Novel immunologic classification of aspergillosis in adult cystic fibrosis

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Background: Patients with cystic fibrosis (CF) demonstrate a wide range of hypersensitivity responses to *Aspergillus*, beyond allergic bronchopulmonary aspergillosis, which require classification.

Objective: This study integrated 2 new methods of *Aspergillus* detection—sputum galactomannan (GM) and real-time PCR—alongside established serologic markers, to reclassify aspergillosis in CF.

Methods: A total of 146 adult patients with CF had serologic tests (ImmunoCap total IgE, specific *Aspergillus fumigatus* IgE, and specific *A fumigatus* IgG), sputum real-time *Aspergillus* PCR, and sputum GM. Patients were classified by using latent class analysis.

Results: Both RT-PCR and GM were more sensitive than culture in detecting *Aspergillus* in sputum (culture 37%, RT-PCR 74%, and GM 46%). Intraassay and interassay reproducibility of PCR and GM was excellent. Latent class analysis of triazole-naive patients identified a nondiseased group and 3 disease classes: class 1 (n = 49, 37.7%) represented patients with or without positive RT-PCR but no immunologic response to *A fumigatus* and negative GM (nondiseased); class 2 (n = 23, 17.7%) represented patients with positive RT-PCR, elevated total and specific *A fumigatus* IgE/IgG, and positive GM (serologic allergic bronchopulmonary aspergillosis); class 3 (n = 19, 14.6%) represented patients with or without positive RT-PCR, elevated *A fumigatus* IgE (not IgG), and negative GM (*Aspergillus* sensitized); and class 4 (n = 39, 30%) represented patients with positive RT-PCR, elevated *A fumigatus* IgG (not IgE), and positive GM (*Aspergillus* bronchitis).

Conclusions: Three distinct classes of aspergillosis in CF were identified by latent class analysis by using serologic, RT-PCR, and GM data. This novel classification will facilitate improved phenotyping, pathogenesis studies, and management evaluations. (J Allergy Clin Immunol 2013;132:560-6.)

Key words: Aspergillus fumigatus*, cystic fibrosis, bronchitis, allergic bronchopulmonary aspergillosis, polymerase chain reaction, galactomannan*

Aspergillus fumigatus causes significant morbidity in patients with cystic fibrosis (CF). Allergic hypersensitivity in CF forms an immunologic spectrum from lone IgE-mediated sensitization to allergic bronchopulmonary aspergillosis (ABPA), affecting up to 65% and 15% of adult patients with CF, respectively.[1,2] Consensus guidelines for the management of ABPA do not embrace patients with *Aspergillus* sensitization and/or colonization.[3] Most available evidence points toward a positive link between greater lung function decline and sensitization to *A fumigatus*, but there are no randomized studies of the effect of anti-inflammatory or antifungal agents.[4-7] The effect of *A fumigatus* colonization is more debatable, and defining colonization is nonstandardized.[8-11] The prevalence of *A fumigatus* in CF sputum samples varies widely with culture technique, from 6% to 57%.[12,13] Therefore, a major obstacle in assessing the clinical impact of colonization or infection is the reliable detection of *Aspergillus* from sputum.[14] Positive sputum culture has not been linked to the development of ABPA or sensitization and is not included in diagnostic criteria. An additional separate clinical entity has also recently been proposed, "*Aspergillus* bronchitis," but is based on observations in just 6 patients persistently growing *A fumigatus* who had improvements in clinical status and lung function following antifungal therapy.[15] No serologic or sputum markers have been found that distinguish patients with colonization from those sensitized or with *Aspergillus* bronchitis/infection.

A diagnostic classification system of *Aspergillus* hypersensitivity and colonization, beyond ABPA, is needed to establish the longitudinal clinical effects of these syndromes and allow future studies of genetics and clinical trials of treatment. Classification requires accurate *Aspergillus* detection and measures of immunologic response. The primary aim of this study was to validate 2 new methods to detect *Aspergillus* in CF sputum—real-time quantitative PCR (RT-PCR) for *Aspergillus* DNA and galactomannan (GM) antigen —and integrate these tests with standard serologic analysis to develop a classification system of

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Abbreviations used
ABPA: Allergic bronchopulmonary aspergillosis
CF: Cystic fibrosis
CV: Coefficient of variation
GM: Galactomannan (a measure of *Aspergillus* growth)
ABPA-S: Serologic ABPA
SPT: Skin prick test

aspergillosis in CF. Secondary aims were to compare baseline clinical differences between classes and monitor changes in body mass index, lung function, and pulmonary exacerbation rates over a 2-year prospective period.

METHODS

Study design

This was a single center, 2-year prospective observational cohort study of adult patients with CF. The study was approved by the South Manchester research ethics committee (07/Q1403/70).

Patients and sample processing

Patients were enrolled from the Manchester adult CF center during outpatient consultations, and all gave written informed consent. Patients enrolled were aged 18 years or older and had a confirmed diagnosis of CF by genetic testing and/or sweat testing. Enrollment was deferred if they had an exacerbation of pulmonary symptoms requiring additional therapy.

Demographics. Patient baseline demographic details were recorded including CF comorbidities, chronic pulmonary infections, inhaled/oral treatments, and lung function. Baseline lung function (FEV_1 and forced vital capacity % predicted) was obtained from patients' annual clinical assessment records, and the best lung function achieved in the year prior to recruitment was recorded. All lung function was performed postbronchodilator, by experienced staff according to European Respiratory Society guidelines.[16] Lung function was recorded again 2 years after enrollment by using the same method. Body mass index was recorded at baseline and 2 years after enrollment. Total days of intravenous antibiotics were prospectively monitored over 2 years as a measure of pulmonary exacerbation rates.

Sputum collection, culture, and DNA extraction. Sputum samples were noninduced, with a minimum volume of 2 mL, refrigerated at 4°C, and processed within 24 hours of collection. An equal volume of Sputasol (Oxoid Ltd, Basingstoke, United Kingdom) was added to each sputum sample, vortexed, and incubated at 37°C for 30 minutes. Sputum culture was then performed according to modified Health Protection Agency National Standards Method BSOP57 (10 μL rather than 1 μL inoculum).[17] The remaining sputum sample underwent further homogenization using sonication as described previously.[18] Culture was repeated after sonication, and 600 μL of sputum was transferred to a sterile microcentrifuge tube for GM detection. DNA was extracted from the remainder of the sample by using the fungal DNA extraction kit MycXtra (Myconostica, Manchester, United Kingdom). Ten sputum samples were split to investigate PCR intraassay reproducibility. Thirty patients gave 2 samples within 9 months to assess reproducibility and chronicity of infection/colonization.

RT-PCR. Samples were prepared in a high efficiency particle arrest-filtered biosafety cabinet. *Aspergillus* DNA was detected and quantified by using a commercial RT-PCR assay, MycAssay Aspergillus (Myconostica), which targets a portion of the 18S ribosomal gene. Cycle threshold (C_t) values less than 38 were positive. Forty DNA extractions were processed twice to investigate PCR interassay reproducibility.

Galactomannan. The Platelia *Aspergillus* enzyme immunoassay (Bio-Rad, Marnes-La-Coquette, France) was used to detect GM in 300 μL sputum samples fully homogenized by Sputasol and sonication (optical density index ≥0.5 positive). Twenty samples were processed twice simultaneously to investigate intraassay reproducibility. Twelve samples were processed daily over 5 days, half were stored at −20°C and half were stored at 4°C between testing, to determine interassay reproducibility.

Allergy tests. Serum samples were tested for eosinophil count, total circulating IgE, specific *A fumigatus* IgG, and 5 specific fungal IgE (sIgE) allergens using the ImmunoCap assay (Phadia, Uppsala, Sweden): *A fumigatus*, *Cladosporium herbarum, Penicillium chrysogenum* (*notatum*), *Candida albicans*, *and Alternaria alternata*. Type 1 immediate hypersensitivity skin prick tests (SPTs) using standard methods were carried out not only to the same panel of fungal allergens but also to 5 common aeroallergens: grass pollen mix, tree pollen mix, *Dermatophagoides pteronyssinus*, and cat and dog dander (Allergopharma, Reinbeck, Germany).[19,20]

Statistics

Data are expressed as means ± SD, and SPSS version 16.0 (SPSS, Inc, Chicago, Ill) was used to compare results from culture, RT-PCR, and GM testing. Results with a P value of less than .05 were considered statistically significant. Latent class analysis was performed by using M*plus* version 6.11 (Muthén & Muthén, Los Angeles, Calif) to detect disease entities on the basis of statistical patterns of association and likelihood between results, which, in turn, gives each patient a probability of membership for each class. Patients on triazole antifungals were excluded, serologic data were log transformed, and both RT-PCR and GM results were converted to binary data. Patients were assigned to classes by using modal probability and then classes were profiled and labeled. Latent classes were compared to look for any differences in demographic, treatment, or clinical characteristics by using Pearson χ^2 tests, ANOVA, and Mann-Whitney U tests.

To create a diagnostic algorithm for future patients, canonical discriminant analysis was performed by using STATA version 11 (StataCorp LP, College Station, Tex). The aim was to first produce linear combinations (canonical variables) of test values that maximally separated the groups. These variables were then used to generate a diagnostic algorithm, using discriminant functions.

RESULTS

One hundred fifty patients consented to participate; 146 completed the study. Baseline clinical characteristics are shown in Table I.

Culture and RT-PCR

Thirty-nine (27%) patients grew *A fumigatus* by culture and 1 additionally grew *A flavus*. In contrast, 108 (74%) patients were RT-PCR positive for *Aspergillus* species. All culture-positive samples were RT-PCR positive; 38 patients were both PCR and culture negative. There was no correlation between colony-forming units and PCR C_t (Spearman correlation coefficient $r = -0.284$). RT-PCR reproducibility was excellent, with an intraassay coefficient of variation (CV) of 1.5% and interassay CV of 1.1% (see Table E1 and the RT-PCR reproducibility section in this article's Online Repository at www.jacionline.org).

Galactomannan

Sixty-eight (46%) patients were GM positive. Of the 108 RT-PCR positive samples, 66 were GM positive and 42 were GM negative. GM reproducibility was excellent, with an intraassay CV of 5% and interassay CV of 9% (see Table E2 and the GM reproducibility section in this article's Online Repository at www.jacionline.org).

Serology and SPTs

SPTs were performed in 115 of the 146 patients; 31 patients either failed to attend appointments or did not consent to testing.

TABLE I. Baseline clinical and demographic details: data are expressed as number (%) or mean ± SD

Baseline clinical characteristic	N = 146
Age (y)	28 ± 9
Male	74 (51%)
F508 del homozygous	83 (57%)
F508 del heterozygous	53 (36%)
BMI (kg/m^2)	21 ± 3
CFRD	52 (36%)
Pancreatic insufficiency (%)	133 (91%)
Oral steroids	26 (18%)
Inhaled steroids	124 (85%)
Azithromycin	140 (96%)
Nebulized antibiotics	120 (82%)
Triazole (excluding fluconazole)	16 (11%)
Chronic *Pseudomonas aeruginosa*	121 (83%)
Chronic *Burkholderia* species	15 (10%)
Chronic MSSA or MRSA	47 (32%)
NTM	7 (5%)
FEV_1 % predicted	53 ± 20
FVC % predicted	70 ± 19

BMI, Body mass index; *CFRD*, cystic fibrosis–related diabetes; *FVC*, forced vital capacity; *MRSA*, methicillin-resistant *Staphylococcus aureus*; *MSSA*, methicillin-sensitive *Staphylococcus aureus*; *NTM*, nontuberculous *Mycobacteria*.

Forty-one (36%) patients demonstrated allergy to 2 or more common allergens, and 53 (46%) patients demonstrated allergy to 2 or more fungal allergens. For fungi, positive *A fumigatus* SPT results were most common (65%). Positive SPT results to other fungi were fewer but still common: *Penicillium chrysogenum* (notatum) 49%, *Alternaria alternata* 34%, *Candida albicans*, and *Cladosporium herbarum* 21%. The level of agreement between fungal SPTs and sIgEs was highest for *A fumigatus* antigens (see Table E3 in this article's Online Repository at www.jacionline.org). Spearman correlation coefficient to compare the levels of sIgE with the size of SPT wheals showed the strongest correlation for *A fumigatus* ($r = 0.728$, $P < .005$).

Latent class analysis

Latent class analysis was performed by using data from 130 triazole-naive patients (Table E4 in this article's Online Repository at www.jacionline.org shows results for the 16 patients on triazoles). Patients on oral steroids (n = 15) were included because only 2 were taking these for a prior diagnosis of ABPA, with the remainder taking low maintenance doses for arthiritis and CF-associated breathlessness. Three goodness-of-fit criteria were applied for model selection, looking for parsimony (see Table E5 in this article's Online Repository at www.jacionline.org). Bayesian information criterion was the only index that provided a clear indication of the optimum number of classes. Class profiling with clinical correlation revealed little difference between 4 and 5 classes, and therefore 4 classes was deemed optimal.

Average values of the latent class variables are displayed in Table II. Examination of the classes according to current knowledge would suggest the following: Class 1 represents nondiseased CF patients with or without *Aspergillus* in sputum but negative GM and no measurable immunologic response; class 2 represents serologic ABPA (ABPA-S) with all immunologic markers being high, positive RT-PCR, and positive GM; class 3 represents *Aspergillus* IgE-sensitized patients with or without *Aspergillus* in sputum but negative *A fumigatus* IgG and negative GM; and class 4 represents *Aspergillus* airway infection/"bronchitis" with negative IgE markers but positive *A fumigatus* IgG, RT-PCR, and GM.

Fig 1 demonstrates that no one marker is able to distinguish all categories and SDs are wide. However, classes 1 and 3 appear separated from classes 2 and 4 by GM, sIgG, and RT-PCR. Classes 2 + 4 compared with classes 1 + 3 had significantly higher sIgG levels (classes 2 + 4 median, 103; classes 1 + 3 median, 40; n = 130; Mann-Whitney $Z = 9.29$; $r = 0.8$; $P < .001$), significantly higher GM levels (classes 2 + 4 median, 2.30; classes 1 + 3 median, 0.10; n = 130; Mann-Whitney $Z = 9.68$; $r = 0.9$; $P < .001$), and significantly more *Aspergillus* DNA (C_t [value 38 − C_t]; classes 2 + 4 median, 9.0; classes 1 + 3 median, 2.1; n = 130; Mann-Whitney $Z = -6.32$; $r = 0.6$; $P < .001$).

Receiver operating characteristic curves for GM, sIgG, and PCR were calculated to determine cutoff values distinguishing classes 2 + 4 from classes 1 + 3. GM performed best, with an area under the curve of 0.991 (SE, 0.006; 95% CI, 0.98-1.00). A cutoff index of 0.5 or more gave a 98% sensitivity, 96% specificity, 95% positive predictive value, and 99% negative predictive value (Fig 2). Specific IgG also performed well and distinguished classes 2 + 4 from classes 1 + 3, with a cutoff of 75 mg/L yielding a sensitivity of 90%, specificity of 96%, positive predictive value of 95%, and negative predictive value of 92%.

Class 2 (ABPA-S) and class 3 (sensitized) can be clearly separated for the first time by using sIgG and GM. A GM index of more than 0.5 gives 96% sensitivity and 100% specificity (area under the curve, 0.989), while an sIgG of more than 75 mg/L gives 96% sensitivity and 90% specificity.

sIgE also has value: class 2 is separated from class 4 by using sIgE; a value of more than 3.7 kUA/L gives 100% sensitivity and specificity for belonging to class 2 rather than class 4. Similarly, class 1 is separated from class 3 by using sIgE; a value of more than 2.0 kUA/L gives a sensitivity of 90% and a specificity of 96% for belonging to class 3 rather than class 1. Further information on total IgE and sIgE separating classes can be seen in Fig E1 in the Online Repository at www.jacionline.org.

Baseline clinical characteristics of latent classes

Six baseline clinical characteristics were found to be statistically different between the latent classes (Table III) (Table E6 in the Online Repository at www.jacionline.org details nonsignificant differences in clinical characteristics). Class 2 used more oral corticosteroids ($Z = 3.1$, $P < .01$). Doses ranged from 5 to 10 mg. Of the 15 patients taking steroids, 9 were in class 2 and 4 were in class 1. Class 2 had more patients with pancreatic sufficiency ($Z = 3.2$, $P < .01$), but there were no significant differences found for functional classes of CF mutation. Class 2 and class 3 had more patients with allergy to 2 or more common allergens (class 2, $Z = 2.4$, $P < .05$, and class 3, $Z = 2.1$, $P < .05$) and more patients with allergy to 2 or more fungal allergens (class 2, $Z = 4.3$, $P < .001$, and class 3, $Z = 2.8$, $P < .01$). Class 2 had higher peripheral blood eosinophil counts ($F_{3,126} = 7.14$, $P < .001$, $r = 0.15$). Class 2 mean, 0.51×10^9/L; SE, 0.14; class 1 mean, 0.18×10^9/L; SE, 0.02 ($P < .001$), class 3 mean, 0.23×10^9/L; SE, 0.03 ($P = .012$); class 4 mean, 0.18×10^9/L; SE, 0.02 ($P < .001$). Finally, there was also a significant difference in baseline body mass index between the classes (ANOVA $F_{3,126} = 2.72$, $P = .047$). However, despite reaching statistical significance, the actual differences in mean scores between the classes was

TABLE II. Average values of latent class variables for 130 triazole-naive patients

	Class 1 (n = 49)	Class 2 (n = 23)	Class 3 (n = 19)	Class 4 (n = 39)
Average class probability	0.981	0.996	0.921	0.992
Median sIgG (mg/L) (SD)	36 (20)	112 (132)	47 (24)	98 (29)
Median sIgE (kUa/L) (SD)	0 (0.6)	13.2 (19.0)	5.2 (3.9)	0 (1.0)
Median tIgE (kUI/L) (SD)	28 (28)	690 (1230)	170 (98)	35 (157)
Mean RT-PCR C_t value (38 − C_t)* ± SD	2.6 ± 3.3	8.2 ± 4.0	3.7 ± 3.3	7.1 ± 4.0
Probability of positive PCR	0.497	1.000	0.778	1.000
Mean GM index ± SD	0.161 ± 0.19	2.544 ± 2.04	0.135 ± 0.17	2.960 ± 2.11
Probability of positive GM	0.004	0.946	0.000	1.000

sIgG, Specific *Aspergillus* IgG; *tIgE*, total IgE.
*The RT-PCR C_t limit of blank is 38. To facilitate comparisons between GM and RT-PCR, the actual C_t value is shown as 38 − C_t, and so higher numbers reflect increasing *Aspergillus* DNA concentrations.

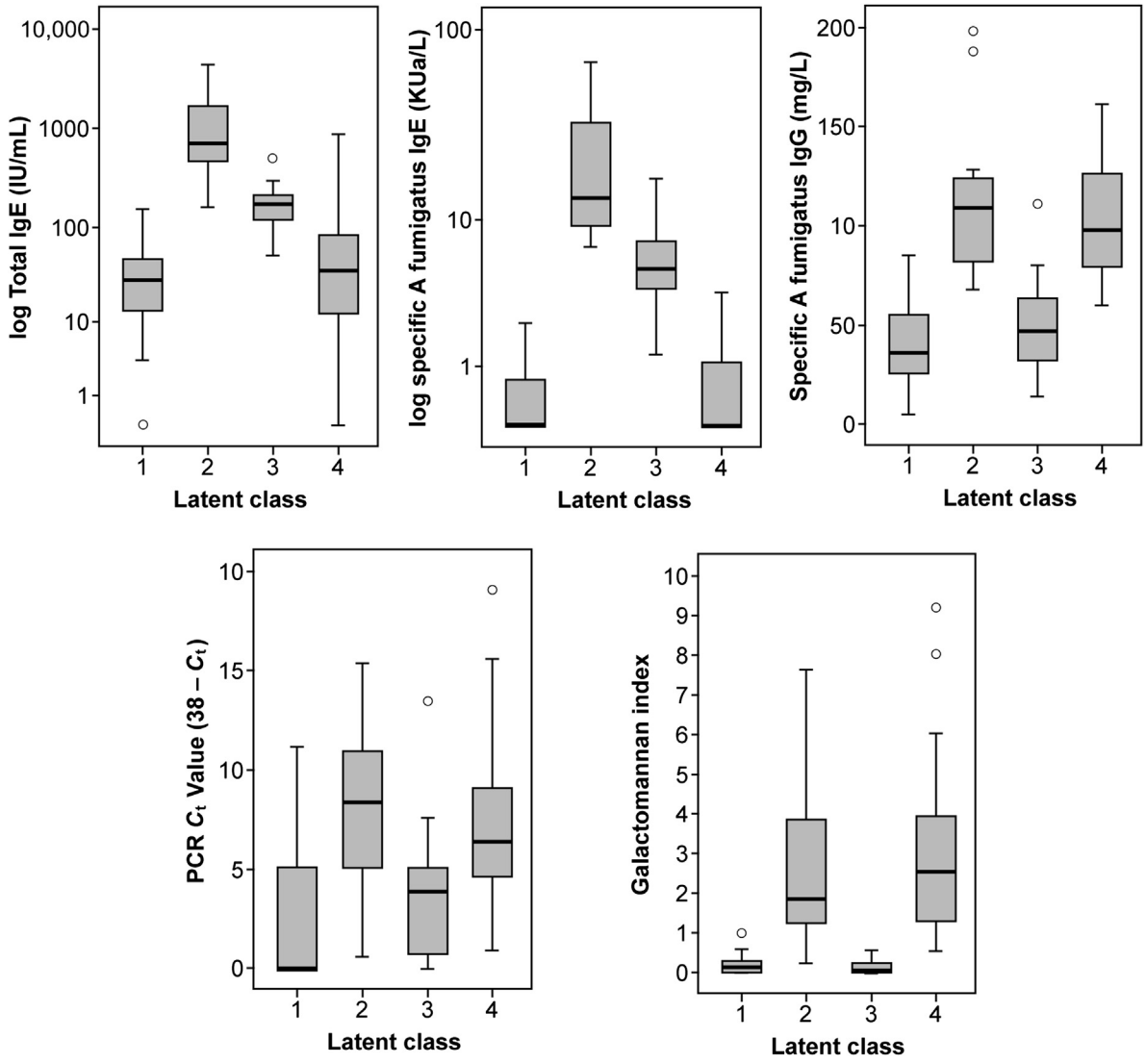

FIG 1. Boxplot distributions of immunologic, RT-PCR, and GM levels by latent class in 130 triazole-naive adult patients with CF.

small: class 1, 22.2; class 2, 20.3; class 3, 22.0; and class 4, 21.3. This resulted in a small effect size ($r = 0.06$), and *post hoc* tests revealed no statistically significant differences between groups.

Prospective clinical data

There were no differences between classes in change of body mass index ($F_{3,126} = 0.36$, $P = .82$) or days of intravenous antibiotics received ($F_{3,126} = 0.20$, $P = .90$) over the 2-year

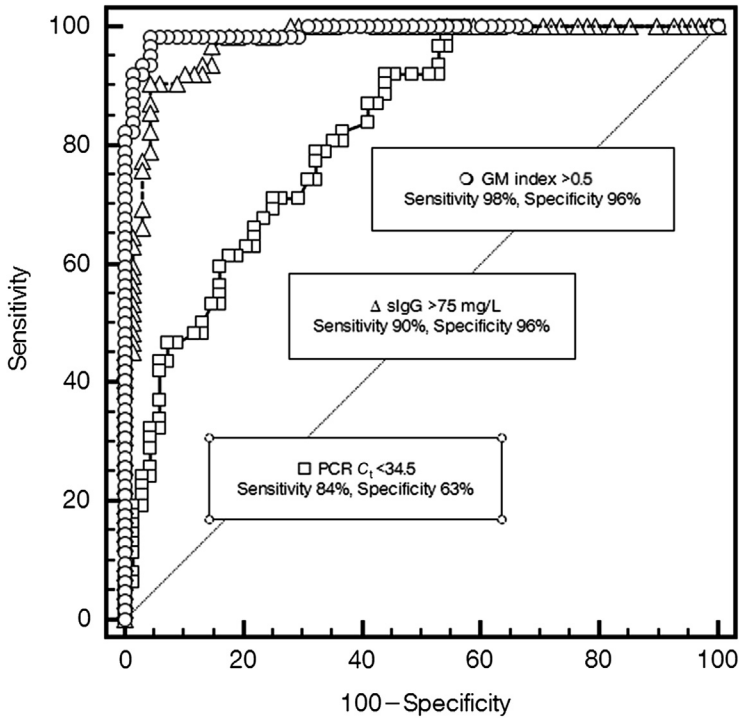

FIG 2. ROC-curve analysis separating classes 2 + 4 from classes 1 + 3. ROC curve showing the performance of GM (optical index >0.5), sIgG (cutoff >75 mg/L) and real-time *Aspergillus* RT-PCR (C_t cutoff <34.5) for distinguishing both class 2 (ABPA) and class 4 (*Aspergillus* bronchitis) from classes 1 and 3. *ROC,* Receiver operating characteristic.

prospective monitoring period. Lung function decline was measured by decline in FEV_1 percent predicted and decline in forced vital capacity percent predicted. Data were not normally distributed, and a square root transformation was done. One-way ANOVA revealed a significant difference in FEV_1 % predicted decline between the classes ($F_{3,126} = 4.4$, $P = .005$, $r = 0.32$) but no significant differences in forced vital capacity decline ($F_{3,126} = 1.45$, $P = .23$). *Post hoc* comparisons were done by using Fisher least significant difference test with Bonferroni adjustment of the calculated P value. FEV_1 decline in all 3 disease classes was significantly greater than in the nondiseased class 1 (mean, 3.16; SE, 0.60): class 2 mean, 6.57; SE, 1.32 ($P = .018$); class 3 mean, 6.16; SE, 1.09 ($P = .018$); and class 4 mean, 5.41; SE 0.83 ($P = .036$). Analysis of covariance demonstrated that none of the baseline clinical differences between classes had an impact on lung function independently or as covariant.

Canonical discriminant analysis

Two canonical discriminant function coefficients were optimal to separate classes and can be used to create a diagnostic algorithm to predict class membership for future patients (see Figs E2 and E3 in the Online Repository at www.jacionline.org). Canonical discriminant analysis revealed that dropping GM and sIgE had the greatest impact on class assignment.

DISCUSSION

This study demonstrates the presence of 3 distinct classes of aspergillosis in adult patients with CF with respect to multiple biomarkers of *Aspergillus* infection and immunologic response to that infection or exposure: ABPA-S, sensitization, and *Aspergillus* infection/bronchitis.

This study moves the field forward in several ways and suggests that it is time to revise the 9-year-old consensus statement.[3] First, we have provided a quantitative evidence base on which to make the diagnosis of ABPA-S, which until now has not been possible. Confirmation of our findings is required, particularly the cutoffs for tIgE and aspergillus-specific IgE, as levels might have been reduced slightly by patients on oral corticosteroids. These diagnostic criteria will facilitate direct study of this group of patients genetically and with antifungal or immunologic therapy. Second, *Aspergillus* sensitization without ABPA can be clearly separated by negative GM and sIgG markers, suggesting a different pathophysiologic process. Our sensitive measures of *Aspergillus* infection indicate modest levels of *Aspergillus* DNA without GM in this group, and so airway *Aspergillus* infection is likely low; microbiome and biofilm studies combined with host genetics would be particularly helpful in this group to better understand this perplexing association. Our data along with 4 other studies suggest that there is a significant link, perhaps not causal, between *Aspergillus* sensitization and falling FEV_1 in CF, which needs urgent investigation and treatment strategies.[4-7] This is also true in severe asthma where *Aspergillus* sensitization is associated with more airflow limitation and more bronchiectasis.[21,22] Third, *Aspergillus* bronchitis in CF has been recently described on the basis of the exclusion of other pathogens, failure to respond to multiple antibiotics, and antifungal clinical response with biomarker improvement.[15] Remarkably, we found that 30% of our patients have this serologically distinctive entity, with high infection loads

TABLE III. Baseline clinical data that were significantly different among the 4 latent classes

Clinical characteristic	Pearson χ^2 or ANOVA *F*	*P* value
BMI	$F_{3,126} = 2.72$	.047
Oral corticosteroids	$\chi^2(3) = 13.3$	.04
Pancreatic sufficiency	$\chi^2(3) = 13.5$	.004
Common allergy (≥2 SPTs)	$\chi^2(3) = 23.3$	<.001
Fungal allergy (≥2 sIgEs)	$\chi^2(3) = 62.2$	<.001
Blood eosinophil count	$F_{3,126} = 7.18$	<.001

All nonsignificant comparisons are shown in Table E6. Data were compared by using 1-way ANOVA for continuous data and Pearson's χ^2 test for categorical data.
BMI, Body mass index.

in sputum demonstrated by high DNA levels, GM, and sIgG. Invasive, pseudomembranous, and necrotizing *Aspergillus* tracheobronchitis occur in immunocompromised patients.[23] However, a more indolent form of *Aspergillus* bronchitis has been described both histologically and clinically[23-26] and is characterized by mild inflammation of the mucosa, sometimes with superficial erosions and ulcers but without penetration of hyphae beyond the lamina propria. The major difficultly is that patients present with symptoms indistinguishable from bacterial CF exacerbations: breathlessness, cough, wheeze, hemoptysis, and obstructing mucous. This gives great value to the use of immunologic/sputum markers for diagnosis and possibly treatment monitoring as, although it is partially responsive to antifungal therapy, it often relapses.[15,27]

RT-PCR is more sensitive than standard culture to detect *Aspergillus* in noninduced sputum samples.[18] While treatment-naive patients in classes with ABPA-S and bronchitis consistently have detectable *Aspergillus* DNA, many patients with and without *Aspergillus* sensitization are also RT-PCR positive. This is in keeping with *Aspergillus* colonization rates found in healthy controls and demonstrates the need for additional markers of *Aspergillus* growth and infection because PCR detects live and dead organisms as well as spores.[28] Spores are relatively inert to the immune system because of surface hydrophobins.[29] The use of RT-PCR C_t values partially distinguishes ABPA-S and bronchitis, but SDs are large. RT-PCR therefore may be more useful for monitoring treatment responses through negativization than diagnosis (Table E4). Furthermore, RT-PCR allows species identification and direct assessment of resistance, unlike GM.[28]

GM is a cell-wall component of *Aspergillus* made up of a mannan core and side units containing galactofuranosyl.[30] GM is secreted by *Aspergillus* hyphae in the early logarithmic growth phase and is also a major component of biofilms, but is not released by dormant spores.[31-34] *In vitro* models of the lung show that GM levels correspond to hyphal invasion across endothelium into alveoli.[34] This is the first study to investigate the use of GM in sputum samples from patients with CF. Sputum sonication not only allows reproducible RT-PCR[18] but also liquifies sputum sufficiently to enable GM detection. Sensitivity of GM detection is higher in bronchoalveolar lavage than serum samples.[35] We found homogenized sputum simple to process for GM with good reproducibility. GM clearly differentiated ABPA-S and bronchitis patients from those with positive PCR in class 1 (simple colonization) or sensitization, a distinct diagnostic advantage over RT-PCR. Certain intravenous antibiotics generate false-positive GM results in serum and bronchoalveolar lavage samples, but this was not a problem in this study because no patient was receiving intravenous antibiotics. GM has the advantage of wide international laboratory availability.

sIgG, as measured by the ImmunoCap assay, mirrored GM in its ability to distinguish those with ABPA-S and *Aspergillus* bronchitis. This test is widely available, is relatively cheap, and is reproducible.[36] A study by Barton et al[37] in 2008 suggested a cutoff of 90 mg/L for separating patients with ABPA from those with *A fumigatus* sensitization or controls. However, there was a clear overlap in the distribution of values with outliers in the control group. This study shows that by recognizing *Aspergillus* bronchitis as a clinical entity, a level of more than 75 mg/L can identify patients with ABPA-S or bronchitis with a 90% sensitivty and a 96% specificity. It has not been established whether sIgG falls with treatment, but 12 of the 13 patients in this study taking antifungal treatment long term, with good serum drug levels, had sIgG levels of less than 75 mg/L. The identification of this class of patient poses questions about the role of IgG antibodies in ABPA and bronchitis. In bronchitis, the etiology is infective, and so the high sIgG levels likely represent a T_H1-driven response. The detection of high sIgG levels in ABPA may represent both type 3 hypersensitivity and a T_H1-driven response to infection. It is noteworthy that a positive association between IgG antibodies to *A fumigatus* and worse lung function in CF was observed in 1988 by Forsyth et al[38] who postulated a different disease mechanism to IgE-mediated hypersensitivity.

A key aim of care for patients with CF is to minmize lung function decline. Previous studies linked lung function decline to ABPA, *Aspergillus* sensitization, and possibly *Aspergillus* colonization.[4,5,7,9] While not powered to detect changes in lung function, less decline in FEV_1 over 2 years was seen in patients without evidence of *Aspergillus* infection or hypersensitivity (with or without simple colonization detected by RT-PCR). However, a limitation of this study is that repeated measures of RT-PCR and GM were made in only 30 patients over 9 months and none throughout the 2-year study period. The natural history of *Aspergillus* infection in CF is poorly studied, but patients are likely to switch between classes, and we have demonstrated acquisition of *Aspergillus* (5 of 30 patients) and reduction in signal spontaneously without triazole therapy. Therefore, prospective longitudinal data on class membership and lung function are required. In addition, this study did not attempt to phenotype patients; symptoms, precise exacerbation rates, and radiologic finding were not recorded. Further studies of these individual classes are vital.

Canonical discriminant analysis allowed the formulation of a diagnostic algorithm for future patients. However, this is impractical for most clinics and requires the routine use of GM and RT-PCR tests. This class prediction model may be useful for clinical trials of therapy and genetic studies of aspergillosis in patients with CF.[39,40] It seems likely that the use of sIgG, using a cutoff of 75 mg/L, could become more clinically useful in practice for investigating and monitoring patients clinically suspected to have *Aspergillus* disease.

In summary, this study has allowed the identification of 3 distinct classes of aspergillosis in adult patients with CF using established serologic techniques combined with RT-PCR and GM antigen detection in sputum. Further studies are required using these new markers alongside current markers to monitor class membership and clinical responses to treatment.

We are indebted to our clinical colleagues at the Manchester Adult CF Centre for their support and clinical advice during this study.

Clinical implications: The identification of these 3 distinct immunologic classes of aspergillosis in adult cystic fibrosis—serologic ABPA, sensitization, and *Aspergillus* infection/bronchitis—will facilitate direct studies of phenotypes, genetics, and management approaches.

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RT-PCR REPRODUCIBILITY

Sample processing

It was noted that for 10 patient samples the sediment obtained after homogenization and centrifuge, in preparation for DNA extraction, was too large to be processed via 1 extraction bead beating tube. The sample was therefore split, and the top and bottom phases of the sediment were processed separately. In 7 out of the 10 samples, both aliquots were positive but in 3 only the bottom phase was positive.

Intraassay reproducibility within a homogenized sputum sample (n = 10)

For the same volume of sputum, there were minimal differences in sputum weight after full homogenization with sonication (Wilcoxon signed-rank test $Z = -0.51$, $P = .959$). Positive samples were positive in each aliquot, and negative samples were negative in each aliquot. C_t differences between the split sample ranged from 0.2 to 1.4 (Table E1), but there was no statistical significance between the 2 samples (Wilcoxon signed-rank test $Z = -0.140$, $P = .889$). The overall CV was 1.5%.

Interassay reproducibility within a DNA extraction sample (n = 40)

All 26 negative samples remained negative ($C_t > 38$), and all 14 positive samples remained positive. C_t values varied between the 2 aliquots by less than 1 RT-PCR cycle (Wilcoxon signed-rank test $Z = -1.291$, $P = .20$). The overall CV was 1.1%.

Reproducibility over 9 months

Of the 30 patients who provided 2 sputa within 9 months, 18 remained PCR positive whereas 7 remained RT-PCR negative. Of the patients who remained positive, 15 had rising RT-PCR signal and 3 had a fall in RT-PCR signal (Wilcoxon signed-rank test $Z = -3.41$, $P < .001$) (Table E2). Five patients became PCR positive over the 9 months.

GM REPRODUCIBILITY

Intraassay reproducibility within a sputum sample (n = 20)

Testing of 2 aliquots from the same sputum sample was done for 20 patient samples. Nine samples were positive in both aliquots, and 11 samples were negative in both aliquots. The overall CV was 5%.

Interassay reproducibility within a sputum sample over 1 week (n = 12)

Twelve patient samples were tested each day over 5 days. Half were frozen at −20°C between testing, and half were refrigerated at 4°C between testing. Eight positive samples remained positive over the 5 days regardless of whether they were low or high positives. Four negative samples remained negative. Reproducibility was not affected by storage conditions, and the overall CV for all patient samples was 9% and the CV for control samples was 5%.

Reproducibility over 9 months

The same 30 patient sputum samples used for RT-PCR reproducibility were used for GM reproducibility (Table E2). Fourteen positive samples remained positive over the 9 months, and 11 had a rise in GM index (mean, 3.9) and 3 had a fall in GM index (mean, 4.6) (Wilcoxon signed-rank test $Z = -2.29$, $P = .02$). Nine negative samples remained negative. Five negative samples became positive; of note, the same 5 samples became PCR positive. Finally, 2 positive samples became negative, associated with a falling RT-PCR signal.

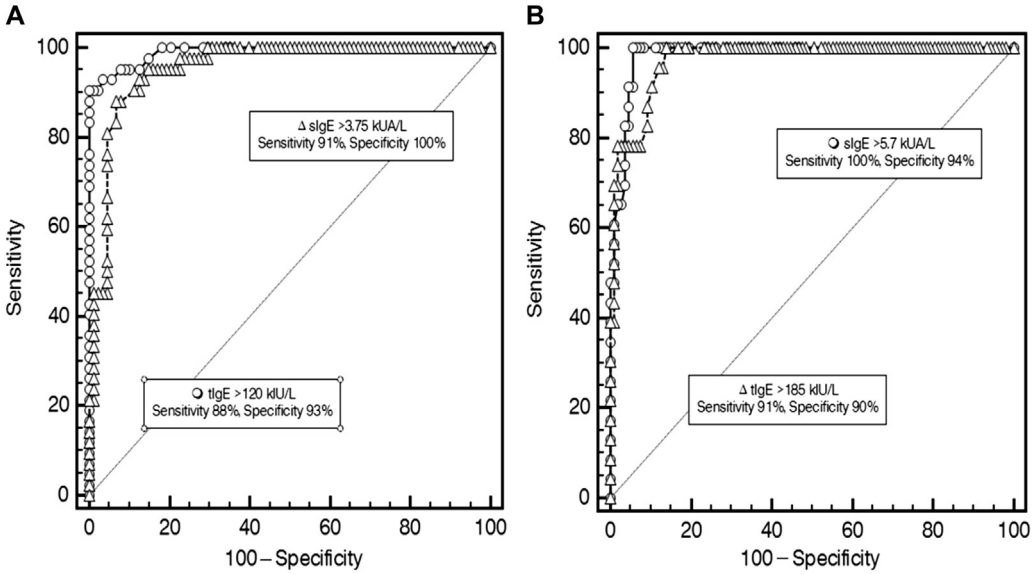

FIG E1. ROC-curve analysis of sIgE and tIgE performance in class separation. **A,** ROC-curve analysis of sIgE (AUC 0.99) and tIgE (AUC 0.96) separating class 2 (ABPA-S) plus class 3 (sensitized) from class 1 (nondisease) plus class 4 (*Aspergillus* bronchitis). **B,** ROC-curve analysis of sIgE (AUC 0.98) and tIgE (AUC 0.97) separating class 2 (ABPA-S) from all other classes. Classes 2 (ABPA-S) and 3 (sensitized) are separated from each other by using tIgE (AUC, 0.91) and sIgE (AUC, 0.90); tIgE > 400 kUI/L 78% sensitivity, 95% specificity; sIgE > 8.5 kUA/L 78% sensitivity, 79% specificity. Applying consensus criteria to class 2 (ABPA-S) shows that a value of >500 kUI/L (minimum diagnostic criteria for ABPA) separates class 2 from all other classes with a sensitivity of 70% and a specificity of 99%, whereas a level of >1000 kUI/L (classic ABPA) gives a sensitivity of 39% and a specificity of 100%. ROC-curve analysis shows that the optimum level is >185 kUI/L, giving 91% sensitivity and 90% specificity (AUC 0.97). *AUC*, Area under the curve; *ROC*, receiver operating characteristic; *tIgE*, total IgE.

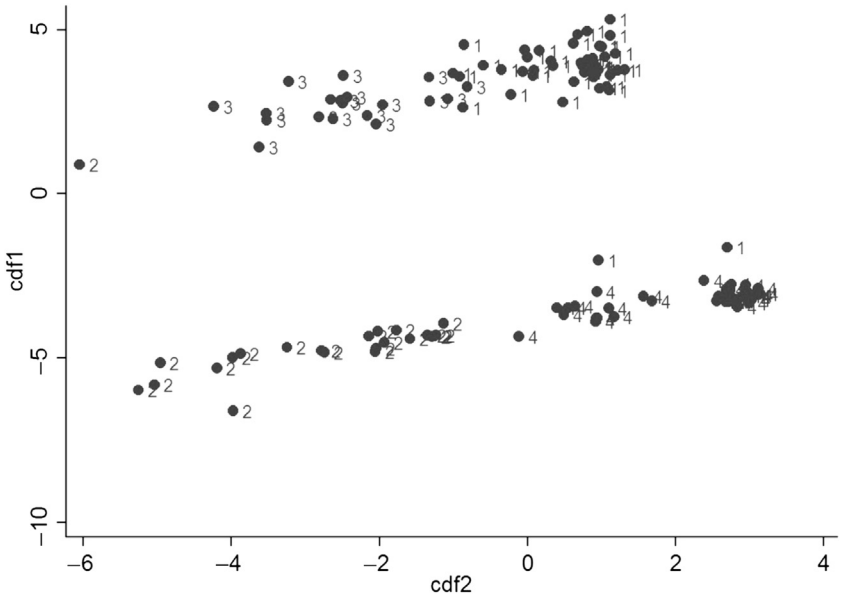

FIG E2. Canonical discriminant functions. cdf1 and 2 are calculated for each patient and then represented by a *dot* on the graph. Each *dot* is labeled by the latent class they were originally assigned to. *cdf1*, Canonical discriminant function 1; *cdf2*, canonical discriminant function 2.

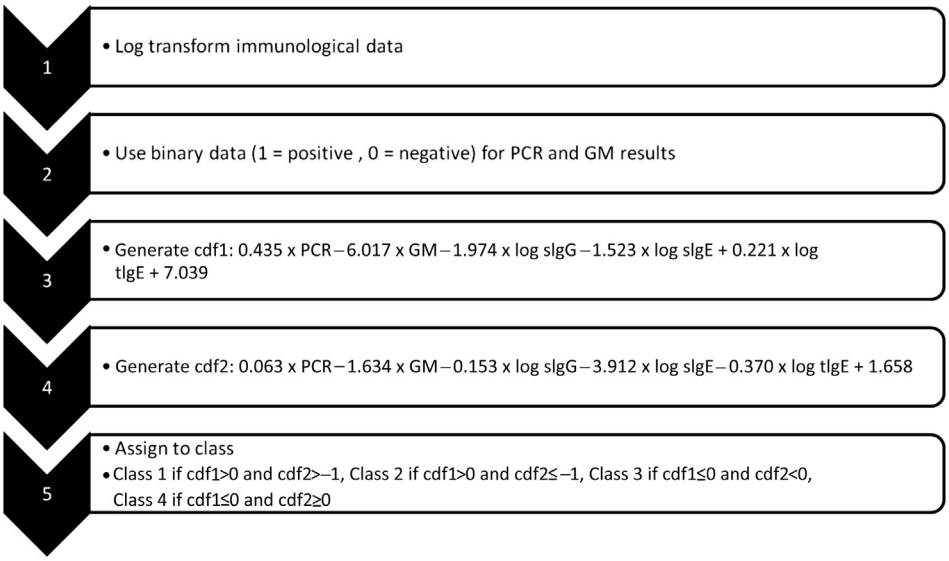

FIG E3. Diagnostic algorithm to predict class membership for future patients. On comparison of this algorithm to the original latent class membership, using the 130 patients not on triazoles, 4 class 1 patients would be misclassified, 2 into class 3 and 2 into class 4. One patient would move from class 2 to class 3, giving an overall estimated error rate of 0.03. The effect of dropping variables from the diagnostic rule, using linear discriminant analysis, is as follows: Drop RT-PCR, introduction of small error rate in class 3 but overall 5 patients still misclassified and error rate increases to 0.039. Drop tIgE, introduction of error in class 3, 6 patients misclassified, and error rate increases to 0.044. Drop sIgG, increased error rate in class 1, 6 patients misclassified, and error rate increases to 0.036. Drop GM, error in all classes, 13 patients misclassified, and error rate increases to 0.091. Drop sIgE, error in all classes, 19 patients misclassified, and error rate increases to 0.14. *tIgE*, Total IgE.

TABLE E1. RT-PCR reproducibility when splitting a sputum sample

Patient	Weight 1st sample (g)	RT-PCR C_t value 1st sample	Weight 2nd sample (g)	RT-PCR C_t value 2nd sample	C_t value difference
1	8.35	33.7	8.25	34.7	1.0
2	7.68	30.1	7.55	29.4	0.7
3	9.10	34.6	9.22	35.5	0.9
4	9.50	37.8	9.51	36.6	1.2
5	8.42	>38	8.35	>38	0
6	9.07	>38	9.08	>38	0
7	9.20	33.6	9.42	34.9	1.3
8	8.87	30.0	8.66	29.6	0.4
9	8.80	37.7	8.81	36.3	1.4
10	7.79	31.8	7.88	32.0	0.2

TABLE E2. RT-PCR and GM results for 30 patients over 9 months

Patient	RT-PCR C_t 1* (38 – C_t)	RT-PCR C_t 2* (38 – C_t)	C_t difference	GM index 1	GM index 2	GM difference
1	0	4.2	−4.2	0.373	0.571	+0.198
2	0	4.1	−4.1	0.283	2.887	+2.604
3	0	2.4	−2.4	0.116	1.260	+1.144
4	0	2.5	−2.5	0.176	0.844	+0.668
5	0	5.5	−5.5	0.163	4.250	+4.087
6	9.0	12.7	−3.7	0.503	5.024	+4.521
7	3.9	10.7	−6.8	1.131	8.734	+7.604
8	8.4	9.6	−1.2	0.769	6.057	+5.288
9	9.1	10.9	−1.8	1.728	5.306	+3.578
10	4.5	8.2	−3.7	3.640	4.064	+0.424
11	4.8	8.8	−4.0	1.330	6.308	+4.978
12	6.9	9.1	−2.2	2.048	4.513	+2.465
13	10.4	11.0	−0.6	0.700	4.980	+4.280
14	3.4	9.7	−6.3	0.522	1.485	+0.963
15	9.6	10.5	−0.9	0.572	8.469	+7.897
16	9.4	9.5	−0.1	1.035	1.446	+0.411
17	5.8	7.4	−1.6	0.088	0.445	+0.357
18	8.4	10.0	−1.6	7.646	2.836	−4.810
19	4.4	8.0	−3.6	4.200	2.433	−1.767
20	2.8	8.9	−6.1	0.026	0.000	−0.026
21	15.6	4.4	+11.2	8.136	1.000	−7.136
22	10.0	1.4	+8.6	0.549	0.242	−0.307
23	10.6	6.9	+3.7	3.275	0.439	−2.836
24	0	0	0	0.145	0.000	−0.145
25	0	0	0	0.067	0.000	−0.067
26	0	0	0	0.309	0.000	−0.309
27	0	0	0	0.175	0.000	−0.175
28	0	0	0	0.039	0.000	−0.039
29	0	0	0	0.387	0.081	−0.306
30	0	0	0	0.009	0.072	+0.063

*The RT-PCR limit of blank is 38. To facilitate comparisons between GM and RT-PCR, the actual C_t value is shown as 38 − C_t, and so higher numbers reflect increasing *Aspergillus* DNA concentrations.

TABLE E3. Performance of ImmunoCap sIgEs compared with SPTs

	Percent in total agreement	Sensitivity of sIgE (%)	Specificity of sIgE (%)	McNemar's χ^2 (*P* value)	Gwet's AC1
Aspergillus fumigatus	90	91	87	1.00	0.86
Alternaria alternata	85	81	87	.21	0.76
Cladosporium herbarum	86	50	92	1.00	0.82
Candida albicans	89	55	97	.15	0.85
Penicillium chrysogenum	79	81	78	.09	0.60

Comparison of the performance of fungal ImmunoCap sIgEs with SPTs. *A fumigatus* ImmunoCap IgEs have the highest sensitivity.

TABLE E4. RT-PCR and GM results for patients on triazole therapy

Patient	Diagnosis	Triazole	Time on triazole	Drug levels	RT-PCR	GM
1	ABPA	Itraconazole	5 y	Therapeutic	NEG	NEG
2	ABPA	Itraconazole	6 y	Therapeutic	NEG	NEG
3	ABPA	Itraconazole	5 y	Therapeutic	NEG	NEG
4	ABPA	Voriconazole	7 mo	Therapeutic	NEG	NEG
5	ABPA	Itraconazole	6 mo	Therapeutic	NEG	NEG
6	ABPA	Itraconazole	6 y	Therapeutic	NEG	NEG
7	ABPA	Itraconazole	5 y	Therapeutic	NEG	NEG
8	ABPA	Itraconazole	3 mo	Therapeutic	NEG	NEG
9	ABPA	Itraconazole	2 y	Therapeutic	NEG	NEG
10	ABPA	Itraconazole	1 y	Therapeutic	POS	NEG
11	ABPA	Itraconazole	2 y	Therapeutic	POS	NEG
12	ABPA	Voriconazole	2 y	Therapeutic	POS	NEG
13	Recurrent thrush	Itraconazole	Intermittent	Subtherapeutic	POS	POS
14	ABPA	Itraconazole	5 y	Subtherapeutic	POS	POS
15	ABPA	Itraconazole	4 y	Subtherapeutic	POS	POS
16	ABPA	Itraconazole	14 mo	Therapeutic	POS	POS

NEG, Negative; *POS*, positive.

TABLE E5. Latent class analysis—summary of goodness-to-fit indices

Number of classes	logL	AIC	BIC
2	−411.913	851.826	893.596
3	−368.369	777.392	837.064
4	−341.347	734.694	812.268
5	−324.788	713.576	809.051
6	−318.075	712.150	825.527
7	−311.495	710.990	842.269
8	−302.992	705.894	855.164

AIC, Akaike's information criterion; *BIC*, Bayesian information criterion; *logL*, the natural logarithm of the likelihood.

TABLE E6. Baseline clinical characteristics with nonsignificant differences among latent classes

Clinical characteristic	Pearson χ^2 or ANOVA *F*	*P* value
Sex	$\chi^2(3) = 0.4$	.95
Age	$F_{3,126} = 1.80$	.15
F508 homozygous	$\chi^2(3) = 2.8$	.42
Inhaled steroid (beclomathasone equivalent dose)	$F_{3,126} = 1.09$	.36
CFRD	$\chi^2(3) = 2.7$	.45
Pseudomonas aeruginosa	$\chi^2(3) = 2.8$	.43
Burkholderia species	$\chi^2(3) = 4.5$	.21
MSSA	$\chi^2(3) = 3.2$	.36
MRSA	$\chi^2(3) = 3.1$	.37
NTB	$\chi^2(3) = 0.6$	.89
Nebulized antibiotic	$\chi^2(3) = 5.4$	.15
Azithromycin	$\chi^2(3) = 3.4$	.34
Dornase alpha	$\chi^2(3) = 8.1$	.07
HTS	$\chi^2(3) = 0.9$	.81
Baseline FEV_1	$F_{3,126} = 0.47$	.70
Baseline FVC	$F_{3,126} = 0.84$	.47

CFRD, Cystic fibrosis–related diabetes; *FVC*, forced vital capacity; *HTS*, hypertonic saline; *MRSA*, methicillin-resistant *Staphylococcus aureus*; *MSSA*, methicillin-sensitive *Staphylococcus aureus*; *NTB*, nontuberculous Mycobacteria.