of ABPA. Consequently, sensitized individuals should have their serology results closely monitored for increases in total IgE or *Af*-sIgE that may indicate ABPA. Despite the lack of effect of azole treatment on the sensitization state eradication therapy to reduce fungal bioburden may be recommended. If sensitization is a prerequisite for ABPA, corticosteroids may be considered at the early sensitization stage to reduce the likelihood of developing ABPA. A longitudinal study with adequate numbers should be performed to assess the benefits of systemic corticosteroid administration on clinical outcomes in *Aspergillus*-sensitized individuals without ABPA. 

Timely detection of *Af* sensitization and CF-ABPA equips clinicians to deliver a targeted approach to CF-*Af* therapy to improve clinical outcomes.
Acknowledgments
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TABLE I. Simplified classification of *Aspergillus*-associated disease in CF.

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<tr>
<th>Cut-off</th>
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<th>sIgE</th>
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<sup>a</sup>Determined as described in the methods section. <sup>b</sup>Local cut-off value. <sup>c</sup>As proposed by Baxter *et al.*<sup>11</sup>
Figure legends

**Figure 1.** Frequency of Af-positive sputum cultures in non-sensitized PWCF (CF Af-sensitized-; n = 25) and Af-sensitized (CF Af-sensitized+; n = 23) in the two year period preceding the BAT. PWCF were screened for Af in sputum as a part of routine care. Data are presented as means ± standard error of mean. **p<0.01.

**Figure 2.** Af-stimulated CD203c levels in non-sensitized, Af-sensitized and ABPA PWCF. (A) PWCF were classified as non-sensitized (n = 25) or Af-sensitized (n = 23). (B) The Af-sensitized cohort was further divided into those with (Af-sensitized+ ABPA+; n=7) or without ABPA (Af-sensitized+ ABPA-; n=16). Data are presented as Tukey box plots and the median is represented by the middle line. *p<0.05, ***p<0.001.

**Figure 3.** Correlation of Af-stimulated CD203c levels with FEV₁ and BMI. Af-stimulated CD203c levels correlated with (A) FEV₁ (r² = 0.6409, P = 0.0002) and (B) BMI (r² = 0.3009, P = 0.0278) in Af-sensitized (n = 16) but not in non-sensitized PWCF (FEV₁; n = 25, r²= 0.0198, P = 0.5027 and BMI; r² = 0.0905, P = 0.1439.).

**Figure 4.** Serologic immune parameters in PWCF according to Af sensitization status. (A) Total IgE, (B) sIgE and (C) sIgG serum levels were measured in non-sensitized (n = 25), Af-sensitized (n = 16) and CF ABPA patients (n = 7). Data are presented as Tukey box plots and the median is represented by the middle line. *p<0.05, ***p<0.001.

**Figure 5.** Effect of itraconazole treatment on Af-stimulated CD203c levels in PWCF. Af-stimulated CD203c levels in blood basophils from PWCF before and after 6 weeks of treatment with oral itraconazole (n = 8, P = 0.4028) are presented as Tukey box plots and the line in the middle of the box represents the median.
Figure 6. Graphical representation of a simplified classification of *Aspergillus*-associated disease using a combination of *Af*-stimulated CD203c, total IgE and sIgE levels. The blue, red and green dots denote non-sensitized (n=25), *Af*-sensitized (n=16) and PWCF with ABPA (n=7), respectively as depicted with 3D scatter plot.

Figure 7. A schematic diagram illustrating the potential stages of *Aspergillus*-associated CF disease with detection methods and possible treatment interventions at each stage. Solid and hashed lines represent established and suggested treatment regimens, respectively.
Figures

Figure 1.

** Frequency of CF+ cultures in 2yrs preceding BAT

CF A sensitized

CF A sensitized+
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure 7.