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SUPPLEMENT ARTICLE

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The myriad challenges of respiratory fungal infection in cystic fibrosis

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Abstract

Fungal infection in cystic fibrosis (CF) is a recognized challenge, with many areas requiring further investigation. Consensus definitions exist for allergic bronchopulmonary aspergillus in CF, but the full scope of clinically relevant non-allergic fungal disease in CF—asymptomatic colonization, transient or chronic infection localized to endobronchial mucus plugs or airway tissue, and invasive disease—is yet to be clearly defined. Recent advances in mycological culture and non-culture identification have expanded the list of both potential pathogens and community commensals in the lower respiratory tract. Here we aim to outline the current understanding of fungal presence in the CF respiratory tract, risk factors for acquiring fungi, host-pathogen interactions that influence the role of fungi from bystander to pathogen, advances in the diagnostic approaches to isolating and identifying fungi in CF respiratory samples, challenges of classifying clinical phenotypes of CF patients with fungi, and current treatment approaches. Development and validation of biomarkers characteristic of different fungal clinical phenotypes, and controlled trials of antifungal agents in well-characterized target populations, remain central challenges to surmount and goals to be achieved.

KEYWORDS
Aspergillus spp., *Candida* spp., fungi, *Scedosporium* spp.

1 | INTRODUCTION

One of the more common dilemmas the clinician caring for the patient with cystic fibrosis faces is the unwelcome report from the microbiology lab informing that the patient's respiratory culture contains a fungus; let us say, a species of *Aspergillus*, *Candida*, *Exophiala*, or *Scedosporium*; or perhaps something one has never dealt with before—what is this *Trichosporon*, this *Rasamsonia*? The clinician pauses and thinks, does that mean something significant? Shall I ignore it, or treat it? If so, what might work best? Should I wait or act?

It is an old dilemma, but it is becoming more frequent. While the existence of allergic bronchopulmonary aspergillosis (ABPA) (and occasionally other allergic bronchopulmonary mycoses) have been recognized in cystic fibrosis (CF) for over half a century, the presence of fungi in the respiratory tract without strong evidence of an allergic response still presents this puzzle of what, if any, deleterious role such organisms might be playing. And in recent years a greater diversity of fungi has been increasingly seen in cultures, and particularly now in studies employing molecular methods to probe the respiratory microbiome.

In this review, we will marshal what incomplete and evolving evidence the literature provides to paint what we hope is a broader and more accurate picture of the scope and nature of the fungal presence in the polymicrobial ecology of the CF airway. We will propose an approach to differentiating harmless fungal colonization from clinically significant and harmful fungal infection, and review the various

treatment options available. The need for cooperative research and clinical trials to place CF mycology on firmer footing is not only apparent, but urgent.

2 | EPIDEMIOLOGY

The understanding of the epidemiology of fungal species in the CF airway is evolving. Allergic disease associated with fungal antigens, including the best characterized such syndrome, ABPA, is beyond the scope of this review. ABPA in CF, present in up to 20% of CF patients, carries significant morbidity, and is considered in detail elsewhere.[1–3] National cystic fibrosis registry data in the USA is limited by a lack of standardization in protocols for collecting and analyzing samples. The most commonly described fungi in registry data are yeasts, specifically *Candida* spp., and the filamentous fungus *Aspergillus*. The annual report for the Cystic Fibrosis Foundation Patient Registry in 2013 noted a median prevalence of 11.9% of patients who were culture positive for *Aspergillus* spp., and 7.8% who were positive for *Candida* spp.[4] The United Kingdom (UK) CF Registry data from 2007 to 2012 was analyzed for fungal isolates, and noted the prevalence of *Aspergillus* spp. increased from 6.5% in 2007 to 13.6% in 2012. Also of note, *Scedosporium* spp. increased slightly from 0.07% to 0.68%.[5]

The range of fungal species described in CF is expanding in concert with improved culture techniques and increased application of molecular diagnostics. Figure 1 outlines the wide spectrum of fungi which have been isolated in CF and these are grouped based on the current clinical impressions of chronicity and pathogenicity.[6] The isolation of *Scedosporium* spp. and *Lomentospora prolificans*, *Exophiala dermatitidis*, *Trichosporon mycotoxinivorans*, and *Pneumocystis jirovecii* is increasingly reported. Identification of *P jirovecii* in CF patients is described throughout the world, with regional differences appreciated. French studies describe a prevalence of *P jirovecii* of 2.5–12.5%, while a cohort of Brazilian CF patients had a prevalence of 38.2%.[7–9]

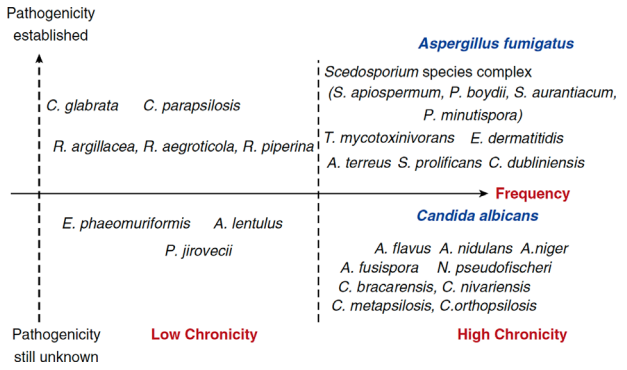

FIGURE 1 Cystic fibrosis fungal biodiversity grouped according to frequency of isolation (*x* axis) and established pathogenicity (*y* axis). The fungi are further divided in terms of chronicity as illustrated. The most frequently isolated filamentous fungi, *Aspergillus fumigatus* and *Scedosporium* species complex, and yeast *Candida albicans* are highlighted. Low-chronicity genera: *A.* = *Aspergillus*; *C.* = *Candida*; *E.* = *Exophiala*; *P.* = *Pneumocystis*; *R.* = *Rasamsonia*. High-chronicity genera: *A.* = *Aspergillus* (*flavus*, *nidulans*, *niger*); *A.* = *Acrophialophora* (*fusispora*); *C.* = *Candida*; *E.* = *Exophiala*; *N.* = *Neosartorya*; *P.* = *Pseudallescheria*; *S.* = *Scedosporium*; *T.* = *Trichosporon*. Reprinted with permission of the American Thoracic Society. Copyright © 2018 American Thoracic Society. Chmiel et al[6]. Annals of the American Thoracic Society is an official journal of the American Thoracic Society

A recent 3-year multicenter study conducted in France, "Muco-Fong," applied a consistent and detailed mycological protocol to CF sputum specimens.[10] In total, 243 CF pediatric and adult patients had sputum specimens that were analyzed using fungal selective media. A total of 81% of patients had at least one fungal species isolated. By far the most prevalent species identified for all age groups were *Candida albicans* (58.8%) and *Aspergillus fumigatus* (35.4%). A large 5-year retrospective study in Germany analyzed over 600 CF patients and over 25 000 throat, sputum, and bronchoalveolar lavage (BAL) specimens for evidence of fungal isolates.[11] *Candida* spp. were the most prevalent fungi isolated, found in 75% of CF patients. *C albicans* (38%) and *Candida dubliniensis* (12%) were the two most common species of yeast identified. *Aspergillus* spp. were isolated in 35% of CF patients, led by *A fumigatus* at 29%. The authors noted no annual or seasonal variations in isolation of *Aspergillus* spp. *E dermatitidis* and *Scedosporium/Lomentospora* spp. were each isolated in 4% of patients over the study period. The first investigation in the United States to apply selective fungal culture media to expectorated sputum from adults with CF carried out 12-month prospective study and noted prevalence rates of *Aspergillus* spp. of 40.8%, *Scedosporium* of 5.2%, *Exophiala* 4.7%, and *Trichosporon* 3.3%.[12] Figure 2 demonstrates that the prevalence of filamentous fungi isolated using fungal selective media is similar across these recent studies.[10–12]

A retrospective analysis of respiratory samples from nine European CF center datasets from 2011 to 2016 supported the epidemiologic findings from these recent studies—despite variability in patient cohorts and diagnostic protocols.[13] Again *C albicans* was the

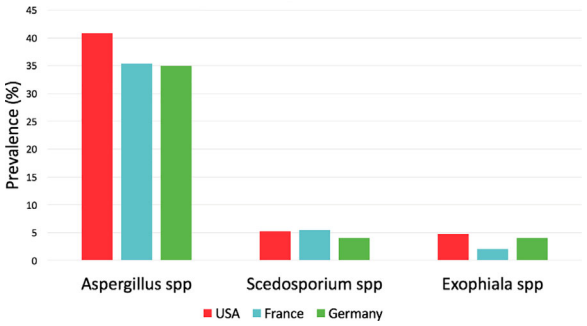

FIGURE 2 Prevalence of the filamentous fungi *Aspergillus*, *Scedosporium*, and *Exophiala* isolated using fungal selective media in CF patients from studies in the USA, France, and Germany (data derived from Refs 10–12)

most prevalent yeast found; the mean number of positive patients detected each year was between 33.8% and 77.9%. The primarily filamentous fungus isolated was *A fumigatus* with rates between 3.9% to 42.4%. *Scedosporium/Lomentospora* spp. were found to have a prevalence rate of 2–5% in the majority of countries studied. *E dermatitidis* prevalence ranged between 0% and 18.3% and *Trichosporon* spp. was reported between 0% and 3.5%.

The prevalence of fungi in the airways of CF patients has traditionally been based on conventional fungal culture isolation and identification techniques, however, the recent application of culture-independent molecular methods has revealed many additional fungal taxa not previously appreciated; for example, analysis of sputum specimens from adult CF patients found that more than 60% of species identified in CF airways were absent in cultures.[14] In a series of recent culture-independent studies, the most commonly identified fungal taxa included: *Aspergillus* spp., *Candida* spp. *Penicillium*, *Malassezi*, with many other rare taxa of unknown clinical significance.[14–16] Figure 3 illustrates the multitude of fungal species identified by molecular methods, and the differences that may constitute a unique lung mycobiome in CF patients.[17]

3 | RISK FACTORS

Existing literature highlights an array of risk factors associated with the identification of fungal species in CF specimens.

A single-center 10-year retrospective cohort study of pediatric and adult CF patients in the United States assessed risk factors for the isolation of fungi on respiratory cultures.[18] The prevalence of filamentous fungi identified was 41.3%, with *A fumigatus* accounting for 36.3%—consistent with other described populations. Decreased lung function was a notable risk factor for identification of filamentous fungi. For every 10% drop in forced expiratory volume in 1 s (FEV_1) below 100% predicted, the odds of identifying filamentous fungi was increased by 5%. This finding is supported by other studies showing the association between *A fumigatus* and lower FEV_1% predicted.[19]

The use of chronic oral antibiotics, and inhaled antibiotics, is associated with the isolation of fungi from sputum samples.[12,20] In particular, chronic azithromycin therapy may be associated with increased risk for *Aspergillus* isolation.[20,21]

Several clinical studies have investigated the relationship between *Pseudomonas* and *Aspergillus* with differing findings. A retrospective cohort study of children with CF examined the relationship between *A fumigatus* and *P aeruginosa* on lung function, and noted an association with lower pulmonary function.[19] In contrast, a retrospective cohort study of children and adults with CF with *A fumigatus* colonization found no significant difference in lung function in patients with concurrent chronic *P aeruginosa*.[22]

The association of inhaled corticosteroids and older age with fungal isolation is not quite as clearly defined—with studies showing both decreased and increased rates of fungal isolation.[18,20,23] Recent work also suggests white race and pancreatic insufficiency are associated with the risk of persistent *Aspergillus* isolation.[20]

The identification of risk factors associated with the recovery of fungi in the CF respiratory tract is useful, though lung function data in particular prompts the question of whether the presence of fungi is a marker, or an etiology, for more severe or progressive disease.

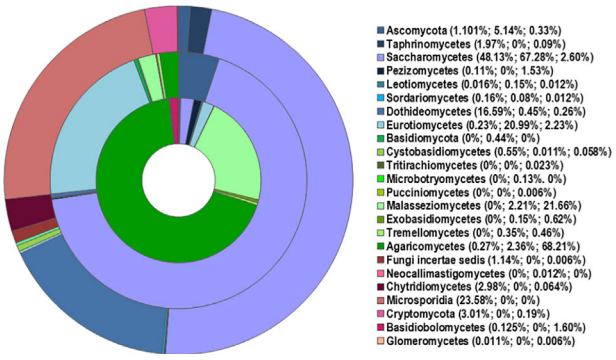

FIGURE 3 Distribution of fungal classes (in % of relative abundance) in the sputum of healthy individuals (outer ring) and patients with CF (middle ring) and asthma (inner ring), based on published pyrosequencing investigations. The percentages on the legend correspond to each class identified in healthy, CF, and asthma populations (from the outer to inner rings, respectively). Reads that were not identified as class level are group at phylum levels (*Ascomycota*, *Basidiomycota*). Classes less than 0.1% are not represented in the rings; the class named "Fungi incertae sedis" refers to unclassified fungi. Source: Nguyen et al[17]

4 | HOST-PATHOGEN INTERACTIONS

Complex host-pathogen interactions exist between innate and adaptive immune defenses of the CF lung and fungal species. Ongoing research is beginning to elucidate the many components that the influence these intricate and dynamic relationships.[24,25]

Of the fungal taxa in the CF airway, the pathogenicity and virulence factors of *A fumigatus* are the best described. In an excellent review Kwon-Chung and Sugui[26] explain why this species is one of the most commonly isolated. *A fumigatus* thrives at body temperature, but it can also tolerate wide variations in temperature, and is equipped with secondary metabolites and efflux pumps which act as adaptive defense mechanisms. In large part due to their hydrophobicity, *A fumigatus* conidia are particularly easy to disperse. Once dispersed, the small size of the conidia (2 to 3 μm) allow them to easily reach the lower respiratory tract. *A fumigatus* has several mechanisms which allow environmental adaptation and evasion in the immune compromised host. For example, both *Aspergillus* and *Scedosporium* spp. have antioxidant mechanisms to defend from reactive oxygen and nitrogen species produced by phagocytic cells.[27]

CF airways may be particularly susceptible to the survival of inhaled fungal spores. Cystic fibrosis transmembrane conductance regulator (CFTR) dysfunction leads to reduced chloride and bicarbonate secretion which alters airway mucus properties, and leads to a decrease in the pH of airway-surface liquid, which in turn hinders endogenous antimicrobial activity. These properties lead to ineffective

mucociliary transport, and provide an environment for a cycle of inflammation and infection.[28] Furthermore, research suggests that CFTR is a key component in directing epithelial cellular response to *A fumigatus*.[29] In the presence of a CFTR mutation, *A fumigatus* may lead to an increased inflammatory response.

Investigation of the interactions between pathogenic bacteria and fungi in the CF airways are a current topic of intense interest, particularly with respect to the relationship between *Pseudomonas* and *Aspergillus*. In vitro work suggests competitive inhibition of *A fumigatus* biofilm formation by small molecules produced by *P aeruginosa*.[30] However, the degree of inhibition seen in vitro appears to be dependent on the source, phenotype, and growth conditions of the *P aeruginosa*.[31,32] *P aeruginosa* interactions with fungi are not limited to *Aspergillus*, with studies also showing inhibition of *Scedosporium* and *Lomentospora* spp., and inhibition of biofilm development with *Candida* spp.[33,34] This work prompts consideration for further investigation into understanding how antibacterial therapies alter the microbiological milieu, and may provide opportunity for fungal colonization and infection.[35]

Studies examining the immune response of CF patients to fungi offer further insight into understanding the wide array of clinical phenotypes observed. The cytokine interleukin (IL)-10 appears to play a role in infection and inflammation in the CF lung. Genetic polymorphisms that alter the production of IL-10 may be related to colonization of *A fumigatus*.[36] Researchers have used fungal-specific serum antibodies to differentiate fungal sensitization from infection.[37] T cell-based diagnostic techniques offer promise in more clearly teasing apart the roles of fungi in CF patients. Further analysis of pro-inflammatory fungal T cell responses may help classify if immune responses are protective from infection or pathogenic to the host.[38] In this vein, recent work points to the induction of regulatory T-cells with a pro-inflammatory Th17-like phenotype in human PBMCs exposed to *A fumigatus*—a response that can shift the balance from a protective to deleterious effect. Toll-like receptor 2 (TLR2) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4) appear to regulate the induction of T-cells, suggesting they may offer a therapeutic target.[39] Interestingly, blood basophil priming and activation has been shown in allergic but not infective phenotypes of aspergillosis in CF.[40,41]

Hope exists for the ongoing development and validation of biomarkers that can guide and monitor therapeutic interventions based on this steadily increasing understanding of fungal virulence and host-pathogen interactions.

5 | DIAGNOSTIC METHODS

The first step in evaluating fungi in the CF airway is a consistent and accurate approach to evaluating respiratory samples. Prior to ascribing differences in geography or patient population as risk factors for isolating fungi, first, analysis of the laboratory methods must be considered. The variability in detection of fungi is highlighted by a retrospective study which noted different prevalence rates for fungal isolation from the same cohort of patients in UK.[42] Patients from the same CF cohort had specimens processed at two centers with different mycological methods throughout the study period, leading to the understanding that laboratory methods rather than geographical or demographic features were driving the difference in prevalence.

Culture-based detection methods are the primary approach for isolating fungi. Guidelines for processing respiratory samples are not standardized, despite the importance of controlling for numerous variables that can affect successful isolation. These factors include the source of the specimen (OP swab, sputum, BAL), volume of specimen, preparation of specimen, choice of culture media, and temperature and duration of incubation.[43]

The site of sampling may play an important role in the rate of isolation of fungi. A retrospective cohort analysis of pediatric CF respiratory samples compared the prevalence of *A fumigatus* in cough swabs, sputum, and BAL specimens.[44] BAL samples detected *A fumigatus* in 29% of specimens, compared to 14% of sputum specimens, and 0.8% of cough swabs. The optimal method for sampling the airway for microbiological studies continues to be refined, with consideration for the risks and benefits of more invasive techniques, and the increasing understanding of the spatial variation in upper and lower airway microbiota.[45]

The volume of respiratory samples used for culture is noted to be a possible confounder in comparing prevalence rates across centers.[42] There is evidence to suggest isolation of *Aspergillus* is more successful with higher volume of specimen plated.[46]

The properties of CF sputa pose unique challenges for diagnostic methods. The highly viscous quality of the sputum has led to the recommendation for the homogenization of specimens. A study on CF patients in Austria compared the results of mycological cultures following chemical homogenization of CF sputum to native, unprocessed sputum, and found homogenization increased sensitivity of culture detection from 72.4% to 96.2%.[47] Other work has suggested the addition of sonication to chemical homogenization of CF sputum to improve the sensitivity of *Aspergillus* PCR testing when compared to traditional culture.[48]

The importance of fungal specific media for culture-based detection has long been described.[49] Recent work highlights the differences in prevalence rates noted by the choices of culture media. A study comparing three laboratory methods (non-selective conventional culture, mycological culture with fungal-selective culture media supplemented with antibiotics, and direct DNA extraction from sputum samples) in 77 adults with CF in Northern Ireland revealed that conventional microbiological culture media can fail to identify fungi in the context of bacterial overgrowth.[50] In this study conventional bacterial culture isolated fungi in 18% of patients. Specific mycological culture fared much better isolating fungi in 78% of patient samples. DNA extraction detected fungi in 100% of patients examined. The authors of the study postulated the low rate of recovery using standard culture was secondary to the overgrowth of gram-negative organisms and the relatively short length of incubation.

These findings were supported by a recent CF cohort study in the United States which compared selective fungal media to traditional bacterial culture for the identification of *Aspergillus*, *Scedosporium*,

Trichosporon, and *Exophiala* species in adults with CF.[12] Of the 184 samples identified with one or more of these fungal species, bacterial culture detected fungal species in only 26% of these samples, with three other fungal selective media having much higher recovery rates, from 63% to 65.8%.

The recent multicenter MucoFong project in France applied a standardized mycological protocol to sputum specimens.[10] All samples were pretreated with a mucolytic, examined and plated on six semi-selective culture media, in context of the previous observations noting that the presence of antibiotics in media can play an important role. All samples were incubated for 3 weeks and checked twice weekly.

While a shorter period of incubation may be sufficient for some fungal species, emerging organisms may require much longer culture duration; ultimately the authors of the MucoFong study recommended an incubation time of 16 days.[10] However, other authors have concluded 7 days may be long enough.[12]

The frequency of sampling is an additional consideration. Two mycology-focused studies conducted with CF patients in France described prevalence rates of *A fumigatus* and *S apiospermum* observed. In one study, a single sputum specimen was collected, and in the other, a longitudinal design was employed with repeat sampling. In the longitudinal study, the recovery of both fungal species more than doubled.[51]

In addition to culture-based methods, molecular approaches are increasingly being applied and excellent reviews of molecular methods exist elsewhere.[43] Multiple polymerase chain reaction (PCR)-based techniques exist, with regional differences in implementation. PCR techniques rely upon use of internal transcribed spacer ribosomal sequences common to all fungi, followed generally by secondary analysis for species specific identification. Real-time PCR (RT-PCR) methods for single fungal species identification may increase sensitivity for detection of fungi compared to culture alone.[37] Difficulties exist in choosing specific PCR targets, and differentiating between polymicrobial infections. Next-generation sequencing approaches hold promise in better describing fungal community members and their interactions. Lastly, genotyping of fungal strains can be applied to describe transmission patterns between patients and from environmental etiologies, as well as elucidating longitudinal patterns of colonization within patients.[52] Figure 4 outlines techniques for fungal identification across both culture- and molecular-based methods.[43]

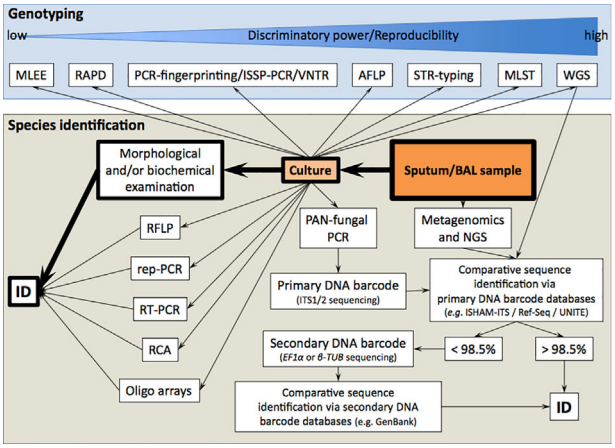

FIGURE 4 Suggested culture- and molecular-based methods for fungal identification and genotyping in a cystic fibrosis setting. This pragmatic approach highlights the yet still central position of culture methods but also indicates alternative molecular techniques, as well as the range of genotyping methods and their discriminatory power and degree of reproducibility. Reprinted by permission from Springer Customer Service Centre GmbH: Springer Nature, Mycopathologia. Chen et al.[43] © Springer Science 2018

6 | CLINICAL DIAGNOSIS

Once fungi are isolated from the lung, the next question that follows is whether these microbes are contaminants, transiently recovered, colonizers, or pathogens. The clinical outcomes related to presence of bacteria in the CF lung may be either protective or deleterious, and this understanding has influenced thinking about the isolation of fungi.[53] Definitions for *Aspergillus* colonization and infection have been proposed, though there remain no standardized definitions.

The persistence of *Aspergillus* spp. in the lung is of fundamental importance. Influenced by the Leeds criteria for characterizing chronic *P aeruginosa*, authors have suggested 50% or more of sputum samples over 6–12 months, or similarly, two or more samples in 1 year, are the minimum frequency of isolation acceptable to make either diagnosis.[21,22,35,54,55] The more challenging question is differentiating colonization versus infection.

Several studies have described CF patients with *Aspergillus* colonization, attempting to answer the question of whether a pattern of colonization caries morbidity, and leads to worsening pulmonary function, or in effect, infection. The body of work describing the association between pulmonary function and *Aspergillus* colonization is inconsistent, with several studies revealing no difference in pulmonary function, while others noting a decline in pulmonary function.[19,21,22,56] The literature on *Candida* spp. parallels these variable outcomes. *C albicans*, the most common species isolated, is generally thought of as non-pathogenic. This being said, when identified as a colonizer of the CF airway *C albicans* is associated with increased rates of decline in FEV_1 in some studies; while an association with preserved lung function was found in another study.[53,57,58]

Two recent clinical studies of patients with *A fumigatus* colonization illustrate the use of pulmonary function and chest imaging to address this conundrum.

A case-control study of CF patients in Greece compared 20 patients with *A fumigatus* chronic colonization versus 60 control patients, and analyzed the difference in FEV_1 between the two groups across two different periods of time.[56] First, the authors were able to look backwards and compare lung function between case and control in the 4 years prior to the onset of colonization. They found a statistically different lower baseline FEV_1 for the patients who would go on to be colonized with *A fumigatus*, suggesting lower lung function

is a risk factor for colonization. Then, looking forward, they described the 7-year period after colonization when the colonized group had significantly lower lung function—leading to the suggestion that colonization with *A fumigatus* is also associated with a more rapid decline in lung function.

A prospective study compared 16 CF patients colonized with *Aspergillus* spp. with 16 matched non-*Aspergillus* CF patients, with respect to pulmonary function and high resolution computed tomography (HRCT).[59] The authors noted no differences in pulmonary function, however, they did find more severe mosaicism suggestive of air trapping, leading them to posit that *Aspergillus* infection may lead to subclinical lung parenchymal injury, and thus may suggest consideration for eradication therapy.

Taken together it appears colonization may exist without evidence of lung function decline, however, in other contexts colonization can progress to a clinical decline which is consistent with infection.

A pathologic distinction between colonization and infection in CF fungal lung disease has been described. A review of autopsies of 63 CF patients from 1982 to 1987 in the United States (Ohio) identified fungi in 14 patients.[60] The fungal species isolated included: *Aspergillus* spp., *Candida* spp., and *Histoplasma capsulatum*. These 14 patients were divided into three groups: five with fungal colonization; five with localized infection; four with disseminated fungal disease. The histologic description of the colonization group included hyphae within alveolar macrophages; and fungi in the airway or in alveolar exudates. Fungal infection was described as fungi associated with mucosal inflammation, either in a focal distribution, consistent with bronchitis or bronchiolitis, or more widely distributed, consistent with invasive disease. The authors noted the difficulty of translating these histologic findings to clinical care, given that pathologic diagnosis is limited by the morbidity of open lung biopsy, and that transbronchial biopsy may miss localized areas of inflammation and infection. Ultimately, the authors recommended clinical findings as the diagnostic criteria for infection.

Aspergillus infection in CF is most frequently described as *Aspergillus* bronchitis. The diagnosis of *Aspergillus* bronchitis was initially suggested based on the clinical observations in six CF patients from both Israel and the UK. The patients shared the following four features: *A fumigatus* positive respiratory cultures, respiratory exacerbations that did not respond to antibiotics, the absence of ABPA, and improvement in symptoms and pulmonary function with antifungal therapy.[61]

A 2-year prospective cohort study of adults with CF in the UK set out to further characterize and classify the spectrum of aspergillosis in CF using several biomarkers.[37] The authors combined real-time quantitative PCR (RT-PCR) for *Aspergillus* DNA and galactomannan (GM) from sputum with serologic testing, which included total IgE (tIGE), specific *A fumigatus* IgE (sIGE) and specific *A fumigatus* IgG (sIgG). Based on these diagnostic tests, they divided patients into four groups: non-diseased, ABPA, *Aspergillus* sensitized, and *Aspergillus* bronchitis. Of the 130 patients who were triazole-naïve, 30% were described as having *Aspergillus* bronchitis. Patients with *Aspergillus* bronchitis had elevated levels for *Aspergillus* DNA, GM, and sIgG; and absent or low values for tIgE and sIgE. Thus, the addition of RT-PCR, GM, and sIgG to the initial diagnostic criteria for *Aspergillus* bronchitis may allow better discrimination between *Aspergillus* colonization and infection. That being said, in current clinical practice sputum processing challenges limit the application of GM and RT-PCR testing, leaving sIgG as the most promising candidate for multicenter evaluation.

A recent single center prospective study in Germany combined the clinical, microbiologic, and immunologic criteria for *Aspergillus* bronchitis noted in the two studies above, and applied this to a cohort of 22 CF patients.[62] Two patients (9%) met criteria for *Aspergillus* bronchitis based on these definitions. Of note, neither patient had new pulmonary infiltrates on chest imaging. The authors note that the absence of longitudinal data leaves many questions about how patients may transit between different classifications over time.

In addition to bronchitis, invasive fungal disease remains a diagnostic and therapeutic challenge in CF. A recent excellent review noted that, in the absence of consensus criteria, empiric criteria to define a "highly probable" invasive pulmonary fungal infection include:

1. Increased sputum production.
2. Multiple isolation of the same fungal species from sputum or BAL (at least two culture-positive samples in 6 months).
3. Pulmonary infiltrate(s) on chest CT scan or X-ray.
4. Treatment failure with antibiotic therapy (two and more antibiotic treatments, duration 2 or more weeks).
5. Unexplained lung function decline.
6. Exclusion of new/other bacteria.
7. Exclusion of allergic ABPA.[63]

Overall the risk for invasive fungal disease in CF is low, however, the CF lung transplant population is particularly vulnerable. The identification of risk factors for invasive pulmonary aspergillosis (IPA) in CF lung transplant recipients is essential. In a single-center retrospective study of 93 CF lung transplant patients in Canada, pre-transplant *Aspergillus* colonization was common, with 70% of patients effected.[64] Intraoperative *Aspergillus* culture was positive in 39% of patients, and 22% of patients went on to develop IPA post-transplant. Intraoperative isolation of *Aspergillus* was associated to a fourfold risk of IPA, pointing to the importance of microbiologic testing and a high level of suspicion in this high-risk group.

A marrying of microbiologic, immunologic, and clinical diagnostic criteria across the spectrum of CF fungal lung disease remains much needed. In Table 1, we propose criteria to consider when making this challenging differential diagnosis.[35,60,63]

7 | TREATMENT

The limitations of the definition of fungal infection in CF both complicates therapeutic decision making and hinders clinical trials for antifungal therapies. The majority of the literature on the use of antifungals describes non-CF, usually immunocompromised

TABLE 1 Proposed diagnostic criteria for fungal colonization and infection in CF lung disease

Criteria	Colonization	Infection
Major criteria		
Microbiology	Minimum of 2 cultures within 12 months and ≥50% in 1 year	
Clinical symptoms	None	New signs and symptoms and/or worsening pulmonary function
Exclude fungal allergy	No evidence of fungal sensitization or ABPA	
New bacterial pathogens	May be present	May be present as co-morbidity
Chest imaging	No change	New infiltrate or airway thickening
Response to antibiotics	Clinical improvement	None
Response to antifungal therapy	None	Clinical improvement
Minor criteria		
Serology (fungal specific IgG)	Ab negative or detected in normal range	Ab elevated
Galactomannan (sputum or BAL)	Negative	May be positive
Bronchoscopy BAL Endobronchial biopsy Brush biopsy Mucosal biopsy Transbronchial biopsy	Fungi within alveolar macrophages or airway/alveolar exudates	Fungi within alveolar macrophages or airway/alveolar exudates Mucosal erythema and/or ulceration Invasion of mucosa by fungal hyphae with acute inflammation

Using published literature (Refs 35, 60, 63), we have crafted a table of diagnostic considerations for differentiating fungal colonization from infection. The term "infection" is used to describe a clinical spectrum from fungal-associated bronchitis to invasive fungal disease.

populations, making it challenging to extrapolate this work. The most commonly used classes of antimycotics in CF lung disease are azoles, polyenes, and echinocandins. These medications are used most often as monotherapies, though they can also be used in combination across several classes.

Azoles are the most studied class antifungals in CF. Triazoles, including itraconazole voriconazole, and posaconazole, are used as therapeutic agents across the spectrum of CF fungal disease, from colonization to invasive fungal infection. To date, no studies using recently approved isavuconazole in CF patients have been published.

With regard to *Aspergillus* colonization, two small studies have described outcomes following the use of itraconazole, with some divergent findings.

A randomized placebo-controlled clinical trial investing whether the use of itraconazole for CF patients chronically colonized with *A fumigatus* would improve clinical outcomes, was undertaken across nine outpatient Canadian centers.[65] Thirty-five patients where included in the study, and received 24 weeks of itraconazole (n = 18) or placebo (n = 17). No effect was observed in the treatment group for the primary outcome, rate of pulmonary exacerbation needing IV antibiotics, or for secondary outcomes including time to first exacerbation, change in lung function from baseline, and quality of life. Of note, this study was limited by the fact that drug levels were obtained in 78% of patients, and 43% of this group had subtherapeutic itraconazole levels. More importantly, this multicenter study was grossly underpowered (target >300 patients based on power calculation) due to difficulty in enrollment.

A study in Ireland assessed the in vitro and in vivo effects of itraconazole therapy for 6 weeks on *Aspergillus*-induced pulmonary inflammation in 13 CF patients colonized with *A fumigatus*, and without ABPA.[66] In vitro itraconazole therapy improved epithelial vitamin D receptor expression while decreasing the fungal metabolite gliotoxin and decreased Th2 cytokines IL-5 and IL-13, which were presumed responsible for inflammation. In vivo itraconazole treatment reduced *A fumigatus* colonization, improved baseline mosaic patterns noted on HRCT, improved respiratory symptoms, decreased exacerbations, and stabilized pulmonary function over a year follow-up.

Taken together, the differences in results in these two studies illustrate the challenges of comparing different outcome measures, and classifying patient populations. Treatment of a patient colonized with a fungus may be expected to have less efficacy than treatment of a patient with fungal infection. Open label treatment of patients diagnosed with *Aspergillus* bronchitis using itraconazole has been described as beneficial, though these are small studies, with a variable duration of therapy (from 2 weeks to more than 2 years) and limited clinical outcome measures.[61,62]

Voriconazole is the first-line therapy recommended for treatment for IPA across all populations.[67] A recent consensus recommendation for the treatment of IPA in CF supported the use of voriconazole as first line therapy, with posaconazole also noted as a consideration.[63] Furthermore, in CF lung transplant patients with *Aspergillus* colonization the preemptive use of voriconazole therapy for 3 months was linked to a reduction in IPA.[68] Triazoles are also recommended in management of *Scedosporium* spp. and *L prolificans*, *E dermatitidis* and *T mycotoxinivorans* infections.[63,69–71]

Triazole therapies are preferable in that they are the only oral medications to treat fungi in CF. However, this class is limited by pharmacokinetic challenges which may contribute to toxicity and azole resistance.[72] Similar to other antimicrobial agents, obtaining therapeutic drug levels is of particular importance for CF patients receiving triazoles, with both subtherapeutic and supratherapeutic levels commonly described.[72,73]

The hepatic metabolism of triazoles through the cytochrome P450 pathway can lead to significant drug interactions. Inducers of hepatic enzymes such as rifampin can decrease levels of itraconazole and voriconazole. Azole inhibition of hepatic enzymes can increase levels of medications including tacrolimus and corticosteroids. Interactions of triazoles with CFTR modulators is of particular interest to the CF community.[74] CYP3A4-mediated ivacaftor and tezacaftor/ivacaftor metabolism is inhibited by itraconazole and voriconazole, leading to increase ivacaftor and tezacaftor exposure, and a recommendation to decrease dosages.[75,76] In combination therapy with lumacaftor/ivacaftor a dose reduction is also recommended when starting or resuming therapy in a patient receiving triazoles. In this case dose adjustment is temporary, as lumacaftor induces CYP3A4 which may counteract the inhibition of triazoles at steady-state while also decreasing triazole levels.[74,77]

While azoles are generally well tolerated, important adverse effects exist. Hepatotoxicity is the primary side effect associated with all azoles. Voriconazole is also notable for reports of vision changes, neurologic toxicity, and photosensitivity.[78] Phototoxicity is of particular concern in CF patients.[79] In the lung transplant population, voriconazole is associated with an increased risk for cutaneous squamous cell carcinoma.[80] Posaconazole may have a preferable side effect profile when compared to voriconazole, particularly with respect to photosensitivity.[81]

Azole resistance in fungi is of increasing concern. Two large single center studies in Denmark and France revealed similar rates of reduced *A fumigatus* susceptibility to itraconazole of 4.5% (6/133) and 4.6% (6/131), respectively.[82,83] Reduced susceptibilities were strongly associated with previous itraconazole therapy. In addition, there is increasing consideration for the development of azole resistance secondary to the use of fungicides in agriculture and environmental exposures.[84] Authors of the French study went on to note that isolates with reduced susceptibilities to itraconazole also had reduced susceptibility to posaconazole, and half of the isolates had reduced susceptibility to voriconazole.[82]

A prospective study of CF patients in Germany, which analyzed resistance patterns of *Scedosporium* spp. and *L prolificans*, noted great variability in minimum inhibitory concentrations, though overall high levels of resistance, particularly from *L prolificans*.[70] Voriconazole had the greatest in vitro activity of the three triazoles tested. This work suggests the importance of assessing local resistance patterns, particularly if a patient is failing to show treatment response.

The application of the polyene antimycotic amphotericin B, in both aerosolized and intravenous forms, is described in CF. Aerosolized amphotericin B is commonly used as prophylaxis in CF lung transplant patients.[68,85] There is ongoing interest in applying this nebulized antifungal in the treatment of CF patients with ABPA, and other emerging fungal species, though this literature is limited.[86,87] Intravenous liposomal amphotericin B is reserved for the treatment of severe invasive fungal disease, with literature again largely limited to case studies.[87] For azole-resistant *A fumigatus*, liposomal amphotericin B is a consideration.[63] Use of amphotericin B is limited in CF in large part secondary to the noted adverse effects which include nephrotoxicity, acute infusion-related reactions, and electrolyte abnormalities. While many *Aspergillus* spp. are generally susceptible to amphotericin B, several fungal species have high levels of resistance, most notably *Scedosporium* spp. and *T mycotoxinivorans*.[70,71]

Echinocandins (caspofungin, anidulafungin, and micafungin) are commonly used to treat invasive candidiasis in non-CF patients, while in CF they are increasingly described in use as combination therapy for the management of invasive fungal disease.[87] This class is generally well tolerated with similar rates of hepatotoxicity and infusion reactions across the three drugs. In cases of azole-resistance authors have recommended combination therapy with an echinocandin in addition to triazoles.[87]

Synergy between classes of antimycotics when used in combination to treat fungal species is described, supporting clinical observations. Organisms with multidrug resistance patterns like *L prolificans* have shown lower MICs with double therapy with amphotericin B and anidulafungin, and triple therapy with the addition of voriconazole.[88] Combination therapy may also be necessary in cases of multiple simultaneous invasive fungal infections.[89]

Lastly, the impact of CFTR modulators on fungi in the CF airway may hold promise. The G551D Observational (GOAL) Study followed changes is prevalence of CF airway microbes in patients before and after treatment with ivacaftor.[90] The study found that ivacaftor was associated with a reduction of culture positivity of *P aeruginosa* and *Aspergillus* spp. in the months following initiation of ivacaftor. Longer follow-up studies are in progress.

8 | CONCLUSIONS

With use of proper mycological culture protocols, it is now clear that fungi are present in the CF respiratory tract of most patients. A substantial minority of patients, perhaps 20%, will be adversely affected by true infection and may benefit from antifungal therapy. The poor state of current understanding mandates a large research agenda. There is a pressing need to standardize mycology culture protocols in much the same way that the CF community has done in monitoring our patients for pathogenic bacteria. Similarly, the risks and benefits of fungal eradication protocols should be considered for evaluation in clinical trials. As the data generated from molecular detection and identification methods accumulates, we need to integrate this complex information into a broader strategic approach to fungal disease in CF. We need to more clearly understand the impact of CFTR modulator therapy upon lung microbiology including impact upon fungal colonization and infection. We need to better unravel the inter-kingdom interactions occurring in the airways of CF patients

harboring fungi along with bacteria and viruses, so that unintended consequences of well-meaning interventions do not unpleasantly surprise us. We need to conduct well-designed observational studies to refine current biomarkers and discover new ones that can aid understanding of fungal pathogenicity and monitor patient clinical status. Validation and standardization of biomarkers will aid efforts to properly allow characterization of patients into categories of allergic and infective phenotypes, which themselves may be dynamic and change over time. And not least, controlled multicenter clinical trials are needed to work out the clinical utility of antifungal therapy in appropriately defined populations of patients likeliest to benefit from targeted antimicrobial therapy.

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