Background: Colonization by *Aspergillus fumigatus* in patients with cystic fibrosis (CF) can cause *A fumigatus* 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 subjects, a response that can be readily measured by using flow cytometry. Objective: We sought to identify *A fumigatus* sensitization in patients with CF by using the basophil activation test (BAT). Methods: Patients with CF attending Beaumont Hospital were screened for study inclusion. BAT was used to identify *A fumigatus* sensitization. Serologic (total and *A fumigatus*-specific IgE), pulmonary function, and body mass index measurements were performed. Results: The BAT discriminates *A fumigatus*-sensitized from nonsensitized patients with CF. Persistent isolation of *A fumigatus* in sputum is a significant risk factor for *A fumigatus* sensitization. Levels of the *A fumigatus*-stimulated basophil activation marker CD203c inversely correlated with pulmonary function and body mass index in *A fumigatus*-sensitized but not nonsensitized patients with CF. Total and *A fumigatus*-specific IgE, but not IgG, levels are increased in *A fumigatus*-sensitized patients with CF and ABPA when compared with those in *A fumigatus*-sensitized and nonsensitized patients with CF without ABPA. Itraconazole treatment did not affect *A fumigatus* sensitization.

Conclusion: Combining the BAT with routine serologic testing allows classification of patients with CF into 3 groups: nonsensitized, *A fumigatus*-sensitized, and ABPA. Accurate and prompt identification of *A fumigatus*-associated clinical status might allow early and targeted therapeutic intervention, potentially improving clinical outcomes.

Key words: Allergic bronchopulmonary aspergillosis, *Aspergillus fumigatus*, basophil activation test, body mass index, CD203c, cystic fibrosis, flow cytometry, FEV1, itraconazole, sensitization

Cystic fibrosis (CF) is an inherited disorder characterized by recurrent polymicrobial pulmonary exacerbations and chronic neutrophil-dominated inflammation. Dysfunctional CF transmembrane conductance regulator protein leads to impaired mucociliary clearance and colonization of the lungs of patients with CF by bacteria and fungi. Much focus has been given to the role of bacteria in the airways of patients with CF; however, an increasing recognition of fungi has emerged. *Aspergillus fumigatus* is the most commonly isolated fungus in patients with CF, with a prevalence of up to 60%. The fungus is associated with a range of manifestations in patients with CF, most commonly allergic bronchopulmonary aspergillosis (ABPA) and, less commonly, aspergillomas and invasive pulmonary aspergillosis.

ABPA affects between 2% and 15% of patients with CF, and recurrent episodes affect pulmonary function. It manifests as an allergic, hypersensitive, Th2 CD4+ cell–driven response to *A fumigatus*, and the diagnosis of ABPA in patients with CF is particularly challenging because of overlapping clinical, immunologic, and radiologic features that are similar to those of a pulmonary exacerbation. To address this, consensus conference criteria were published to aid clinicians in diagnosing CF with ABPA. Despite this, inherent weaknesses in these criteria exist. They can be difficult to use in the CF setting, and many patients with CF-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 treatment of CF with ABPA. Hence there is a growing need for a simplified updated classification to robustly diagnose ABPA, facilitating earlier intervention to improve clinical outcomes.

Sensitization is an immunologic phenomenon defined by the production of specific IgE (sIgE). It can arise from a combination of genetic predisposition and allergen exposure. Recent reports show that *A fumigatus* sensitization is associated with a greater decrease in lung function and an increased rate of...
Abbreviations used
ABPA: Allergic bronchopulmonary aspergillosis
BAT: Basophil activation test
BMI: Body mass index
CF: Cystic fibrosis
GM: Galactomannan
ROC: Receiver operating characteristic
sIgE: Specific IgE
sIgG: Specific IgG
SPT: Skin prick test

pulmonary exacerbations in patients with CF. The basophil activation test (BAT) is a novel technique that measures upregulation of CD203c on stimulation with the specific allergen to which a patient is sensitized. CD203c is an ectonucleotide pyrophosphatase/phosphodiesterase expressed on basophil surfaces, which are important effector cells in type II immune responses. CD203c can be rapidly measured by means of flow cytometry and has been proposed as a diagnostic tool in patients with atopic disease, including peanut, drug, and wasp venom allergy.

It has been shown that basophils are primed and hyperresponsive to A fumigatus allergen stimulation in patients with CF and ABPA. In the current study the BAT to A fumigatus was used to identify A fumigatus sensitization in a CF cohort, and results were correlated with key CF-clinical measurements. Furthermore, A fumigatus–stimulated CD203c, in conjunction with commonly available immunologic parameters, was examined for use in the classification of patients with CF into nonsensitized, sensitized, and ABPA groups.

METHODS
Patient recruitment
We prospectively recruited 48 patients with CF to the study between October 2012 and 2014. As control subjects, 11 healthy volunteers without CF 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 (see the Methods section in this article’s Online Repository at www.jacionline.org) and serum sampling were performed on the day of the BAT. Total circulating IgE, sIgE, and specific IgG (sIgG) levels to A fumigatus were determined by using the ImmunoCAP assay (Phadia, Uppsala, Sweden). Patients’ demographics are outlined in Table E1 in this article’s Online Repository at www.jacionline.org. Quarterly sputum samples were routinely collected for standard microbiological evaluation, including fungi. Exclusion criteria were pregnancy, lung transplantation, peanut allergy, and age less than 16 years.

Cohort characterization
The CF with ABPA cohort were given diagnoses per previously published consensus criteria. sIgE levels to Aspergillus species were used as an alternative to skin prick tests (SPTs), which are interchangeable in consensus criteria. To differentiate between A fumigatus–sensitized and nonsensitized patients with CF, an arbitrary cutoff (1.36) was set at 3 SDs greater than the mean value of the stimulation index of the healthy control population, with the stimulation index defined as the ratio between A fumigatus– and PBS-stimulated CD203c values.

BAT
Samples were processed, as previously described. Briefly, venous blood was collected in S-Monovette EDTA blood tubes (Sarstedt, Numbrecht, Germany) and centrifuged at 400g for 10 minutes at 4°C. The supernatant was further centrifuged at 3000g for 10 minutes at 4°C, and 97 μL of platelet-free plasma was added to 100 μL of erythrocyte/leukocyte pellet and incubated for 30 seconds at 37°C. Samples were incubated with 3 μL of A fumigatus extract (Mediwiss Analytic GmbH, Moers, Germany) for 10 minutes at 37°C. PBS and peanut extract (Mediwiss Analytic GmbH) were used as controls. Untreated basophils were also evaluated. After washing, cells were stained with fluorescence isothiocyanate–labeled mouse anti-human CD3, HLA-DR, CD41a, and CD66b; peridinin-chlorophyll-protein-complex Cy5.5–labeled mouse anti-human CD123 (BD Biosciences, San Jose, Calif); and phycoerythrin-labeled mouse anti-human CD203c (BioLegend, San Diego, Calif) at saturating concentrations. The LIVE/DEAD FixableNear-IR Dead Cell stain kit (Invitrogen, Carlsbad, Calif) 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 minutes on ice. After centrifugation at 490g for 5 minutes, cells were resuspended in 2.5 mmol/L EDTA containing 5% FCS and analyzed by means of flow cytometry on a BD FACS Calibur (BD Biosciences) equipped with BD CellQuest Pro Software. Compensation was performed with CalibrBeads (BD Biosciences), and at least 200 basophils per sample were analyzed. Data were analyzed with FlowJo software (TreeStar, Ashland, Ore).

Statistical analysis
Statistical analysis was performed with GraphPad PRISM 4.0 software (GraphPad Software, San Diego, Calif). Data were tested for normality by using the Kolmogorov-Smirnov test. Normal data were compared by using a 2-tailed independent Student t test, and for nonnormal data, the Mann-Whitney U test was performed. Differences were considered significant at a P value of less than .05.

RESULTS
The BAT discriminates between nonsensitized and A fumigatus–sensitized patients with CF
Flow cytometry was used to measure basophil activation in response to A fumigatus. Basophils were gated as the Live/Dead CD3−HLA-DR−CD41a−CD66b−CD123+ population and evaluated for CD203c expression after A fumigatus extract stimulation (see Fig E1 in this article’s Online Repository at www.jacionline.org). PBS and peanut extract were used as nonoffending and immunogenic controls, respectively. An arbitrary cutoff of 1.36 was determined by using the stimulation index of the control subjects without CF (mean ± SD, 1.018 ± 0.114; n = 11) to differentiate between A fumigatus–sensitized and nonsensitized patients with CF. A positivity threshold of 3 SDs greater than the healthy mean of the stimulation index was determined as before. By using this cutoff, 23 (47.9%) of the 48 recruited patients with CF were A fumigatus sensitized. The ability of sIgE to distinguish between sensitized and nonsensitized subjects has been previously proposed by using a cutoff of 0.35 kU/L. The diagnostic performance of the BAT was examined against sensitization status determined with sIgE (A fumigatus sensitization cutoff, 0.35 kU/L; ImmunoCAP assay) by using receiver operating characteristic (ROC) curve analyses. The area under the ROC curve was 0.9134 (P < .0001, see Fig E2 in this article’s Online Repository at www.jacionline.org), indicating the excellent discriminative ability of the BAT between nonsensitized and A fumigatus–sensitized patients with CF and corroborating the strength of A fumigatus–stimulated CD203c levels as an indicator of A fumigatus sensitization in patients with CF.
A fumigatus–sensitized patients with CF have higher frequencies of A fumigatus–positive sputum cultures compared with nonsensitized patients with CF

Patients with CF were screened for the presence of Aspergillus species in their quarterly sputum samples as part of routine care, amounting to 8 sputum samples from each patient in the 2-year period preceding the BAT. Fourteen (60.9%) of 23 A fumigatus–sensitized patients, but only 6 (24.0%) of 25 nonsensitized patients with CF grew A fumigatus on at least 1 occasion in the 2-year period preceding the BAT. A fumigatus–sensitized patients with CF displayed higher frequencies of A fumigatus sputum isolation compared with nonsensitized patients with CF ($P = .0038$, Fig 1).

Candida albicans colonization was also assessed to verify the specificity of the BAT to $A$ fumigatus. In contrast to $A$ fumigatus, 19 (82.6%) of 23 $A$ fumigatus–sensitized and 17 (68.0%) of 25 nonsensitized patients with CF 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 (see Fig E3 in this article’s Online Repository at www.jacionline.org), illustrating the specificity of the BAT to $A$ fumigatus. Similar results were observed when colonization with Pseudomonas aeruginosa was investigated (see Fig E4 in this article’s Online Repository at www.jacionline.org). No significant difference in $A$ fumigatus–stimulated CD203c levels between patients with nonmucoid ($P = .3263$) or mucoid ($P = .2351$) $P$ aeruginosa compared with their noncolonized counterparts was noted. In summary, BAT to $A$ fumigatus is specific, and patients with CF with an increased frequency of $A$ fumigatus in sputum are more likely to have sensitization to $A$ fumigatus.

A fumigatus–stimulated CD203c levels are increased in blood basophils from $A$ fumigatus–sensitized compared with nonsensitized patients with CF

$A$ fumigatus–sensitized patients with CF had significantly higher $A$ fumigatus–stimulated CD203c values compared with healthy control subjects ($P < .0001$) and nonsensitized patients with CF ($P < .0001$; Fig 2, A). Interestingly, $A$ fumigatus–stimulated CD203c values were significantly higher in $A$ fumigatus–sensitized patients with CF with ABPA than $A$ fumigatus–sensitized patients with CF without ABPA ($P = .0408$; Fig 2, B). This demonstrates that the BAT to $A$ fumigatus distinguishes $A$ fumigatus sensitization from nonsensitization along a quantitative spectrum and could also be potentially used in the diagnosis of patients with ABPA within the $A$ fumigatus–sensitized cohort.

CD203c expression levels inversely correlate with FEV$_1$ and body mass index of $A$ fumigatus–sensitized patients with CF

$A$ fumigatus sensitization has previously been shown to be associated with worse lung function in patients with a variety of pulmonary diseases, including asthma, chronic obstructive pulmonary disease, and CF.\cite{16,17,27,28} Therefore we explored the relationship of $A$ fumigatus–stimulated CD203c levels with lung function (FEV$_1$) and body mass index (BMI), both established determinants of mortality in patients with CF.\cite{29} We observed a significant correlation of $A$ fumigatus–stimulated CD203c levels with decreasing FEV$_1$ in $A$ fumigatus–sensitized patients ($n = 16$, $r^2 = 0.6409, P = .0002$; Fig 3, A). Interestingly, no correlation between $A$ fumigatus–stimulated CD203c values and FEV$_1$ in nonsensitized patients with CF was detected ($n = 25$, $r^2 = 0.0198, P = .5027$; Fig 3, A). Patients with CF with ABPA receiving corticosteroid treatment were excluded from this analysis because of the potential interference of corticosteroid therapy with CD203c levels (see Figs E5 and E6 in this article’s Online Repository at www.jacionline.org). BMI was also shown to inversely correlate with $A$ fumigatus–stimulated CD203c levels in $A$ fumigatus–sensitized ($n = 16$, $r^2 = 0.3009, P = .0278$) but not nonsensitized patients with CF.

**FIG 1.** Frequency of $A$ fumigatus ($Af$)–positive sputum cultures in nonsensitized patients with CF ($CF Af$ sensitized−; $n = 25$) and $A$ fumigatus–sensitized patients ($CF Af$ sensitized+; $n = 23$) in the 2-year period preceding the BAT. Patients with CF were screened for $A$ fumigatus in sputum as part of routine care. Data are presented as means ± SEMs. **$P < .01$.**

**FIG 2.** $A$ fumigatus ($Af$)–stimulated CD203c levels in nonsensitized patients, $A$ fumigatus–sensitized patients, and patients with CF and ABPA. A, Patients with CF were classified as nonsensitized ($n = 25$) or $A$ fumigatus sensitized ($n = 23$). B, The $A$ fumigatus–sensitized cohort was further divided into those with ($CF Af$ sensitized+ ABPA+; $n = 7$) or without ($CF Af$ sensitized+ ABPA−; $n = 16$) ABPA. Data are presented as Tukey box plots, and the median is represented by the middle line. MFI, Mean fluorescence intensity. *$P < .05$ and ***$P < .001$. 
Together, these data illustrate that *A. fumigatus*–stimulated CD203c levels correlate with key clinical parameters in patients with CF, including FEV₁ and BMI, confirming the clinical effect of the *A. fumigatus*–sensitized state.

Total IgE and *A. fumigatus* sIgE, but not sIgG, levels are increased in sera of *A. fumigatus*–sensitized patients with ABPA. Total IgE and *A. fumigatus* sIgE and sIgG levels were evaluated in serum samples from nonsensitized patients, *A. fumigatus*–sensitized patients, and patients with CF-ABPA. As described above, the stimulation index threshold was used to distinguish sensitized (>1.36) from nonsensitized (<1.36) patients. The patients with CF-ABPA were defined as those meeting classical consensus criteria for ABPA. Total IgE levels were significantly higher in patients with CF-ABPA (n = 7) when compared with those in nonsensitized and *A. fumigatus*–sensitized patients with CF (n = 25, P < .0001 and n = 16, P < .0001, respectively; Fig 4, A). Similarly, when sIgE levels were assessed, significantly higher levels were observed in patients with CF-ABPA than in nonsensitized and *A. fumigatus*–sensitized patients with CF (P = .0004 and P < .0001, respectively; Fig 4, B). *A. fumigatus*–sensitized patients with CF without ABPA had higher sIgE levels than nonsensitized patients with CF (P < .0001). No increase in sIgG levels was observed in the CF-ABPA group when compared with the *A. fumigatus*–sensitized and nonsensitized patients with CF; however, *A. fumigatus*–sensitized patients with CF without ABPA had higher sIgG levels than nonsensitized patients with CF (P = .0302; Fig 4, C). Therefore total serum IgE and sIgE levels, but not sIgG levels, are useful in aiding the detection of CF-ABPA and in differentiating it from *A. fumigatus* sensitization.

**Antifungal therapy does not alter *A. fumigatus*–stimulated CD203c expression levels on basophils**

Recent work by our group has shown that elimination of *A. fumigatus* bioburden with itraconazole (400 mg/d for 6 weeks) improves clinical outcome. BAT to *A. fumigatus* was performed to investigate the effect of fungal eradication on *A. fumigatus* sensitization. Although we have previously shown that itraconazole reduces the fungal load in sputum, no change in

**n = 25, r² = 0.0905, P = .1439; Fig 3, B.** Together, these data illustrate that *A. fumigatus*–stimulated CD203c levels correlate with key clinical parameters in patients with CF, including FEV₁ and BMI, confirming the clinical effect of the *A. fumigatus*–sensitized state.

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Total IgE and *A. fumigatus* sIgE and sIgG levels were evaluated in serum samples from nonsensitized patients, *A. fumigatus*–sensitized patients, and patients with CF-ABPA. As described above, the stimulation index threshold was used to distinguish sensitized (>1.36) from nonsensitized (<1.36) patients. The patients with CF-ABPA were defined as those meeting classical consensus criteria for ABPA. Total IgE levels were significantly higher in patients with CF-ABPA (n = 7) when compared with those in nonsensitized and *A. fumigatus*–sensitized patients with CF (n = 25, P < .0001 and n = 16, P < .0001, respectively; Fig 4, A). Similarly, when sIgE levels were assessed, significantly higher levels were observed in patients with CF-ABPA than in nonsensitized and *A. fumigatus*–sensitized patients with CF (P = .0004 and P < .0001, respectively; Fig 4, B). *A. fumigatus*–sensitized patients with CF without ABPA had higher sIgE levels than nonsensitized patients with CF (P < .0001). No increase in sIgG levels was observed in the CF-ABPA group when compared with the *A. fumigatus*–sensitized and nonsensitized patients with CF; however, *A. fumigatus*–sensitized patients with CF without ABPA had higher sIgG levels than nonsensitized patients with CF (P = .0302; Fig 4, C). Therefore total serum IgE and sIgE levels, but not sIgG levels, are useful in aiding the detection of CF-ABPA and in differentiating it from *A. fumigatus* sensitization.

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CD203c levels before and after itraconazole treatment was observed (n = 8, P = .4028; Fig 5). Additionally, no difference in baseline CD203c expression was observed (n = 8, P = .3829; see Fig E5, A). This shows that decreasing the fungal burden in patients with CF undergoing antifungal therapy with itraconazole does not affect A fumigatus 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 3 days), a trend toward efficacy of treatment was observed, with A fumigatus–stimulated CD203c values halving (57.6%) 1 week after treatment initiation (P = .1604, see Fig E6). However, this trend decreased to 30% after 1 month and before the next infusion of corticosteroids (n = 4, P = .2672; see Fig E6), which is suggestive of the transient effect of corticosteroid therapy with time.

### DISCUSSION
The role of bacteria in the lungs of patients with CF has been extensively studied, and potential roles for fungi are beginning to emerge. Recently, much focus has been given to the wide spectrum of A fumigatus–associated morbidities in patients with CF, including A fumigatus sensitization and ABPA. Both of these clinical states are associated with poorer clinical outcomes. For this reason, identifying patients with A fumigatus sensitization with or without ABPA is important to ensure appropriate and timely therapeutic intervention and minimize pulmonary effects. In this study we focused on the basophil activation marker CD203c as an indicator of A fumigatus sensitization and assessed its effect on important clinical measures of CF disease.

Sensitization to A fumigatus has previously been associated with poorer lung function, a finding confirmed by this study. Gernez et al recently reported that basophils from patients with CF and ABPA are primed and hyperresponsive to stimulation with A fumigatus extract. In the current study we used the BAT to identify the A fumigatus sensitization status of our CF cohort. From a logistic viewpoint, the BAT to A fumigatus can be performed in less than 4 hours by using a maximum of 2 mL of whole blood. This can be taken in the same blood draw as the routinely measured immunologic 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 center practice.

Almost half of the patients studied were A fumigatus sensitized, which is in accordance with previous studies (31% to 61%) that used serology and SPTs alone. Increased serum IgE levels to A fumigatus 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 patients with CF. Nevertheless, both of these tests harbor disadvantages. Measuring levels of sIgE to A fumigatus is an easily accessible test for the clinician and convenient for the patient. However, it has been shown that sIgE 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.

SPTs are often laborious and time consuming because the full investigation can take up to 72 hours to complete. Furthermore, subcutaneous injection of the antigen will induce swelling, itching, and reddening at the site of injection in an allergic patient and is therefore a source of discomfort to the patient. The SPTs can be subjective for the attending clinician and open to interpretation because some suggest a wheal diameter of greater than 3 mm and others recommend a diameter of greater than 4 mm for a positive result. Accumulating reports have...
shown striking differences in individual patients between SPT responses and serologic 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{13} Of note, \textit{A fumigatus} sIgE levels correlated significantly with CD203c levels (see Fig E7 in this article’s Online Repository at www.jacionline.org). The BAT is a specific and reliable measure of IgE-dependent response in sensitized subjects because (1) the mast cells are tissue bound, (2) basophil accessibility is easy, and (3) that the upregulation of basophil surface markers can be conveniently measured by means of flow cytometry. By comparing CD203c levels with the already established cutoff sIgE value of 0.35 kUA/L\textsuperscript{16} to distinguish between sensitized and nonsensitized patients, ROC curve analysis further confirmed the usefulness of the BAT as an indicator of \textit{A fumigatus} sensitization in patients with CF. Persistent and prolonged exposure to the fungus is a significant risk factor for \textit{A fumigatus} sensitization because sensitized patients display a higher frequency of \textit{A fumigatus} isolation from sputum culture. Although a study by Baxter et al\textsuperscript{15} showed no correlation between \textit{A fumigatus} colonization and sensitization, it is conceivable that these different outcomes might be related to methodological differences in defining \textit{A fumigatus} sensitization, colonization, and the microbiological techniques used. Interestingly, the \textit{A fumigatus}–sensitized ABPA cohort had higher \textit{A fumigatus}–stimulated CD203c values than \textit{A fumigatus}–sensitized patients without ABPA, illustrating that levels of measured CD203c might allow differentiation between \textit{A fumigatus} sensitization and CF with ABPA.

We and others have previously shown that persistent carriage of \textit{A fumigatus} in the airways of patients with CF is associated with radiologic abnormalities, including more severe bronchiectasis and mosaic pattern perfusion, and has an effect on pulmonary function.\textsuperscript{16, 17} \textit{A fumigatus} sensitization is also associated with lung function decrease and increased duration of intravenous antibiotic treatments.\textsuperscript{15, 17} In line with this, our data illustrate a significant inverse correlation between \textit{A fumigatus}–stimulated CD203c values with FEV\textsubscript{1} and BMI. These findings are in accordance with previously published studies suggesting a negative effect of \textit{A fumigatus} sensitization in patients with CF.\textsuperscript{15-17} \textit{A fumigatus} sensitization likely facilitates CF with ABPA, and multiple factors, including degree of fungal exposure, mucus viscosity, immune status, atopy, and age, might 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 α have also been described to associate with ABPA development.\textsuperscript{40-42} Longitudinal studies are needed to confirm that \textit{A fumigatus} sensitization routinely precedes ABPA.

Baxter et al\textsuperscript{11} have recently proposed a novel classification of aspergillosis in adults with CF. Using a combination of serologic (total IgE and \textit{A fumigatus} sIgE and sIgG), RT-PCR, and galactomannan (GM) data, they distinguish between nonsensitized subjects, patients with CF with ABPA, patients with \textit{A fumigatus} sensitization, and patients with \textit{A fumigatus} bronchitis. In accordance with their findings, we found that patients with CF-ABPA had higher total IgE and \textit{A fumigatus} sIgE levels compared with nonsensitized and \textit{A fumigatus}–sensitized patients without ABPA. Our study showed no difference in sIgG levels between patients with CF with or without ABPA. Additionally, when applied to our CF population, both the GM assay and RT-PCR for \textit{A fumigatus} detected a high number of \textit{A fumigatus}–positive sputum samples (data not shown), preventing adequate differentiation between the cohorts according to the aforementioned classification. This high positive might 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{23, 42} 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 nonsensitized subjects, \textit{A fumigatus}–sensitized patients, and patients with CF-ABPA. We used a combination of \textit{A fumigatus}–stimulated CD203c and total IgE and \textit{A fumigatus} sIgE levels (Table I) to identify ABPA, avoiding the need for RT-PCR, GM, or \textit{A fumigatus} sIgG testing that is not always accessible in CF centers. Increased \textit{A fumigatus}–stimulated CD203c values (>1.36) combined with increased serum sIgE (>1.45 kUA/L) and total IgE (>185 kU/L) levels correctly identified all cases of CF with ABPA based on the consensus conference criteria. Of note, the sIgE cutoff (1.45 kUA/L) used in the proposed classification system for identification of CF with ABPA is higher than the sIgE cutoff (0.35 kUA/L) routinely used to identify sensitization alone. Furthermore, the total IgE cutoff herein has been considerably reduced compared with the consensus values (minimum, 500 kU/L), which is in line with a previously published ROC curve analysis demonstrating that the optimum level for ABPA diagnosis is greater than 185 kU/L, producing 91% sensitivity and 90% specificity.\textsuperscript{11}
The inclusion of the reduced level in our proposed classification system was validated when 1 patient meeting our classification criteria for CF with ABPA (ie, increased CD203c, total IgE, and A fumigatus sIgE levels), but not the consensus conference criteria (total IgE >50 kU/L), had ABPA, as defined by consensus conference criteria shortly after the study conclusion. A graphic representation of our classification is depicted in Fig 6.

Our previous work has shown that itraconazole alleviates fungal burden in patients with CF colonized with A fumigatus. In this study itraconazole administration did not influence A fumigatus–stimulated CD203c levels, suggesting that fungal eradication from the lung does not affect A fumigatus sensitization once established. Taking into consideration that persistent A fumigatus colonization is a significant risk factor for sensitization, early itraconazole intervention might be warranted to clear colonization, reduce exposure, and thereby minimize the risk of sensitization to A fumigatus. Acute ABPA flares can be treated with intravenously administered pulsed methylprednisolone at 10 to 15 mg/kg/d for a 3-day period every month. Methylprednisolone was administered to 4 patients with CF with ABPA in this study, and a trend toward reduction in A fumigatus–stimulated CD203c values was observed after treatment. However, this trend was not significant (P = .1604), warranting future longitudinal study with increased numbers to examine the effect of corticosteroid treatment on A fumigatus sensitization and potentially the use of the BAT to monitor treatment responses. Additionally, future work should also examine the effect of anti-IgE and anti–IL-13 strategies in the context of CD203c levels and CF with ABPA.

In conclusion, we propose a novel and simplified means of identifying sensitization to A fumigatus by using A fumigatus–stimulated CD203c values. Using the BAT, we show that increased incidence of A fumigatus colonization is associated with A fumigatus sensitization and that the latter state affects lung function. The practical applicability of the BAT to A fumigatus in the clinical setting includes an evaluation of corticosteroid–stimulated CD203c values for patients positive for A fumigatus on at least 2 occasions within the preceding 2 years (Fig 7). If the BAT result is negative, itraconazole treatment can be offered for fungal eradication; however, a positive result indicates sensitization and a potential increased future risk of ABPA. Consequently, sensitized patients should have their serologic results closely monitored for increases in total IgE or A fumigatus sIgE levels that might indicate ABPA. Despite the lack of effect of azole treatment on the sensitization state, eradication therapy to reduce fungal bioburden can be recommended. If sensitization is a prerequisite for ABPA, corticosteroids can be considered at the early sensitization stage to reduce the likelihood of having ABPA. A longitudinal study with adequate numbers should be performed to assess the benefits of systemic corticosteroid administration on clinical outcomes in A fumigatus–sensitized patients without ABPA. Timely detection of A fumigatus sensitization and CF with ABPA equips clinicians to deliver a targeted approach to CF A fumigatus therapy to improve clinical outcomes.

We sincerely thank the patients with cystic fibrosis and healthy volunteers for taking part in this study.


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METHODS
Pulmonary function testing
Results of pulmonary function tests, including FEV₁, were determined by using the MicroLab Spirometer (CareFusion, San Diego, Calif) after appropriate calibration and reported as relative measurements (percent predicted). Three representative pulmonary function tests meeting American Thoracic Society/European Respiratory Society criteriaE1 were performed at each study visit, and the averages were used for analyses.

REFERENCE
FIG E1. Gating strategy for monitoring cell-surface expression of the basophil activation marker CD203c. A, Leukocytes were chosen based on forward scatter (FSC) and side scatter (SSC). B-D, Basophils were selected as the CD3−HLA-DR−CD41a−CD66b−Live/Dead− and CD123+ population (Fig E1, B) and monitored for CD203c expression levels after 10 minutes of stimulation with PBS, peanut, and A fumigatus (Af) extract (Fig E1, C and D). Shown are representative histograms from nonsensitized (Fig E1, C) and A fumigatus-sensitized patients with CF (Fig E1, D). All samples were adequate for flow gating strategy and analysis.
FIG E2. ROC curve analyses of the diagnostic performance of the BAT against sensitization status, as determined by sIgE levels. *A fumigatus* sIgE levels of greater than 0.35 kU/L have been considered indicative of sensitization, and this cutoff value was used to bisect our cohort into sensitized and nonsensitized patients based on their sIgE levels. The area under the ROC curve of 0.9134 (P < .0001) indicates the excellent discriminating ability of the BAT between nonsensitized (n = 33) and *A fumigatus*-sensitized (n = 15) patients with CF who were defined by using the *A fumigatus* sIgE levels (cutoff, 0.35 kU/L).
FIG E3. Frequency of *Candida albicans*-positive cultures in nonsensitized (CF Af sensitized−; n = 25) and *A fumigatus* (Af)-sensitized patients with CF (CF Af sensitized+; n = 23) in the 2-year period preceding the BAT. Patients with CF were screened for the presence of *C albicans* in their sputum samples as part of routine care. Culture data presented here were collected in the 2-year period preceding the BAT, and the differences between the 2 groups were nonsignificant (*P* = .0981). Data are presented as means ± SEMs. n.s., Not significant.
FIG E4. Effect of *Pseudomonas aeruginosa* colonization on *A fumigatus*-stimulated CD203c levels. Patients with CF (n = 48) were screened for the presence of nonmucoid and mucoid *P aeruginosa* in their sputum as a part of routine care. There was no significant difference in *A fumigatus*-stimulated CD203c levels between patients with nonmucoid (*P* = .3263) or mucoid (*P* = .2351) *P aeruginosa* compared with their noncolonized counterparts. Data are presented as means ± SEMs. n.s., Not significant.
FIG E5. Baseline CD203c levels in blood basophils before and after treatment with itraconazole or methylprednisolone. Baseline surface CD203c levels in blood basophils were evaluated by means of flow cytometry before and after 30 days of treatment with oral itraconazole ($P = .3829$, $n = 8$; A) and before and 7 ($P = .3402$, $n = 4$) and 30 days (and $P = .1660$, $n = 4$) after treatment with intravenous methylprednisolone (B). Data are presented as Tukey box plots, and the middle line represents the median. IV, Intravenous; MFI, mean fluorescence intensity.
A fumigatus–stimulated CD203c levels in blood basophils before and after treatment with methylprednisolone. A fumigatus–stimulated surface CD203c levels in blood basophils were evaluated by means of flow cytometry before and 7 days ($P = .1604, n = 4$) and 30 days ($P = .2672, n = 4$) after treatment with intravenous (IV) methylprednisolone. Data are presented as Tukey box plots, and the middle line represents the median. MFI, Mean fluorescence intensity.

**FIG E6.**
FIG E7. Correlation of total IgE levels (A), sIgE levels (B), and specific/total IgE ratios (C) with CD203c levels in our CF cohort (n = 48). Total and sIgE levels are shown in logarithmic scale.
### TABLE E1. Characteristics of the patients enrolled in this study

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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</thead>
<tbody>
<tr>
<td><strong>Baseline clinical characteristics (n = 48)</strong></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>28 ± 10</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>29 (60.4%)</td>
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<tr>
<td>Female</td>
<td>19 (39.6%)</td>
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<tr>
<td>ΔF508 homozygous</td>
<td>20 (41.7%)</td>
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<tr>
<td>ΔF508 heterozygous</td>
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<tr>
<td>BMI (kg/m²)</td>
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<tr>
<td>CFRD</td>
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<tr>
<td>IGT</td>
<td>7 (14.6%)</td>
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<td>FEV₁ (% predicted)</td>
<td>54 ± 23</td>
</tr>
<tr>
<td>FVC (% predicted)</td>
<td>75 ± 25</td>
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</tbody>
</table>

Data are presented as numbers (percentages) or means ± SDs.

*CFRD*, CF-related diabetes; *FVC*, forced vital capacity; *IGT*, impaired glucose tolerance.