FUNGAL RESPIRATORY INFECTIONS IN CYSTIC FIBROSIS

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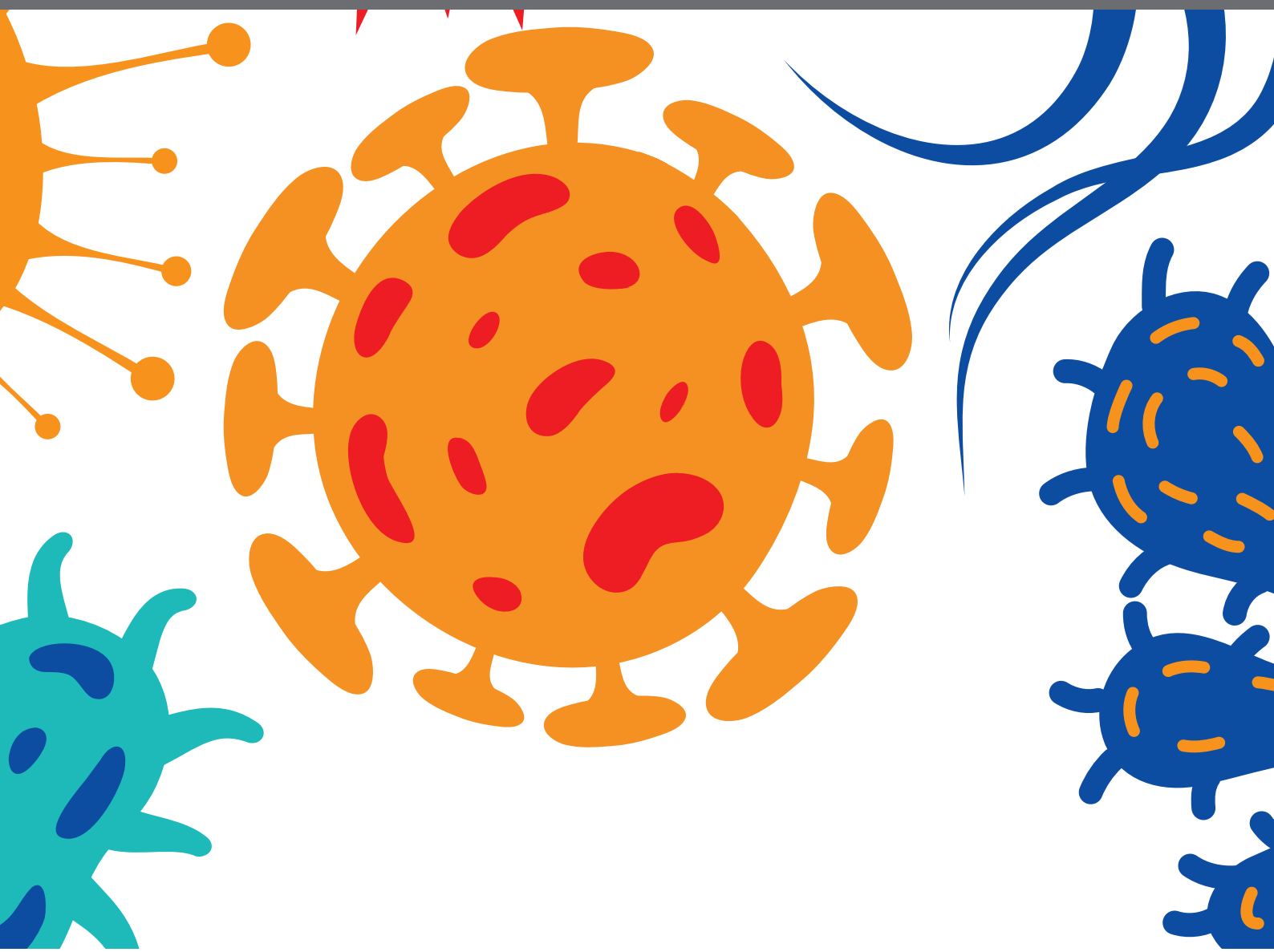

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FUNGAL RESPIRATORY INFECTIONS IN CYSTIC FIBROSIS

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EDITORIAL
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Editorial: Fungal Respiratory Infections in Cystic Fibrosis

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Editorial on the Research Topic

Fungal Respiratory Infections in Cystic Fibrosis

Cystic fibrosis (CF), which predominantly affects Caucasian populations (Europe, Australia, North of America), is an inherited autosomal recessive genetic disease caused by mutations in the *CFTR* (Cystic Fibrosis Transmembrane conductance Regulator) gene that encodes a chloride channel located at the apical surface of numerous epithelial cell types. Many organs are therefore affected by this disease, but the outcome essentially depends on the extent of pulmonary involvement and damage. Indeed, mutations in the *CFTR* gene result, in the respiratory tract, in the thickening of the bronchial mucus and impairment of mucociliary clearance that together allow the entrapment of inhaled bacteria and desiccated yeast cells and filamentous fungal spores and provide a suitable environment for their proliferation. Thus the respiratory tract of CF patients often becomes colonized by various microorganisms, causing recurrent pulmonary exacerbations, which lead to a persistent inflammatory reaction and progressive deterioration of lung function. Bacteria, notably *Pseudomonas aeruginosa*, are the major cause of these infections. As a consequence, special attention has been paid to the prevention and treatment of bacterial infections. Progress in this field, including advances in the diagnosis of the disease as well as in the nutritional status of the patients, have progressively led to a significant increase in life expectancy. However, in addition to bacteria, many fungal species can also colonize the CF lung, potentially leading to respiratory infections, which increase in frequency in parallel with the increase in life expectancy. Although still controversial, there is now accumulating evidence that chronic colonization of the airways by fungi contributes to clinical or functional pulmonary deterioration. Even more problematic, in patients undergoing lung or heart/lung transplantation, this fungal colonization of the airways may lead to severe and often fatal disseminated infections because of the ability of these thermotolerant fungi to rapidly disseminate in a susceptible host and, for some of these pathogens, because of their low innate susceptibility or frank resistance to current antifungal agents.

Among the prominent fungal species colonizing the CF airways, *Candida albicans* for yeasts and *Aspergillus fumigatus* for filamentous fungi predominate. However, other fungi are increasingly

reported in this context, including *Scedosporium* species, *Aspergillus terreus, Exophiala dermatitidis*, species in the *Rasamsonia argillacea* complex, and *Lomentospora prolificans*. In addition, pioneering studies of the microbiota have suggested an even greater diversity of the fungal flora colonizing the CF airways. Unfortunately, unlike bacterial infections, our knowledge about the epidemiology and pathogenesis of fungal respiratory infections in CF remains scarce and sustained research on this topic is needed. Therefore, we are delighted to introduce the Research *Topic "Fungal Respiratory Infections in Cystic Fibrosis"* in Frontiers in Cellular and Infection Microbiology to highlight some tremendous advances in this field.

For this Research Topic, we have collected twelve articles, including eleven original research studies and one review.

First, Bonnet et al. provided an excellent compilation of recent data about *Pneumocystis jirovecii* in CF. This review clearly demonstrates that knowledge in the field is still fragmented, and this topic remains an open area of investigation. Of importance, a multicenter prospective study using standardized methods for *P. jirovecii* screening in CF patients including lung transplant recipients, and MLST/NGS analysis of the *P. jirovecii* genome combined with patient-age stratification, CFTR genotyping, and microbiome/mycobiome analysis is warranted and long overdue.

Alongside this review, a series of three original articles deal with various aspects of the biological surveillance of fungal respiratory infections in the context of CF. First, Grehn et al. provide evidence that frequent contacts with pets may be a risk factor for allergic bronchopulmonary aspergillosis (ABPA) in patients with CF, suggesting that such information should be requested when questioning the patient about social history and lifestyle, especially for *A. fumigatus*-sensitized patients or patients with recurrent ABPA. In another original study, Grehn et al. show that urban life should be considered as a risk factor for airway colonization by *A. fumigatus*. This study has the merit of making us aware of the influence of environmental factors on the clinical course of CF patients, and this information also should be collected during patient interviews. Finally, this series of articles ends with the report from Guegan et al. that underscores a huge variability in the degree of azole resistance in *A. fumigatus* depending on the azole drug, the patient origin, and the clinical setting, but also among different isolates from a single patient. In addition to epidemiological data, this study highlights the need for a global reflection that must be engaged towards changing our routine procedures for mycological examination of respiratory secretions from CF patients to include systematic *in vitro* susceptibility testing of *A. fumigatus* isolates to azole antifungals.

Other breakthroughs introduced in this Research Topic concern the diagnosis of fungal respiratory infections in CF patients. For example, the report from Eschenhagen et al. demonstrates that the presence of serum-specific anti-*A. fumigatus* IgG in CF patients may be a useful marker for acute ABPA and *A. fumigatus* pneumonia, but not for *A. fumigatus* bronchitis, although it should only be interpreted together with other biological markers. A standardized multicenter longitudinal study should be conducted on a larger CF cohort since better knowledge of underlying immunological mechanisms may improve its clinical utility. In addition, Patel et al. investigated the relationships between some airway biomarkers, including the leukocyte differential cell count and the level of neutrophil elastase, interleukin-8, galactomannan, and tumor necrosis factor receptor type 2 in sputum or bronchoalveolar lavage fluid samples, and fungal culture positivity. Fungal culture positivity was found to be associated with exposure to indoor molds, bronchiectasis, and diminished lung function, but no relationship was seen with the different biomarkers that were evaluated. In their report, Martin-Souto et al. have selected a crude antigenic extract of *S. boydii* to detect IgG antibodies directed towards *Scedosporium* spp. and *L. prolificans* in sera from CF patients. An ELISA test was developed, showing very high sensitivity and specificity, which therefore may improve the serodiagnosis of *Scedosporium/ Lomentospora* infections. This serological method may also be interesting for monitoring the evolution of infection and to monitor the effectiveness of antifungal interventions.

In this Research Topic, we are also delighted to introduce a report from Currie et al., which demonstrates that CFTR modulators may have additional immunomodulatory benefits to prevent or treat *Aspergillus*-induced inflammation in CF. In this study, it is particularly intriguing to observe the comparable effects of CFTR modulators in phagocytes from CF patients and from control individuals, thus raising important questions about their exact mechanism of action.

Another series of investigations compiled in this Research Topic provides interesting information about the pathogenesis of fungal respiratory infections in CF with special emphasis on microbial interactions that may occur in the CF lung. First, Roisin et al. shed light on microbial interactions modulating the susceptibility of pathogens to antimicrobial drugs in polymicrobial biofilms. More specifically, they show that *Stenotrophomonas maltophilia* increased, in these biofilms, the susceptibility of *A. fumigatus* to amphotericin B, whereas *A. fumigatus* protected *S. maltophilia* from levofloxacin. This opens new windows for analyzing in the near future the antimicrobial susceptibility of pathogens in polymicrobial biofilms and to defining the mechanisms underlying such changes in their susceptibility. In the same area, Bertulino de Oliveira et al. have tested the effect of peptidorhamnomannans (PRMs) extracted from *L. prolificans*, *Scedosporium apiospermum, Scedosporium boydii*, and *Scedosporium aurantiacum* on bacterial species relevant to CF (e.g. *P. aeruginosa*, *B. cepacia*, methicillin-resistant *S. aureus*, and *Escherichia coli*). Interestingly, their data suggest that PRMs from *Scedosporium* and *Lomentospora* cell surfaces may play an important role in fungal colonization by disrupting some bacterial populations. In addition, Gonçalves de Almeida et al. have explored the lung microbiome of three young CF patients colonized by fungi. They found a personal signature with low variation in the microbiome across pulmonary exacerbations and a core set of virulence factors and antibiotic resistance genes. This study is important as understanding the microbial community and its interactions is crucial to improving therapeutic interventions by avoiding deleterious therapies that

inadvertently restructure the pathogenic microbiota. Future studies focusing on the influence of fungi on bacterial diversity and microbial interactions in the CF microbiome will be welcome to fulfill the huge knowledge gap concerning the influence of fungi on the evolution of CF pulmonary disease. Last but not least, Le Govic et al. have used both functional genomics and metabolomics approaches to demonstrate that the *S. apiospermum sidD* gene drives the synthesis of a unique extracellular siderophore, N^{α}-methylcoprogen B, that was found to be essential for fungal growth and virulence. This secondary metabolite seems important for iron acquisition from pyoverdine, which might explain the apparent antagonism between *S. apiospermum* and *P. aeruginosa* within the CF lung.

Overall, this series of reports show that sustained research projects aimed at deciphering the epidemiology and pathogenesis of fungal respiratory infections in the context of CF are now ongoing worldwide. This is, of course, an essential prerequisite for faster progress in the prevention, diagnostics (notably for differentiating transient carriage and chronic colonization of the airways), and treatment of these life-threatening infections.

AUTHOR CONTRIBUTIONS

All authors contributed to this editorial. NP and J-PB wrote the initial manuscript. AB and WM revised the manuscript, and all authors approved the submitted version.

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BRIEF RESEARCH REPORT
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CFTR Modulators Dampen *Aspergillus*-Induced Reactive Oxygen Species Production by Cystic Fibrosis Phagocytes

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Excessive inflammation by phagocytes during *Aspergillus fumigatus* infection is thought to promote lung function decline in CF patients. CFTR modulators have been shown to reduce *A. fumigatus* colonization *in vivo*, however, their antifungal and anti-inflammatory mechanisms are unclear. Other treatments including azithromycin and acebilustat may dampen *Aspergillus*-induced inflammation due to their immunomodulatory properties. Therefore, we set out in this study to determine the effects of current CF therapies on ROS production and fungal killing, either direct or indirect by enhancing antifungal immune mechanisms in peripheral blood immune cells from CF patients upon *A. fumigatus* infection. Isolated peripheral blood mononuclear cells (PBMCs) and polymorphonuclear cells (PMNs) from CF patients and healthy volunteers were challenged with *A. fumigatus* following pre-treatment with CFTR modulators, azithromycin or acebilustat. Ivacaftor/lumacaftor treated CF and control subject PMNs resulted in a significant reduction ($p < 0.05$) in *Aspergillus*-induced ROS. For CF PBMC, *Aspergillus*-induced ROS was significantly reduced when pre-treated with ivacaftor alone ($p < 0.01$) or in combination with lumacaftor ($p < 0.01$), with a comparable significant reduction in control subject PBMC ($p < 0.05$). Azithromycin and acebilustat had no effect on ROS production by CF or control subject phagocytes. None of the treatments showed an indirect or direct antifungal activity. In summary, CFTR modulators have potential for additional immunomodulatory benefits to prevent or treat *Aspergillus*-induced inflammation in CF. The comparable effects of CFTR modulators observed in phagocytes from control subjects questions their exact mechanism of action.

Keywords: *Aspergillus fumigatus*, cystic fibrosis, phagocytes, inflammation, CFTR modulators, azithromycin, acebilustat

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INTRODUCTION

Cystic fibrosis (CF) is a life-limiting autosomal recessive disorder characterized by chronic respiratory infections, progressive respiratory disease and respiratory failure (Elborn, 2016). Gene mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) in epithelial cells affects mucus fluid dynamics and pathogen survival (Elborn, 2016; McElvaney et al., 2019).

CFTR is also expressed in immune cells and mutations in this gene are associated with impaired antimicrobial activity and dysregulated inflammatory responses (Moss et al., 2000; Carrabino et al., 2006; Painter et al., 2006; Deriy et al., 2009; Del Porto et al., 2011; Mueller et al., 2011; Zhou et al., 2013; Johansson et al., 2014). Treatment of the bacterial infectious complications in CF patients has traditionally focused on clearance and eradication of the pathogen from the airways, thereby diminishing and preventing airway inflammation (Döring et al., 2012; Ciofu et al., 2013; Addy et al., 2020).

Airway inflammation in CF is a much-debated topic, as underlying mechanisms may be either intrinsic or extrinsic, or a combination of both (Nichols and Chmiel, 2015). Modulating the inflammatory response needs to consider a careful balance aimed at a minimum of inflammation without reducing the antimicrobial activity of the immune system. Management approaches to *Aspergillus* infections in CF have focused on Allergic Bronchopulmonary Aspergillosis (ABPA) by reducing the allergic inflammation induced (Agarwal et al., 2016). First-line treatment with corticosteroids is targeted against the induced inflammation, with a recommendation for antifungal therapy when first-line treatment fails. *Aspergillus fumigatus* is the major fungal pathogen isolated from sputum of far more CF patients than those diagnosed with ABPA (Warris et al., 2019). Studies indicate that infection with *A. fumigatus* in the CF airways can result in increased pulmonary exacerbations, bronchiectasis, and worse respiratory quality of life (Amin et al., 2010; Breuer et al., 2019; Hong et al., 2020). Nevertheless, there is a lack of data on how to manage those infections, e.g., eradication of infection or damping the inflammation, and what the associated risks and benefits of such approaches would be.

Our group previously demonstrated that peripheral blood phagocytes from CF patients show normal antifungal killing in response to *A. fumigatus*, but that this is associated with excessive reactive oxygen species (ROS) production (Brunel et al., 2018). Additionally, we highlighted that the heightened inflammation was correlated with poorer lung function in CF patients. As eradication of *A. fumigatus* from the airways of CF patients is a huge challenge due to the universal presence of *A. fumigatus* in the environment, finding ways to dampen *Aspergillus*-induced inflammation may be more feasible, as long as these strategies do not affect antifungal killing mechanisms.

In our current study, a key objective was to determine in detail the effects of three important CF therapies, azithromycin, acebilustat, and CFTR modulators, on the antifungal immune mechanisms. Azithromycin is a macrolide antibiotic with immunomodulatory and anti-inflammatory properties (Cigana et al., 2006; Legssyer et al., 2006) used as a long-term treatment for CF patients to improve lung function and reduce exacerbations in those with persistent *Pseudomonas aeruginosa* (Mogayzel et al., 2013; Principi et al., 2015). Acebilustat, currently in phase I/II trials in CF patients, is a leukotriene A4 hydrolase (LTA_4H) inhibitor, inhibiting the production of the intracellular lipid mediator leukotriene B4 (LTB4) (Bhatt et al., 2017; Elborn et al., 2017a,b, 2018). LTB4 is a principal chemoattractant for recruiting neutrophils to inflamed sites across the airway epithelium and known to stimulate ROS production and to enhance the NF-κB pathway, thus driving inflammation (Woo et al., 2003). CFTR modulators are the first causative treatment option for CF and have been shown to reduce pulmonary exacerbations in CF patients homozygous for the F508-del mutation (Wainwright et al., 2015). Additionally, ivacaftor reduced colonization and prevalence of *A. fumigatus* in CF patients with a G551D genotype (Heltshe et al., 2015; Frost et al., 2019). Whilst the effects of CFTR modulators on epithelial cell function are reasonably well-understood (Kuk and Taylor-Cousar, 2015), the effects on immune cell function have not been investigated in detail. Assessment of the hypothetical effect of the CFTR modulators on immune cells resulting in a decrease of microbial induced inflammation, is of high value. We present here our results of the effect of these treatments on *Aspergillus*-induced ROS production and fungal killing, either direct or indirect by enhancing antifungal immune mechanisms in peripheral blood phagocytes.

MATERIALS AND METHODS

Human Subjects

Blood samples were donated by adult CF patients attending the Aberdeen Royal Infirmary (Aberdeen, UK) and healthy volunteers recruited from the Institute of Medical Sciences (Aberdeen, UK). All participants provided written informed consent and donated a maximum of 50 mL (CF patients) or 100 mL (healthy volunteers) of blood. This study was approved by East of Scotland Research Ethics Service (18/ES/0154) and the College Ethics Review Board of the University of Aberdeen (CERB/2016/8/1300). All samples were collected according to approved guidelines and procedures. Clinical report forms were provided for each CF patient and included; demographics, genotype, body mass index (BMI), forced expiratory volume in 1 s (FEV_1), *Aspergillus* serology, sputum culture results, co-morbidities, pulmonary exacerbation episodes over the previous 12 months and medications.

A. fumigatus Strains

Thirteen *A. fumigatus* strains were used including 12 clinical strains and the well-characterized laboratory strain AF 293. Clinical isolates from CF patients (10,749, 11,361, 5,923, 7,762, 10,225, 15,115, 10,410, 11,856), patients with chronic infection (1,145, 9,475) and acute infection (11,146, 11,160) were kindly provided by Prof Paul E. Verweij (Centre for Expertise in Mycology, Radboud University Medical Centre, Nijmegen, NL).

A. fumigatus Culture Conditions

A. fumigatus conidia were grown on glucose minimal media for 7 days at 35°C and harvested in phosphate buffer saline (PBS) supplemented with 0.05% Tween-80. Conidia were then filtered through a 40 μm sterile filter and resuspended to the required concentration in RPMI or PBS + Ca^{2+}/Mg^{2+} (0.9 nM Ca^{2+} and 0.49 mM Mg^{2+}).

CFTR Modulators, Azithromycin, and Acebilustat

Ivacaftor and lumacaftor (AdooQ Bioscience, USA) stock solutions were prepared by solubilizing in 100% DMSO (Sigma Aldrich, UK) at 10 mg/mL. CFTR modulator stock solutions (1.6, 3.2, or 6.4 μl) were diluted in 1 mL sterile water. DMSO diluted in sterile water was used as a control in all experiments, at 0.32 or 0.64% when testing direct antifungal activity and 0.16% for ROS assays. Azithromycin dihydrate (Sigma Aldrich, UK) was solubilised in 100% DMSO at 20 mg/mL and stock solutions (1, 2, and 2.5 μl) were diluted in 1 mL of sterile water for ROS assays and RPMI for fungal killing. Acebilustat (MedChemExpress, USA) was solubilised in 100% DMSO at 0.1 mM and diluted to a working concentration of 10 μM in sterile water for ROS assays and RPMI for fungal killing.

Concentrations used in the various experiments were based the IC50 of acebilustat to inhibit LTB4 production, the maximum reported tissue concentrations of azithromycin, and previous *in vitro* studies performed with the CFTR modulators (reviewed by Csanády and Töröcsik, 2019).

Phagocyte Isolation

Whole blood samples were collected in a Vacuette® containing EDTA (Greiner Bio-One) and allowed to cool to room temperature before mixing 1:1 with sterile PBS. Blood was then overlaid on equal parts of Histopaque (10,771 and 11,919; Sigma Aldrich) and separation of the cell fractions was achieved by density gradient centrifugation at 300 g for 30 min at 4°C. The peripheral blood mononuclear cell (PBMC) layer was removed and washed three times in PBS. The fraction containing polymorphonuclear cells (PMN) was treated twice with hypotonic lysis buffer (8.3 mg/ml NH_4Cl and 1 mg/ml $KHCO_3$ in sterile water) to lyse erythrocytes then washed three times with decreased spins to remove thrombocytes. PBMC and PMN pellets were resuspended in RPMI or PBS + Ca^{2+}/Mg^{2+}, counted and adjusted to the required concentration. Viability was tested by using trypan blue exclusion.

Reactive Oxygen Species (ROS) Production

Production of oxygen radicals was evaluated using luminol-based chemiluminescence as previously described (Brunel et al., 2018). Briefly, PBMCs and PMNs were suspended in PBS +Ca^{2+}/Mg^{2+} and plated at 5×10^5 cells/well and left untreated (vehicle control) or pre-treated with ivacaftor (8 μg/ml), lumacaftor (8 μg/ml), ivacaftor+lumacaftor (8 μg/ml of each), acebilustat (0.031 μg/ml) or azithromycin (20 μg/ml) for 1 h at 37°C, 5% CO_2. Cells were then infected with *A. fumigatus* resting conidia (1×10^7/well) and 100 μM luminol was added to each well. Kinetic reads were taken every 180 s for 2 h using a luminescence plate reader (Biotek Gen5™).

Fungal Killing

To assess direct fungal killing of each drug, *A. fumigatus* strains were plated at 5×10^4 conidia per well in RPMI in a 96 U-well plate. Conidia were then incubated with ivacaftor/lumacaftor (16 or 32 μg/ml of each), azithromycin (10, 20, or 50 μg/mL), or acebilustat (0.031 μg/ml) for 18 h or left untreated (vehicle control). To test anti-hyphal activity; conidia were plated and left for 16 h (37°C, 5% CO_2) to allow for gemination prior to incubation with ivacaftor (16 or 32 μg/ml), lumacaftor (16 or 32 μg/ml), or ivacaftor+lumacaftor (16 or 32 μg/ml of each) for 6 h.

To assess enhanced fungal killing by phagocytes, PMNs and PBMCs were plated at 1×10^5 and 5×10^5 cells per well respectively, and left untreated (vehicle control) or pre-treated with ivacaftor (8 μg/ml), lumacaftor (8 μg/ml), ivacaftor/lumacaftor (8 μg/ml of each), acebilustat (0.031 μg/ml) or azithromycin (20 μg/ml) for 1 h at 37°C, 5% CO_2. Cells were then infected with *A. fumigatus* conidia (1×10^5 conidia per well) and left for 18 h at 37°C, 5% CO_2.

Following the indicated incubation times, plates were centrifuged at 2,500 g for 10 min. For phagocyte experiments, media was removed, and cells lysed with 100 μl of saponin (0.005% in MilliQ water; Sigma Aldrich) for 20 min. For the cell-free experiments, fresh media was added. Twice concentrated XTT-menadione solution (XTT salt 200 μg/ml, Invitrogen; menadione crystalline 172 μg/ml, Sigma Aldrich) was added to each well (100 μl diluted 1:2 in media or saponin) and plates left for 2–3 h in the dark at 37°C, 5% CO_2 to allow for reduction of XTT to formazan. Plates were spun at 2,500 g for 10 min and supernatant transferred to a flat-bottomed 96-well plate prior to measuring absorbance at 450 nm using a VersaMax microplate reader.

Statistical Analysis

All data are presented as mean ±SEM. Significance between control subjects and CF patients was analyzed using Mann Whitney *U*-tests. For multiple comparisons between drug treatments a Kruskal–Wallis test was used with Dunn's post-test. Data analysis was carried out using GraphPad Prism V5.04.

RESULTS

Clinical Characteristics of Participants

Ten patients (60% male) participated in this study with a median age of 26 years (range 16–46 years). Six were homozygous for the F508-del mutation. Median FEV_1 was 43.5% predicted (range 15–104.2%) and BMI was 21 (range 17–40). None of the patients received CFTR modulators or antifungals. Four patients had signs of fungal sensitization (*Aspergillus* IgE > 1 kU/L) (**Supplementary Table 1**). Control subjects were between the ages of 20–55 years.

CFTR Modulators and Azithromycin Have no Direct Antifungal Effect

Ivacaftor (16 or 32 μg/ml), lumacaftor (16 or 32 μg/ml), or ivacaftor/lumacaftor (16 or 32 μg/ml of both) did not have a significant effect on the hyphal metabolic activity for both *A. fumigatus* isolates when compared to the vehicle controls (**Figure 1A**). No direct effect was observed on the metabolic activity of an additional 10 clinical isolates and one lab strain (AF293) with all concentrations and combination tested (**Supplementary Figure 1**). When incubating *A. fumigatus* conidia with ivacaftor/lumacaftor (16 or 32 μg/ml of both),

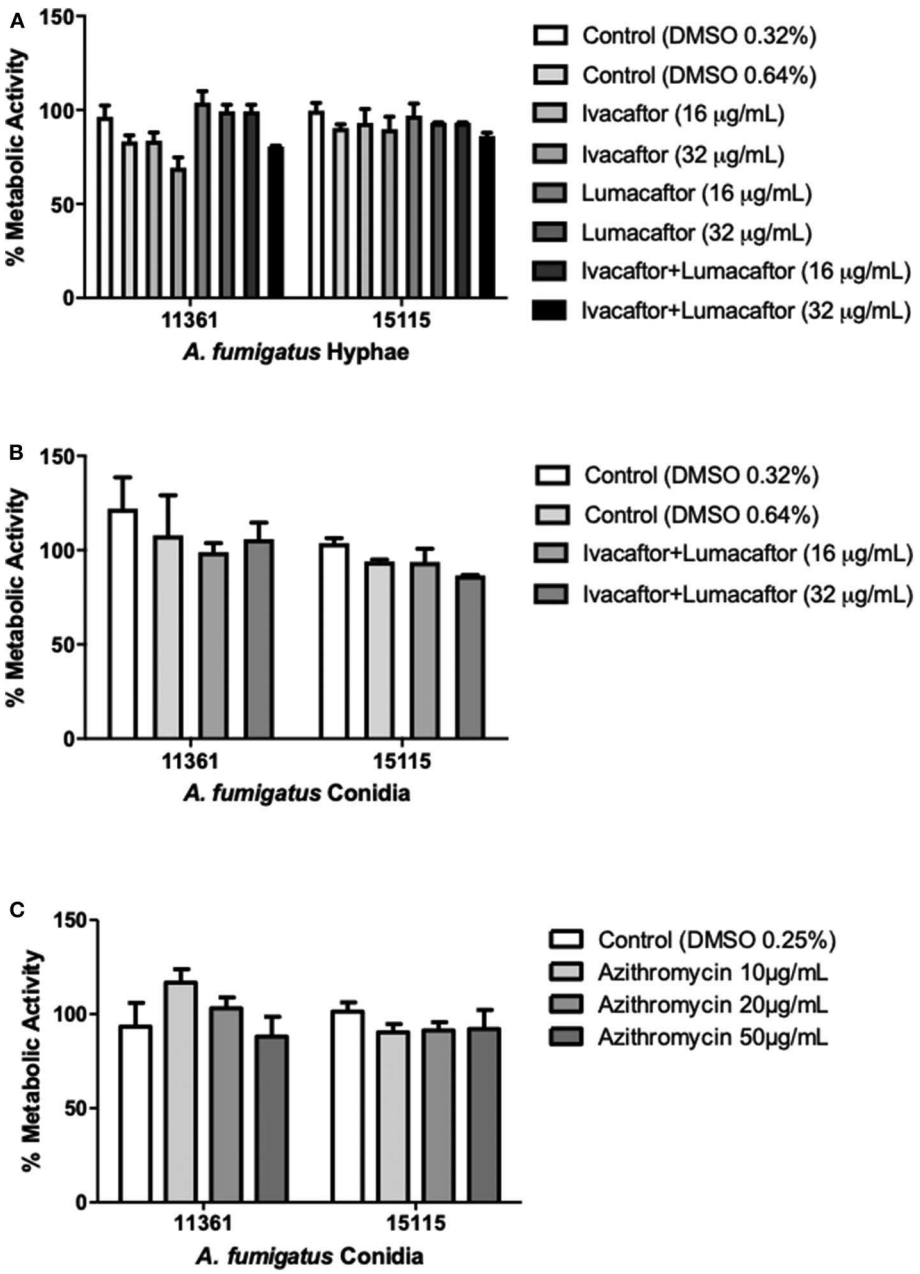

FIGURE 1 | CFTR modulators and azithromycin have no direct antifungal effect. **(A)** Hyphae from 2 clinical *A. fumigatus* isolates (11,361, 15,115) were untreated (DMSO) or treated with ivacaftor (16 or 32 μg/ml), lumacaftor (16 or 32 μg/ml) or ivacaftor/lumacaftor (16 or 32 μg/ml of each) for 6 h prior to measuring metabolic activity. **(B)** Two clinical *A. fumigatus* isolates (11,361, 15,115) were grown in the presence of DMSO or ivacaftor/lumacaftor (16 or 32 μg/ml of each) or **(C)** in the presence of various concentrations of azithromycin for 18 h at 37°C, 5% CO_2. Following the specified incubations metabolic activity was assessed with XTT-menadione. Data are representative of 2 **(A,B)** and 3 **(C)** independent experiments and are presented the mean ±SEM of % metabolic activity compared to *A. fumigatus* grown in RPMI only.

no effect on the metabolic activity was observed when compared to the vehicle controls (**Figure 1B**). Again, no effect was observed for an additional 10 clinical isolates and one lab strain (AF293) (**Supplementary Figure 2**). No effect on fungal metabolic activity was observed for all azithromycin concentrations tested (**Figure 1C**, **Supplementary Figure 3**).

Aspergillus Activated Phagocytes From CF Patients Show Exaggerated ROS Production

Phagocytes from CF and control subjects were co-incubated with *A. fumigatus* conidia and analyzed for ROS production. ROS production after incubation with two different clinical isolates

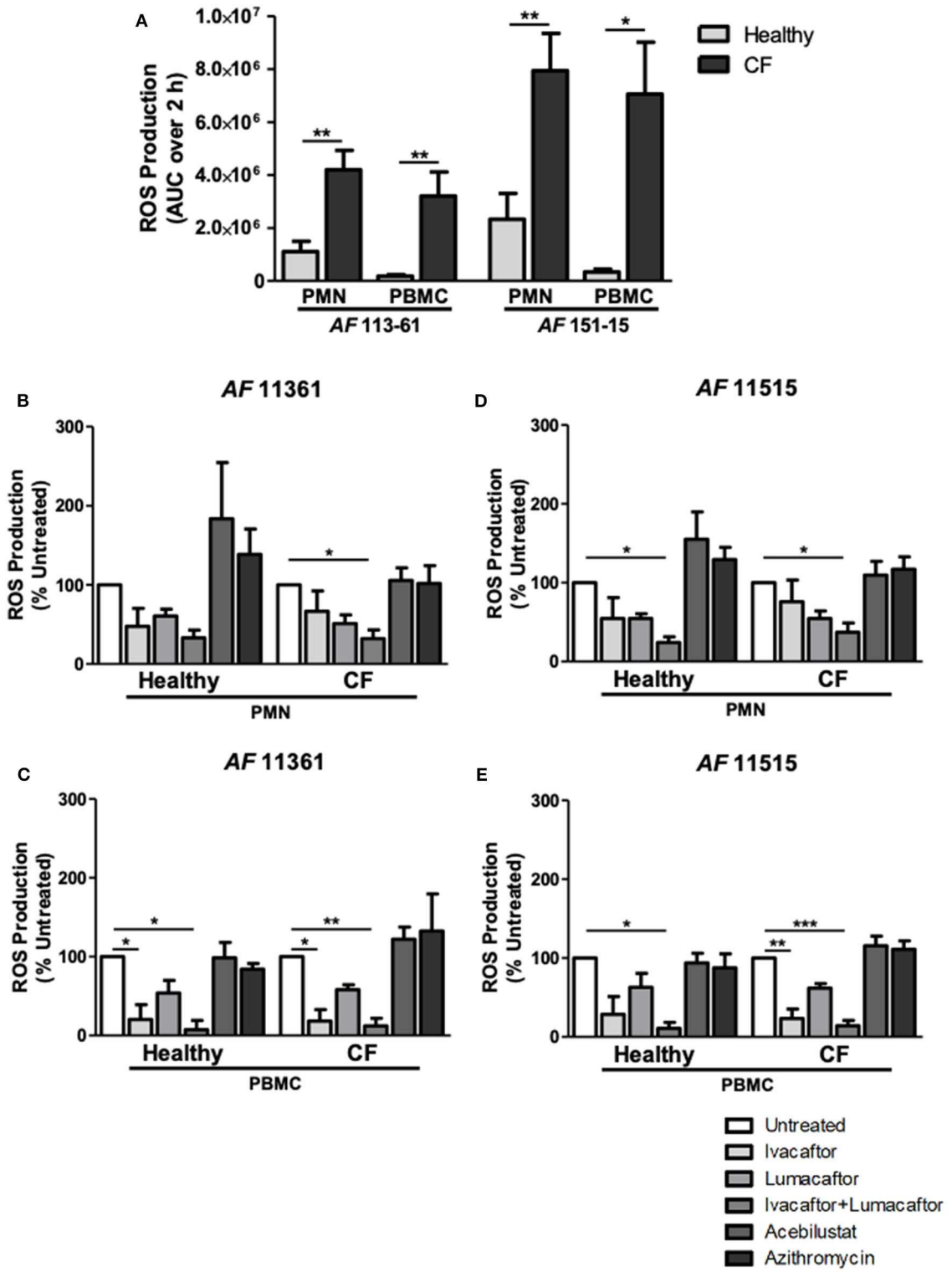

FIGURE 2 | CFTR modulators, but not acebilustat or azithromycin, reduce *Aspergillus*-induced ROS production by both healthy and CF phagocytes. Isolated PMN or PBMC were either untreated (0.16% DMSO) or treated with ivacaftor (8 μg/ml), lumacaftor (8 μg/ml), ivacaftor/lumacaftor (8 μg/ml of both), acebilustat (0.031 μg/ml) or azithromycin (20 μg/ml) for 1 h at 37°C, 5% CO_2 prior to *A. fumigatus* infection (MOI 20). ROS was measured by luminol chemiluminescence. **(A)** ROS production by control and CF PMN and PBMC in response to *A. fumigatus* CF patient isolates 11,361 and 15,115. **(B–D)** ROS production by control and CF PMN in response to two *A. fumigatus* isolates originating from CF patients following pre-treatment with ivacaftor, lumacaftor, acebilustat or azithromycin and **(C–E)** and by control and CF

(Continued)

FIGURE 2 | PBMC. Data presented relative to untreated samples which was set as 100%. Data are presented as mean ±SEM of total ROS produced over 2 h (AUC) for 4–5 control subjects and 5–9 CF patients. Statistical analysis was carried out using **(A)** Mann-Whitney test and **(B–E)** Kruskal-Wallis test. Abbreviations, AUC; area under the curve, PMN; polymorphonuclear cells, PBMC; peripheral blood mononuclear cells, ROS; reactive oxygen species, SEM; standard error of the mean. $^{*}p \leq 0.05$; $^{**}p \leq 0.001$; $^{***}p \leq 0.001$.

of *A. fumigatus* conidia was significantly increased by CF PMN and PBMC when compared to healthy controls (**Figure 2A**) confirming our previous observations (Brunel et al., 2018). CF PMN produced ROS at levels up to 4-fold greater than PMNs from control subjects in response to both *A. fumigatus* isolates ($p \leq 0.01$). *A. fumigatus*-induced ROS production by CF PBMC was 18- to 20-fold higher ($p \leq 0.01$) when compared to cells from control subjects (**Figure 2A**).

CFTR Modulators Reduce *Aspergillus*-Induced ROS by Phagocytes

The viability of the phagocytes with or without the CFTR modulators (alone or in combination at 8 μg/mL) remained around 95% after 6 h incubation. To assess the effect of each treatment on *Aspergillus*-induced ROS production by healthy and CF phagocytes, we normalized each treatment response as a percentage of the untreated control. A decrease in ROS production by CF PMN was observed following pre-treatment with ivacaftor (11,361; −33.4 ± 25.9%, 15,115; −24.1 ± 27.5%) and lumacaftor (11,361; −48.9 ± 11.01%, 15,115; −45.3 ± 9.5%) when compared to untreated controls, although the changes did not reach statistical significance. Ivacaftor/lumacaftor pre-treatment resulted in a significant reduction of *Aspergillus*-induced ROS production by CF PMNs for both strains (11,361; −67.8 ± 10.8%, 15,115; −62.8 ± 11.6%, $p \leq 0.05$) (**Figures 2B,D**).

The same trend was observed in PMN from control subjects; ivacaftor (11,361; −52.5 ± 23.0%, 15,115; −45.3 ± 26.3%) and lumacaftor (11,361; −39.33 ± 8.7%, 15,115; −45.4 ± 6.0%) reduced ROS levels, although not statistically different from untreated PMN. When PMN from control subjects were pre-treated with ivacaftor/lumacaftor, this significantly reduced ROS production in response to both strains (11,361; −66.8 ± 9.7%, 15,115; −75.8 ± 7.1%, $p \leq 0.05$) (**Figures 2B,D**).

For CF PBMC, *Aspergillus*-induced ROS was significantly reduced when pre-treated with ivacaftor (11,361; −81.7 ± 14.6%, $p \leq 0.05$; 15,115; −76.60 ± 11.89%, $p \leq 0.01$), but not when treated with lumacaftor (11,361; −41.9 ± 6.3%; 15,115; −38.2 ± 5.8%). Ivacaftor/lumacaftor pre-treatment significantly attenuated generation of ROS by CF PBMCs (11,361; −87.96 ± 9.7%, $p \leq 0.01$; 15,115; −85.9 ± 6.7%, $p \leq 0.001$) (**Figures 2C,E**).

As observed with CF PBMC, pre-treatment of control PBMC with ivacaftor showed a clear decrease in ROS production (11,361; −79.7 ± 14.6%, $p \leq 0.05$; 15,115 −71.32 ± 22.4%, $p =$ n.s.). Ivacaftor/lumacaftor significantly reduced ROS production by control subject PBMC (11,361; −92.5 ± 11.7%, 15,115; −89.14 ± 7.57%, $p \leq 0.05$ for both strains) (**Figures 2C,E**).

Next, we assessed the differential effects of those treatments based on underlying CFTR genotypes. PMN and PBMC from CF patients homozygous for the F508-del mutation ($n = 6$) treated with ivacaftor/lumacaftor showed a significant reduction in ROS (–54.2 ± 11.0%; $p \leq 0.01$) and (−86.6 ± 5.7%; $p \leq 0.001$, respectively) (**Figure 3**). Ivacaftor alone significantly reduced ROS production by PBMC homozygous for the F508-del mutation (−80.0 ± 8.1%; $p \leq 0.05$), but had no effect on PMN (F508-del/F508-del). Lumacaftor pretreatment of PMN and PBMC resulted in a 33.0% (±11.1%) and 41.35% (±5.3%) reduction, respectively, in the F508-del homozygous group which was not significantly different to untreated cells (**Figure 3**).

Treatment with ivacaftor alone or in combination with lumacaftor showed a significant decrease in *Aspergillus*-induced ROS production by PMN (−93.8 ± 4.5 and −85.9 ± 5.6%, respectively) and PBMC (−96.4 ± 2.2 and −94.7 ± 3.2%, respectively) from CF patients with other CFTR mutations (non F508-del, non G551D) (**Figure 3**).

Pre-treatment with azithromycin (20 μg/ml) or acebilustat (64 nM) had no effect on *Aspergillus*-induced ROS production by PMN or PBMC from either CF patients or control subjects when compared to untreated cells (**Figures 2B–E**).

CFTR Modulators, Acebilustat, and Azithromycin Do Not Impair Fungal Killing by Healthy and CF Phagocytes

Control subject PMN reduced metabolic activity by ∼70% for both *A. fumigatus* isolates (**Figures 4A,C**). In comparison, CF PMN reduced the metabolic activity of the two isolates between 80 and 92% (**Figures 4A,C**). None of the treatments had any effect on killing of the two *A. fumigatus* isolates by both CF and control subject PMN when compared to untreated controls (**Figures 4A,C**). CF PBMC showed an increased killing of both isolates but changes did not reach statistical significance when compared to control subject PBMC (**Figures 4B,D**). No significant differences in antifungal killing were observed associated with a specific treatment given compared to untreated CF and control subject PBMC (**Figure 4B**).

DISCUSSION

We show that the CFTR modulators, ivacaftor, lumacaftor and its combination, are able to downregulate ROS production by human CF phagocytes without compromising their fungal killing ability. Importantly, this effect was not specific to CF cells, indicating potential off-target mechanistic effects of CFTR modulators. To our knowledge we are the first to demonstrate that CFTR modulators have immunomodulatory effects on both CF and control subjects' phagocytes when challenged with *A. fumigatus*. Azithromycin and acebilustat did not affect ROS production or fungal killing by CF or control subjects' phagocytes. Furthermore, CFTR modulators and azithromycin do not directly affect fungal viability.

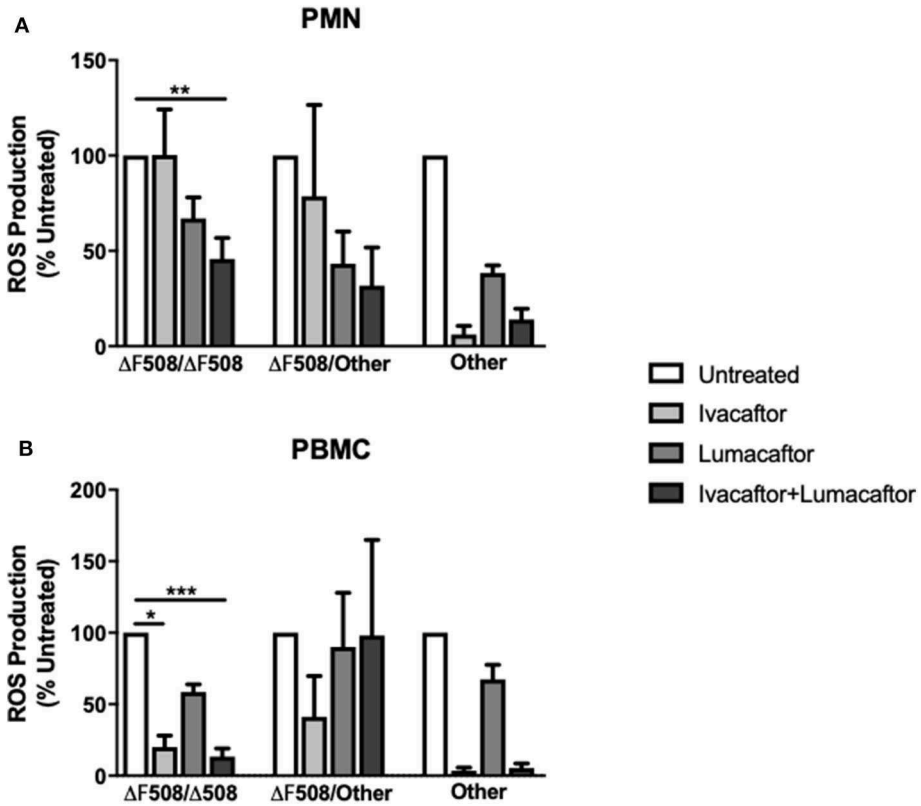

FIGURE 3 | CFTR modulators differentially influence ROS production based on CFTR genotypes and type of immune cells. Isolated PMN or PBMC from CF patients with different underlying CFTR gene mutations were either untreated (0.16% DMSO) or treated with ivacaftor (8 μg/ml), lumacaftor (8 μg/ml), ivacaftor/lumacaftor (8 μg/ml of both) for 1 h at 37°C, 5% CO_2 prior to *A. fumigatus* infection (MOI 20). ROS was measured by luminol chemiluminescence. **(A)** ROS production by CF PMN in response to two clinical *A. fumigatus* isolates from CF patients (11,361 and 15,115) following pre-treatment with ivacaftor, lumacaftor, and **(B)** by CF PBMC. Data presented relative to untreated samples which was set as 100%. Data are presented as mean ±SEM of total ROS produced over 2 h (AUC) for 5–9 CF patients. AUC, area under the curve; PMN, polymorphonuclear cells; PBMC, peripheral blood mononuclear cells; ROS, reactive oxygen species; SEM, standard error of the mean. *$p \leq 0.05$; **$p \leq 0.001$; ***$p \leq 0.001$.

Our study shows that CFTR modulators reduce ROS responses by human CF phagocytes infected with *A. fumigatus*, and that this reduction was statistically significant in PMN and PBMC from patients homozygous for the F508-del mutation treated with ivacaftor/lumacaftor. In addition, pretreatment of PBMC homozygous for the F508-del mutation with ivacaftor alone significantly reduced the *Aspergillus*-induced ROS production. As the number of CF patients heterozygous for the F508-del mutation and those with other mutations (non F508-del, non G551D) were low (both $n = 2$), a proper comparison between the three groups was not possible. Nevertheless, a comparable trend was observed for the phagocytes from CF patients heterozygous for the F508-del mutation. Remarkable is the observation that in the two CF patients with non-F508-del and non-G551D mutations, treatment with ivacaftor alone and in combination with lumacaftor almost completely abolished the *Aspergillus*-induced ROS production in PMN and PBMC. Most of the observed differences can be related to the specific mode of action of ivacaftor and lumacaftor. Lumacaftor acts directly to improve the defective cellular processing and trafficking of the F508-del mutant CFTR channel, with ivacaftor potentiating the gating properties of the mutant CFTR channel caused by a variety of gene mutations (Kuk and Taylor-Cousar, 2015).

The differential effect of ivacaftor pretreatment leading to a significant reduction in *Aspergillus*-induced ROS production by CF PBMC is in sharp contrast with the effect observed on CF PMN. A higher sensitivity of CF PBMC for potentiating the channel function might explain this observation, but needs further research. Improvements of ion fluxes underpinning the clinical efficacy of the CFTR modulators will likely influence the aberrant immune responses and observed hyperinflammation (Hartl et al., 2012; Pohl et al., 2014).

Only limited data is available showing that ivacaftor/lumacaftor can directly modulate CF-related inflammation. Studies performed with *P. aeruginosa* stimulated CF bronchial epithelial cells (homozygous for F508-del) showed that this combination reduces the transcription of CXCL8 and the phosphorylation of p38 MAPK (Ruffin et al., 2018). Human CF monocytes (homozygous for F508-del) stimulated with LPS/ATP before and after patients received treatment with ivacaftor/lumacaftor showed decreased levels of IL-18, TNF and caspase-1 (Jarosz-Griffiths et al., 2020). Excessive

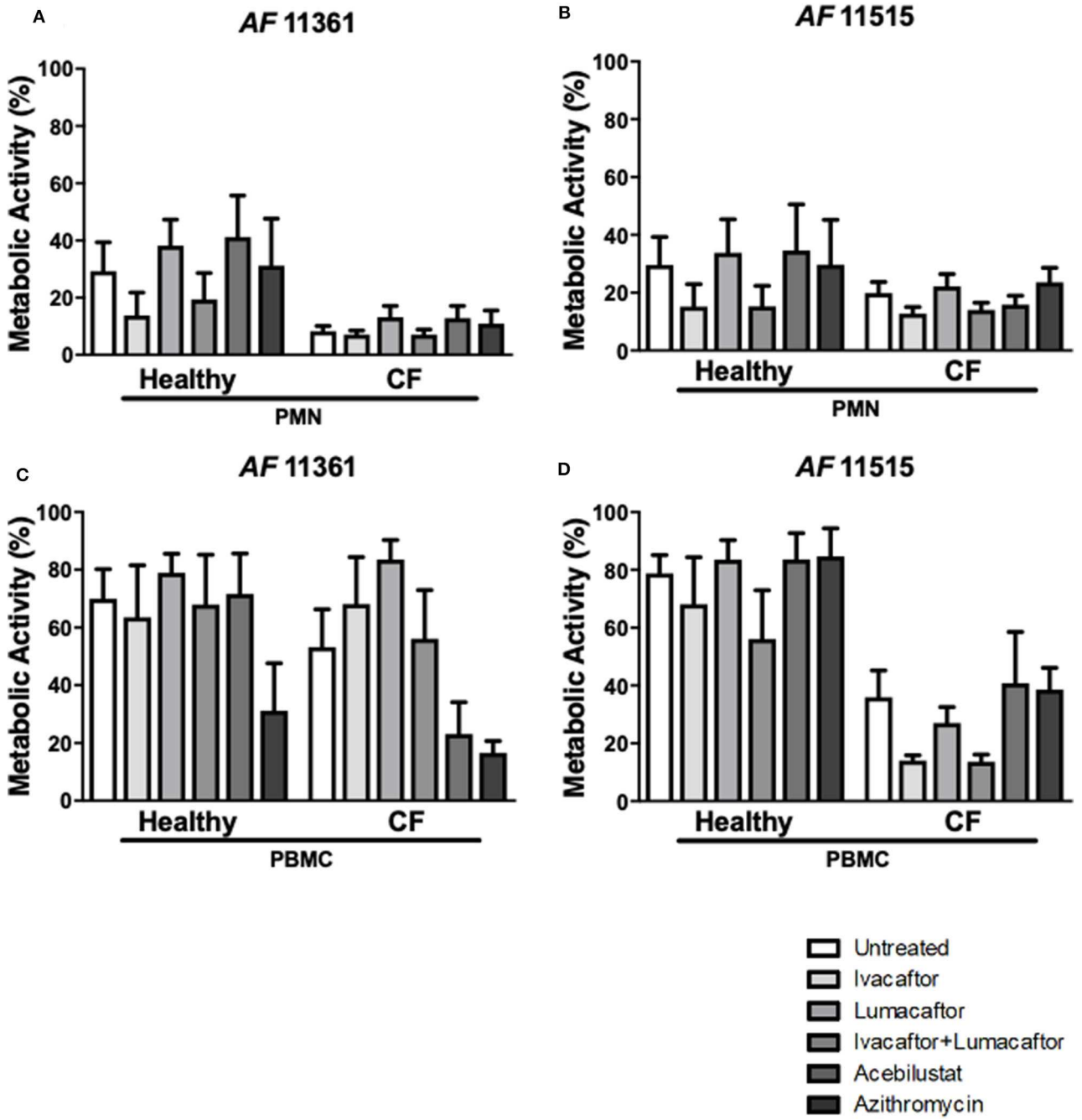

FIGURE 4 | CFTR modulators, acebilustat and azithromycin do not impair fungal killing by healthy and CF phagocytes. Isolated PMN or PBMC were either untreated (0.16% DMSO) or treated with ivacaftor (8 μg/ml), lumacaftor (8 μg/ml), ivacaftor/lumcaftor (8 μg/ml of both), acebilustat (0.031 μg/ml) or azithromycin (20 μg/ml) for 1 h at 37°C, 5% CO_2 prior to *A. fumigatus* infection (PMNs MOI: 1; PBMCs MOI: 0.2). Following 18 h incubation metabolic activity was determined using XTT-menadione. Assessment of fungal metabolic activity following co-incubation with pre-treated control and CF PMN **(A,C)** and PBMC **(B,D)** compared to untreated cells. Data are presented as mean ±SEM for 4–5 healthy controls and 3–6 CF patients. Statistical analysis was carried out using Kruskal–Wallis test. PMN, polymorphonuclear cells; PBMC, peripheral blood mononuclear cells; SEM, standard error of the mean.

ROS production is linked to defective autophagy (Luciani et al., 2011). In CF epithelial cells, stockpiling of large amounts of mutant CFTR leads to increases in aggresomes and ROS production, and autophagy inhibition (Luciani et al., 2010). A comparable phenomenon has been observed in CF macrophages (Abdulrahman et al., 2013). CFTR modulators have been shown to have the ability to target autophagy in CF airway epithelial cells to decrease inflammation in the lung (Luciani et al., 2012)

and might underpin the reduced ROS production as shown in our results.

Remarkably, ivacaftor and lumacaftor also decreased *Aspergillus*-stimulated ROS production by phagocytes from control subjects. Potentiation of the CFTR channel above normal physiological function or yet unknown off-target effects might explain this observation. It is important to acknowledge that the exact mechanisms of action have not been elucidated for both ivacaftor and lumacaftor.

Azithromycin did not affect the ROS production by CF or control subjects' phagocytes in response to *A. fumigatus*. Azithromycin has both immunomodulatory and anti-inflammatory properties (Cigana et al., 2006; Legssyer et al., 2006) and accumulates in neutrophils (Bosnar et al., 2005), but the effect on ROS production has hardly been studied. Bystrzycka et al. (2017) demonstrated a concentration-dependent effect of azithromycin (0.5–50 μg/ml) in decreasing the amount of ROS produced by PMA stimulated healthy human neutrophils. Earlier studies suggest that the effect of azithromycin depends on the stimulus used (Culić et al., 2002; Parnham et al., 2005). Due to the systemic glutathione deficiency in CF patients, azithromycin may be of value to improve the antioxidant activities by CF cells, thereby diminishing the toxic effects of ROS on lung tissue (Roum et al., 1993).

Acebilustat has not been investigated with respect to its influence on ROS production in immune cells. Based on the fact that LTB4 induces ROS production by dHL-60 neutrophils, inhibition of LTA4H by acebilustat would be predicted to diminish ROS production (Woo et al., 2003). However, acebilustat had no effect on ROS production by either CF or control subjects' phagocytes in our study. Its anti-inflammatory properties in the inflamed lung are most likely attributed to its inhibitory effects on neutrophil migration into the airways and the lungs (Woo et al., 2003).

Pretreatment with CFTR modulators was not associated with an enhanced fungal killing by CF and control subjects' phagocytes. This is in contrast with reports that CFTR modulators augment bacterial killing. Ivacaftor has been shown to augment killing of *P. aeruginosa* by CF macrophages (G551D/F508-del) to the same degree as healthy cells (Pohl et al., 2014). Similarly, lumacaftor alone increased killing of *P. aeruginosa* by CF macrophages (homozygous for Phe508del), but no effect was seen on control subjects' monocytes (Barnaby et al., 2018).

Azithromycin pre-treatment of healthy and CF phagocytes did not influence fungal killing. A different observation is reported for bacterial killing, as azithromycin-loaded neutrophils showed more effective killing of *Aggregatibacter* sp. by increased phagocytosis (Lai et al., 2015). Despite reports demonstrating that macrolide antibiotics have *in vitro* antifungal activity against *Aspergillus* species and other fungi (Kim et al., 2003; Hosoe et al., 2006), we did not observe any direct antifungal effect of azithromycin. Previous studies show long term azithromycin treatment can result in increased risk of colonization with *A. fumigatus* in CF patients possibly associated with its inhibitory effect on immune responses (Legssyer et al., 2006; Jubin et al., 2010). Additionally, there is an association between *A. fumigatus* colonization and non-tuberculous mycobacteria (NTM) in CF patients (Coolen et al., 2015). Although long term azithromycin reduces the risk of NTM, it does not suggest additional benefits to prevent *Aspergillus* infections.

Ivacaftor has been shown to have direct antibacterial effects against *Staphylococcus aureus* (MIC 8 μg/ml) and *Streptococcus* spp. (MIC 32 μg/ml), but was not active against *P. aeruginosa* (Reznikov et al., 2014). Similarly, Payne et al. (2017) showed 32 mg/L of ivacaftor resulted in a several log-fold decrease in CFUs with *Streptococcus* spp. and bacteriostatic effects against *S. aureus*, but was ineffective against *P. aeruginosa*. Clinical studies have shown ivacaftor reduces colonization of *A. fumigatus* in CF patients (with at least one copy of G551D mutation) and *P. aeruginosa*, but not *S. aureus* (Heltshe et al., 2015). Using data from the UK CF Registry comparing ivacaftor users and their contemporaneous comparators, reduced prevalence of *Aspergillus* spp., as well as *P. aeruginosa* and *S. aureus*, but not *Burkholderia cepacia* were found (Frost et al., 2019). We show that ivacaftor and/or lumacaftor have no antifungal activity, suggesting that reduced colonization observed clinically is not due to a direct effect. Synergy in bacterial killing by combining CFTR modulators and specific antimicrobials indicates possible additional benefit to treat CF lung infections. Schneider et al. (2016) found that using ivacaftor or ivacaftor/lumacaftor in combination with polymyxin B increases killing of *P. aeruginosa*. Ivacaftor in combination with vancomycin or ciprofloxacin increased the potency of these antibiotics against *S. aureus* and *P. aeruginosa,* respectively (Reznikov et al., 2014). Comparable studies with antifungal drugs are lacking. However, for the most commonly used antifungals, the mold-active azoles, an extra challenge is faced when co-prescribing these two drugs due to drug-drug interactions (Jordan et al., 2016).

In summary, CFTR modulators may have additional immunomodulatory benefits to prevent or treat *Aspergillus*-induced inflammation in CF. The comparable effects of CFTR modulators observed in phagocytes from control subjects questions their exact mechanism of action.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by East of Scotland Research Ethics Service (18/ES/0154) and the College Ethics Review Board of the University of Aberdeen (CERB/2016/8/1300). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AW and DA-J conceived and designed the study. AC and EM performed the experiments and analyzed the data. HW

provided expertise and analysis support. AC, AW, and HW wrote the manuscript. All authors contributed in the revision of the manuscript and approve of submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00372/full#supplementary-material

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Pneumocystis jirovecii in Patients With Cystic Fibrosis: A Review

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Pneumocystis pneumonia (PCP) remains the most frequent AIDS-defining illness in developed countries. This infection also occurs in non-AIDS immunosuppressed patients, e.g., those who have undergone an organ transplantation. Moreover, mild *Pneumocystis jirovecii* infections related to low pulmonary fungal burden, frequently designated as pulmonary colonization, occurs in patients with chronic pulmonary diseases, e.g., cystic fibrosis (CF). Indeed, this autosomal recessive disorder alters mucociliary clearance leading to bacterial and fungal colonization of the airways. This mini-review compiles and discusses available information on *P. jirovecii* and CF. It highlights significant differences in the prevalence of *P. jirovecii* pulmonary colonization in European and Brazilian CF patients. It also describes the microbiota associated with *P. jirovecii* in CF patients colonized by *P. jirovecii*. Furthermore, we have described *P. jirovecii* genomic diversity in colonized CF patients. In addition of pulmonary colonization, it appears that PCP can occur in CF patients specifically after lung transplantation, thus requiring preventive strategies. In other respects, *Pneumocystis* primary infection is a worldwide phenomenon occurring in non-immunosuppressed infants within their first months. The primary infection is mostly asymptomatic but it can also present as a benign self-limiting infection. It probably occurs in the same manner in CF infants. Nonetheless, two cases of severe *Pneumocystis* primary infection mimicking PCP in CF infants have been reported, the genetic disease appearing in these circumstances as a risk factor of PCP while the host-pathogen interaction in older children and adults with pulmonary colonization remains to be clarified.

Keywords: *Pneumocystis jirovecii*, *Pneumocystis* pneumonia, cystic fibrosis, pulmonary colonization, genomic diversity, microbiota, *Pneumocystis* primary infection, lung transplantation

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INTRODUCTION

Pneumocystis jirovecii is a transmissible fungus that causes severe pneumonia in immunocompromised patients (Walzer and Cushion, 2005). However, *Pneumocystis* infections cover a spectrum of presentations, in which PCP represents only a small proportion whereas most *Pneumocystis* infections correspond to mild diseases due to low levels of parasitism

(Morris and Norris, 2012). Indeed, polymerase chain reaction (PCR) assays have revealed that patients without PCP can be infected with a few organisms (Nevez et al., 1999). In these circumstances, the term "pulmonary colonization with *P. jirovecii*" is frequently used (Morris and Norris, 2012). Pulmonary colonization arises in immunocompromised patients as well as immunocompetent patients with underlying lung diseases. The potential role of the fungus as a morbidity cofactor has been suggested (Morris and Norris, 2012). Moreover, these colonized patients may act as infectious sources (Le Gal et al., 2012, 2015a). For these reasons, the characterization of colonized populations as well as that of *P. jirovecii* organisms in colonized populations are required. For instance, it has been established that patients with cystic fibrosis (CF) can be colonized by *P. jirovecii* (Calderón et al., 2010). CF is due to mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene that encodes a chloride channel involved in electrolytic exchanges and is associated with impairment of the mucociliary clearance (Ferec and Cutting, 2012). The resulting thickening of mucus favors pulmonary colonization or infections due to bacteria and fungi (Lipuma, 2010). The first data on pulmonary colonization with *P. jirovecii* in CF patients were obtained in Munich, Germany (Sing et al., 2001). Further studies provided additional data in this field (Respaldiza et al., 2005; Le Gal et al., 2010; Hernandez-Hernandez et al., 2012; Pederiva et al., 2012; Green et al., 2016; Nevez et al., 2018). Moreover, PCP can also occur in CF patients (Royce and Blumberg, 2000; Quattrucci et al., 2005; Solé et al., 2006; Kaur et al., 2016; Wojarski et al., 2018). The objective of this review is to compile available information on *P. jirovecii* and CF worldwide.

PULMONARY COLONIZATION WITH *P. JIROVECII* IN CF PATIENTS

Differences of *P. jirovecii* Prevalence in European and Brazilian CF Patients

In Munich, Germany, sputum specimens from 95 patients were prospectively assayed using a nested-PCR amplifying the mitochondrial large subunit rRNA (mtLSUrRNA) gene (Sing et al., 2001). *P. jirovecii* was detected in seven patients (7.4%). Two patients who were submitted to recurrent sampling remained positive for *P. jirovecii* detection over a 4–6-week period.

In Seville, Spain, sputum or oropharyngeal wash specimens from 88 patients were assayed using a nested-PCR amplifying the mtLSUrRNA gene. *P. jirovecii* was detected in 19 patients (21.6%) (Respaldiza et al., 2005).

In Brest, Brittany, France, sputum specimens from 76 patients were retrospectively assayed using both qPCR and nested-PCR amplifying the mtLSUrRNA gene (Le Gal et al., 2010). *P. jirovecii* was detected in one patient (1.3%). A second study was performed in Rennes, the largest city in Brittany, in the course of which sputum specimens from 86 patients were retrospectively assayed using the same qPCR (Nevez et al., 2018). The fungus was detected in three patients (3.5%).

In a French multicenter study, sputum specimens from 104 patients who lived in the following four cities, Angers, Bordeaux, Dunkirk and Lille, were prospectively analyzed using a conventional PCR combined with a qPCR amplifying the mtLSUrRNA gene (Hernandez-Hernandez et al., 2012). The fungus was detected in 13 patients (12.5%).

In Manchester, United Kingdom, sputum specimens from 111 patients were prospectively assayed using a qPCR amplifying the mtLSUrRNA gene (Green et al., 2016). The fungus was detected in nine patients (8.1%) with a higher frequency in specimens from patients presenting with pulmonary exacerbation than in those from patients without exacerbation (9.2 vs. 2%, $p = 0.03$). One patient, submitted to recurrent sampling, remained positive for *P. jirovecii* detection over a 3-month period.

In Porto Alegre, Brazil, BAL specimens from 34 patients were retrospectively assayed using a nested-PCR amplifying the mtLSUrRNA gene. *P. jirovecii* was detected in 13 patients (38.2%) (Pederiva et al., 2012).

The frequencies of *P. jirovecii* colonization in patients enrolled in these seven studies differ significantly. Specifically, the frequency observed in Brittany, Western France, differed from that was observed in Seville, Porto Alegre, and other French regions, whereas it did not differ from that observed in Munich and in Manchester (Sing et al., 2001; Respaldiza et al., 2005; Le Gal et al., 2010; Hernandez-Hernandez et al., 2012; Pederiva et al., 2012; Green et al., 2016; Nevez et al., 2018) (**Table 1**).

Pulmonary Microbiota and *P. jirovecii* Colonization in CF Patients

Pulmonary microbiota characteristics in CF patients with or without *P. jirovecii* colonization were partially described in four studies. In the Brest study, 74 out of 76 patients were colonized by bacteria and/or fungi (Le Gal et al., 2010). The low incidence of *P. jirovecii* (1.3%) was not compatible with any comparison of microbiota in patients colonized by *P. jirovecii* or not. It is noteworthy, that the patient colonized by *P. jirovecii* was also colonized by *Pseudomonas aeruginosa*. In the Rennes study, 86 patients were colonized by bacteria and/or fungi (Nevez et al., 2018). Likewise, the low incidence of *P. jirovecii* (3.5%), rendered any comparison difficult. The three patients colonized by *P. jirovecii* were also colonized by *Staphyloccus aureus*, *Stenotrophomonas maltophilia, Candida glabrata* (first patient), by *Saccharomyces* sp. and *Haemophilus influenzae* (second patient), by *Aspergillus fumigatus, Candida albicans, P. aeruginosa* (third patient). In the French multicenter study (Hernandez-Hernandez et al., 2012), the presence of *P. aeruginosa* mucoid-strains showed a negative association with *P. jirovecii* presence. In the Manchester study, no difference of microbiota in CF patients with exacerbation and with or without *P. jirovecii* colonization was observed (Green et al., 2016). These studies were based on the results of microbial cultures combined with those of *P. jirovecii* DNA amplification since this fungus is uncultivable. Recently, next generation sequencing (NGS) studies showed that the pulmonary microbiota of CF patients was more complex than initially supposed, considering the results of microbial cultures (Caverly et al., 2019; Cuthbertson et al., 2020;

TABLE 1 | Comparison of characteristics of cystic fibrosis (CF) patients who underwent *Pneumocystis jirovecii* detection in pulmonary specimens [partly reproduced from Nevez et al. (2018)].

	Germany (Munich)	**Spain (Seville)**	**Brittany, France (Rennes and Brest taken together)**	**Other French regions and cities (Pays de la Loire, Angers; South West, Bordeaux; North, Dunkirk and Lille)**	**Brazil (Porto Alegre)**	**United Kingdom (Manchester)**
Number of patients	95	88	162	104	34	111
Median age, years [range]	23.2	15.8 [1–35]	19.0 [3 months-41]	24.0 [15–32]	11.0 [1–35]	31+/– 10 (mean; sd)
Sex ratio M/F	52/43	41/47	81/81	50/54	17/17	59/52
Inclusion period	NA	May 2001-July 2002	February 2005-August 2007	October 2006-March 2009	March 2006 – August 2009	October 2013 – May 2014
Number of specimens	137	88	324	146	NA	226
Pulmonary samples examined for *Pneumocystis jirovecii* detection	Sputa	Sputa (54) and oropharyngeal washes (34)	Sputa	Sputa	BAL	Sputa
Technique of *Pneumocystis jirovecii* detection in pulmonary specimens	Nested PCR targeting mtLSUrRNA gene	Nested PCR targeting mtLSUrRNA gene	Real-time PCR targeting mtLSUrRNA gene	Conventional PCR followed by a real-time PCR targeting mtLSUrRNA gene	Nested PCR targeting mtLSUrRNA gene	Real-time PCR targeting mtLSUrRNA gene
Number of patients with a prior treatment with cotrimoxazole	0	2	NA (Rennes) 30 out of 76 (Brest)	NA	5 (cotrimoxazole or azithromycin in the 6 months preceding sampling)	102
Number of patients colonized with *Pneumocystis jirovecii*	7 (7.4%; CI 95: 2.1–12.7%)	19 (21.6%; CI 95: 12.9–30.1%)	4 (2.5%; CI 95: 0.1–4.9%)	13 (12.5%; CI 95: 6.1–18.9%)	13 (38.2%; CI 95%:21.9–54.5%)	9 (8.1%; CI 95%: 3–13.2%)

BAL, bronchoalveolar lavage; CI, confidence interval; F, female; M, male; mtLSUrRNA, mitochondrial large subunit ribosomal RNA; NA, not available.

Enaud et al., 2020; Soret et al., 2020). Nonetheless, none of these recent studies has specifically focused on the relationship between microbiota and *P. jirovecii* colonization.

P. jirovecii Genomic Diversity in CF Patients

The pioneer study on *P. jirovecii* genotyping in Seville identified three different mtLSUrRNA genotypes in 11 patients colonized by the fungus (Respaldiza et al., 2005). Genotype 1 (85C/248C) was the most prevalent (5/11, 45.5%) followed by genotype 3 (85T/248C) (3/11, 27.3%) and genotype 2 (85A/248C) (2/11, 18.2%). The genotype distribution was similar to that previously described in a Spanish population presenting PCP and chronic pulmonary diseases, but excluding CF (Montes-Cano et al., 2004).

In the multicenter French study, genotype 1 predominated in the overall population (∼60%), consistent with the results of the Spanish study (Respaldiza et al., 2005; Hernandez-Hernandez et al., 2012). However, geographic variations emerged with higher prevalence of genotype 2 in Dunkirk in the absence of genotype 3, whereas genotype 1 predominated in Angers in the absence of genotype 2 (Hernandez-Hernandez et al., 2012). In the other French study performed in Brittany, genotyping was successful in two out of the four patients colonized by *P. jirovecii* (Nevez et al., 2018). A 13-week old infant had a mixed infection with the combination of genotype 2 and genotype 3, associated with internal transcribed spacer (ITS) haplotype Jf. The second patient harbored genotype 2 in the absence of ITS haplotype identification. Both patients harbored a dihydropteroate synthase (DHPS) wild type (threonine55/ proline57). In the Brazilian study, mtLSUrRNA genotyping was successful in 12 patients and also showed a predominance of genotype 1 (5/12, 41.6%) and lower frequencies of genotype 3 (3/12, 25%) and genotype 2 (2/12, 16.6%). In addition, all patients harbored a DHPS wild type. A Spanish study has analyzed the longitudinal distribution of mtLSUrRNA genotypes in CF patients. During a 1-year follow-up, a continuous infection-and-clearance cycle of genotypes was observed with a switch from genotype 1 to genotype 3 (Montes-Cano et al., 2007). The results of another Spanish study were consistent, since a predominance of genotype 3 in CF adult patients was observed (Montes-Cano et al., 2006).

Pneumocystis Primary Infection in CF Infants

Pneumocystis primary infection is a common phenomenon in non-immunosuppressed infants without underlying diseases, with a peak of occurrence between the third and fifth months (Vargas et al., 2001; Larsen et al., 2007; Nevez et al., 2020). It occurs contemporaneously to a self-limiting infection in the course of which the pulmonary tract is colonized by the fungus (Vargas et al., 2001; Larsen et al., 2007; Nevez et al., 2020). Primary infection occurs probably in the same manner in CF infants. Consistently, in the course of the study performed in Rennes, *P. jirovecii* was detected in a 13-week old infant who recovered without specific treatment (Nevez et al., 2018). Nonetheless, two PCP cases in CF infants have been recorded in the literature. The first one concerned a 15-week old HIV-seronegative infant newly diagnosed with CF who underwent a BAL to determine pneumonia etiology. BAL examination revealed *P. jirovecii* asci and pathogens usually associated with CF, such as *S. aureus* and *P. aeruginosa*. The patient recovered after cotrimoxazole treatment (Royce and Blumberg, 2000). The second one concerned a 16-week old HIV-seronegative infant newly diagnosed with CF who underwent a BAL to investigate chronic cough etiology. BAL examination revealed *P. jirovecii* asci and *P. aeruginosa*. The patient recovered after cotrimoxazole treatment (Kaur et al., 2016). The young age of the two infants strongly suggested that they effectively developed *Pneumocystis* primary infection. These two case-reports showed that PCP contemporaneous to severe primary infection occurs in CF infants in the apparent absence of risk factors other than the genetic disease itself.

PNEUMOCYSTIS PNEUMONIA IN CF PATIENTS AFTER LUNG TRANSPLANTATION

Beyond pulmonary colonization, overt PCP occurs in CF patients after lung transplantation due to immunosuppressive therapy. In Spain, in the course of a 14-year follow-up, 57 CF patients underwent lung transplantation. Surprisingly none developed PCP (Solé et al., 2006). In Italy, in the course of a 6-year follow-up, 55 CF patients underwent lung transplantation. Three patients developed PCP (5.4%), two of whom died (Quattrucci et al., 2005). In Poland, in the course of a 7-year follow-up, 21 CF patients underwent lung transplantation. Eight patients (38%) developed PCP (Wojarski et al., 2018). Thus, the risk of PCP in CF patients after lung transplantation appears highly variable.

GENERAL DISCUSSION

There are several hypotheses to explain differences in prevalence of pulmonary colonization with *P. jirovecii* in European and Brazilian CF patients.

Differences may result from technical issues. In the European studies, available specimens were sputa whereas in the Brazilian study it was BALs which provided high-quality cells from the alveolus and consequently a higher sensitivity to *P. jirovecii* detection (Pederiva et al., 2012). A qPCR assay was used in the Brittany study (Nevez et al., 2018) instead of two-step PCR assays which were performed in the other studies (Sing et al., 2001; Respaldiza et al., 2005; Hernandez-Hernandez et al., 2012; Pederiva et al., 2012; Green et al., 2016). Sputum examination or the qPCR assay might have been less sensitive than BAL examination and the two-step-PCR assays.

Climatic parameters may affect *P. jirovecii* presence in CF patient populations and may explain geographical variations of prevalence. The factor of a Mediterranean climate in Seville and a temperate, maritime climate in Brittany has already been discussed (Le Gal et al., 2010; Nevez et al., 2018). Unfortunately, information was mainly obtained from Europe and Brazil, which limited the discussion on putative relationship between geographical location and *P. jirovecii* incidence in CF patients. Nonetheless, geographical variations could be non-independent

factors (Miller et al., 2007, 2018). Indeed, population densities in European regions that differ from each other may affect inter-individual encounters and consequently *P. jirovecii* transmission and acquisition.

A history of cotrimoxazole treatment for bacterial infections may play a role in *P. jirovecii* clearance from the lungs, thus influencing the results of *P. jirovecii* detection. For instance, two out of 88 patients in Seville compared to 30 out of 76 patients in Brest had a history of cotrimoxazole treatment (2.2 vs. 39.4% $p < 0.001$) (Respaldiza et al., 2005; Le Gal et al., 2010). Furthermore, in Manchester, *P. jirovecii* was more frequent in patients not treated with cotrimoxazole (29.7 vs. 0%, $p = 0.03$) (Green, 2015).

Attention should be paid to other patients' characteristics, such as CFTR gene mutations. Two of the three patients in the Rennes study who tested positive for *P. jirovecii* were heterozygous for the F508Del mutation (Nevez et al., 2018). Nonetheless, considering the lack of information in the other studies a putative relationship between the presence of *P. jirovecii* and CFTR mutations remains speculative, requiring further evaluation.

The presence of *P. jirovecii,* its relationship with patients' age, pulmonary microbiota and the severity of CF must be debated. Bacterial infections arise earlier than fungal infections during the disease's progression, both acting as aggravating factors in respiratory dysfunction (Caverly et al., 2019; Delhaes et al., 2019; Cuthbertson et al., 2020). CF patients are initially infected with *H. influenzae* and *S. aureus*, and further develop infections with *P. aeruginosa* or *Burkholderia cepacia* complex. The use of antibiotics to prevent or treat infections by these bacteria, has resulted in improving the clinical outcome and increasing the life expectancy of CF patients (Castellani et al., 2018). Consequently, they are at risk for mycoses (Schwarz et al., 2018) but not for *P. jirovecii* infections, according to our analysis. The median ages of the patients enrolled in the Spanish (15.8 years) and Brazilian (11 years) studies (Respaldiza et al., 2005; Pederiva et al., 2012) were lower than those of the patients enrolled in the French multicenter study (23.5 years) (Hernandez-Hernandez et al., 2012), the Brittany study (19 years) (Nevez et al., 2018), and the British study (mean age, 31 years) (Green et al., 2016). The colonization with *P. jirovecii* may be transient and may occur earlier during CF progression, which is consistent with the presence of *P. jirovecii* in younger Spanish and Brazilian patients and the scarcity of *P. jirovecii* detection in older Breton and British patients.

In vivo competitive inhibition between species and networks between fungal and bacterial kingdoms in CF may exist (Soret et al., 2020). For instance, *P. aeruginosa* may limit the spread of *P. jirovecii* within the lungs. Indeed, in the French multicenter study, the patients colonized by *P. aeruginosa* mucoid strains were less likely to be colonized by *P. jirovecii* (Hernandez-Hernandez et al., 2012). Moreover, *P. jirovecii* colonization was associated with less severe lung disease according to FEV1 values, which appeared consistent with the negative correlation between *P. jirovecii* and *P. aeruginosa* mucoid strains. On the other hand, *P. jirovecii* colonization was associated with *P. aeruginosa* colonization in Brazilian patients (Pederiva et al., 2012). However, it remains unknown whether these patients were specifically infected with mucoid strains. and the relationship between *P. jirovecii* and mucoid strains is only one of the possible hypotheses.

P. jirovecii's role as an aggravating factor on its own is unclear in CF patients, while it has been previously suggested in patients with COPD (Morris and Norris, 2012). The Manchester study may be consistent with this role in CF patients since the presence of *P. jirovecii* was significantly associated with exacerbation (Green et al., 2016), in contrast to the French multicenter study (Hernandez-Hernandez et al., 2012), in which the presence of *P. jirovecii* was associated with less severe lung diseases. We can hypothesize that *P. jirovecii* provokes a local inflammation facilitating subsequent infections such as by *P. aeruginosa*, in the course of which the fungus could be detectable or not (Ulrich et al., 2010; Döring et al., 2011; Hernandez-Hernandez et al., 2012; Pederiva et al., 2012). Finally, whether the fungus's presence is the cause or the result of exacerbation remains an open question.

Most data on microbiota resulted from routine culture and identification. Today, NGS methods allow identifying a higher number of microbial species, and thus to analyze cooperative, competitive and/or adaptive interactions between microorganisms that play a role in CF disease progression (Quinn et al., 2014; Layeghifard et al., 2019; Soret et al., 2020). However, these methods are mainly based on amplification of ITS sequences to analyze mycobiota, *i.e.,* fungal microorganisms. These sequences are present in multicopies in most fungal genomes but are unfortunately present in a single copy in the *P. jirovecii* genome (Nahimana et al., 2000). Thus, despite their potential efficiency, NGS methods may not be sensitive enough to capture *P. jirovecii* sequences present in relative average/low abundance within the mycobiota profiles (Delhaes et al., 2012).

Analyses of the results of *P. jirovecii* genotyping from cross-sectional studies must be made cautiously. Indeed, a continuous colonization/clearance cycle with changes of mtLSUrRNA genotype prevalence, e.g., with a switch of genotype 1 to genotype 3 has been identified (Montes-Cano et al., 2007). Other studies not focusing on CF patients suggest that single-nucleotide polymorphism (SNP) at position 85 is somehow correlated to fungal burden levels and virulence. A putative link between SNP 85T or 85A, high fungal burdens and infection severity has been suggested whereas SNP 85C may be related to low/average burdens and less severe infections (Esteves et al., 2010a,b, 2016). The switch from genotype 1 (85C/248C) to genotype 3 (85T/248C) in CF patients who, nevertheless, do not develop severe infections remains unclear. Genotype 3 may be the most adapted in the course of CF disease progression. It is unfortunate that, in the Manchester study which highlighted significant association of *P. jirovecii* and exacerbation, genotyping was not performed. No DHPS mutant-types were identified in patients from Brittany or Brazil, consistent with the general low prevalence of mutant types previously reported in these two locations (Wissmann et al., 2006; Le Gal et al., 2013a). In the Brittany study, a 13-week old infant at risk for *Pneumocystis* primary infection harbored a combination of mtLSUrRNA genotype 2 and genotype 3, associated with ITS

haplotype Jf and a DHPS wild type (Nevez et al., 2018). No additional data on genotypes in a context of primary infection in CF infants are available. Indeed, the two case reports on severe primary infection that we mentioned above did not provide any information about genotypes (Royce and Blumberg, 2000; Kaur et al., 2016). Conversely, data exist on genotypes in non-CF infants developing primary infection (Totet et al., 2003a,b; Nevez et al., 2020). In Brittany, *P. jirovecii* genotyping was performed in non-immunosuppressed infants developing symptomatic primary infection. MtLSUrRNA genotype 2 was the most frequent (13/21, 61, 9%) whereas, none harbored genotype 1. Thus, the presence of genotype 2 in the aforementioned CF infant may reflect particular geographical characteristics of the fungus in infants living in this region. ITS haplotype Jf has rarely been described in France, whatever the patient population (range, 0–7.5%), whereas Eg is the most frequent haplotype (range, 42.8–64.5%) (Nevez, 2003; Le Gal et al., 2013b, 2015b). The presence of type Jf in this infant could simply be a random event. Finally, all these genotypic characteristics were obtained using a unilocus approach and Sanger's method of sequencing, while more informative assays such as multilocus sequence typing (MLST) combined with NGS are available today (Alanio et al., 2016; Charpentier et al., 2017). Moreover, shotgun metagenomics may represent an alternative method to identify pulmonary mycobiota including *P. jirovecii* organisms (Irinyi et al., 2020).

The detection of *P. jirovecii* does not seem to be predictive of subsequent PCP occurrence since no colonized patients enrolled in the above-mentioned studies developed PCP. In the absence of profound immunodeficiency, the shift from colonization to PCP did not occur. In lung transplant recipients with iatrogenic immunodeficiency, attack rates of PCP have been assessed at 5–5.8% or 6.5–43% in patients with or without chemoprophylaxis, respectively (review in reference Iriart et al., 2015). However, precise data on attack rates of PCP among transplanted CF patients were not provided in this review. The differences in PCP occurrence in CF patients from Spain, Italy, and Poland (Quattrucci et al., 2005; Solé et al., 2006; Wojarski et al., 2018) may be explained by different uses of PCP chemoprophylaxis.

Although rare, PCP can also occur contemporaneously to *Pneumocystis* primary infection in CF infants (Royce and Blumberg, 2000; Kaur et al., 2016) and the medical community should be aware of this. Early impairment of pulmonary function due to the genetic disease, in *P. jirovecii* immune-naïve infants, in the apparent absence of immunosuppression, could be the cause of PCP occurrence. Indeed, this early impairment is objectivized by the presence of *S. aureus* and *P. aeruginosa* in infants a few weeks old, while these bacteria are usually detected in older patients with pulmonary dysfunction and more advanced disease. Conversely, this hypothesis is not consistent with the negative association between mucoid *P. aeruginosa* and *P. jirovecii* suggested in the French multicenter study (Hernandez-Hernandez et al., 2012). Nevertheless, microorganism interactions in CF patients including infants are certainly complex. Moreover, whether PCP prophylaxis in CF infants is required remains an open question.

Finally, data on *P. jirovecii* and CF are fragmented. This topic remains an open field of investigation. A multicenter prospective study comprising identical methods of *P. jirovecii* screening in CF patients including those who have undergone lung-transplantation, with patient-age stratification, CFTR mutation analysis, and MLST/NGS analysis of the *P. jirovecii* genome combined with microbiome/mycobiome examination is warranted.

AUTHOR CONTRIBUTIONS

PB and GN: wrote, corrected, and submitted the manuscript. SL: wrote, corrected, and provided biological data from CF patients in Brest. EC: wrote the paragraphs on genomic diversity. LD: wrote paragraphs on microbiota. DQ: performed biological diagnoses and provided biological data from CF patients in Brest. FR-G: read and corrected the manuscript, wrote the reference section, and provided biological data from Rennes. SR: provided clinical data from CF patients and represents the Réseau Muco-Ouest. All authors contributed to the article and approved the submitted version.

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Modulated Response of *Aspergillus fumigatus* and *Stenotrophomonas maltophilia* to Antimicrobial Agents in Polymicrobial Biofilm

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Introduction: The complexity of biofilms constitutes a therapeutic challenge and the antimicrobial susceptibility of fungal-bacterial biofilms remains poorly studied. The filamentous fungus *Aspergillus fumigatus* (Af) and the Gram-negative bacillus *Stenotrophomonas maltophilia* (Sm) can form biofilms and can be co-isolated from the airways of cystic fibrosis (CF) patients. We previously developed an *in vitro* biofilm model which highlighted the antibiosis effect of Sm on Af, which was dependent on the bacterial fitness. The aim of the present study was to investigate the *in vitro* susceptibility of Af and Sm in mono- or polymicrobial biofilms to five antimicrobial agents alone and in two-drug combinations.

Methods: Af and Sm clinical reference strains and two strains from CF sputa were tested through a planktonic and biofilm approaches. Af, Sm, or Af-Sm susceptibilities to amphotericin B (AMB), itraconazole (ITC), voriconazole (VRC), levofloxacin (LVX), and rifampicin (RFN) were evaluated by conventional planktonic techniques, crystal violet, XTT, qPCR, and viable plate count.

Results: Af planktonic cells and biofilms in formation were more susceptible to AMB, ITC, and VRC than Af mature biofilms. Af mature biofilms were susceptible to AMB, but not to ITC and VRC. Based on viable plate count, a lower concentration of LVX and RFN was required to reduce Sm cell numbers on biofilms in formation compared with mature biofilms. The antibiosis effect of Sm on Af growth was more pronounced for the association of CF strains that exhibited a higher fitness than the reference strains. In Af-Sm biofilms, the fungal susceptibility to AMB was increased compared with Af biofilms. In contrast, the bacterial susceptibility to LVX decreased in Af-Sm biofilms and was fungal biomass-dependent. The combination of AMB (64 μg/mL) with LVX or RFN (4 μg/mL) was efficient to impair Af and Sm growth in the polymicrobial biofilm.

Conclusion: Sm increased the Af susceptibility to AMB, whereas Af protected Sm from LVX. Interactions between Af and Sm within biofilms modulate susceptibility to antimicrobial agents, opening the way to new antimicrobial strategies in CF patients.

Keywords: *Aspergillus fumigatus*, *Stenotrophomonas maltophilia*, polymicrobial biofilm, antimicrobial susceptibility, antifungal agent, antibacterial agent

INTRODUCTION

Biofilm-embedded cells have specific characteristics, which distinguish them from planktonic cells, namely their lower susceptibility to drugs explained in particular by the presence of self-produced protective matrix and the reduction of microbial metabolic activity in the biofilm (Desai et al., 2014; Lebeaux et al., 2014; Flemming et al., 2016).

Many microorganisms are commonly co-isolated from the airways of cystic fibrosis (CF) patients (Botterel et al., 2018; Granchelli et al., 2018), including the filamentous fungus *Aspergillus fumigatus* (Burgel et al., 2016) and the Gram-negative bacillus *Stenotrophomonas maltophilia* (Esposito et al., 2017). Approximatively, 10% of the French CF patients carry in their airways *S. maltophilia*, and 30 % carry *Aspergillus* (French, 2019), with common co-infections, as recently shown in a very large cohort of CF patients (Granchelli et al., 2018). Treatment of these pathogens in chronic respiratory diseases is often difficult due to their multidrug-resistant nature, especially for *S. maltophilia*, and to their biofilm-forming ability (Flores-Treviño et al., 2019). The presence of microbial aggregates and biofilms have already been observed in the respiratory tract of CF patients, which is a favorable environment for biofilm formation (Bjarnsholt et al., 2009; Ramage et al., 2011; Kragh et al., 2014). *S. maltophilia* biofilm was documented in the sputum of CF patients (Høiby et al., 2017), although there is no direct evidence supporting the presence of *Aspergillus* biofilm *in vivo* in CF patients.

The complexity of biofilm structure constitutes a therapeutic challenge since infections are often treated with drugs selected according to the results of susceptibility testing of microorganisms in planktonic form. Furthermore, Keays et al. (2009) showed a better clinical outcome when CF patients were treated with efficient biofilm-targeting agents. The *in vitro* antifungal tolerance of *A. fumigatus* in biofilm has already been reported (Mowat et al., 2007; Seidler et al., 2008; Bugli et al., 2013; Luo et al., 2018). Mowat et al. (2007) showed that amphotericin B, itraconazole, voriconazole, and caspofungin were 1,000 times less efficient on biofilm-life form than on planktonic form. Several studies showed that levofloxacin could be an alternative to treat *S. maltophilia* infections (King et al., 2010; Wu et al., 2013; Herrera-Heredia et al., 2017; Pompilio et al., 2020). This molecule could reduce biofilm biomass (Di Bonaventura et al., 2004; Passerini de Rossi et al., 2009), but some levofloxacin-resistant strains emerge (Wang et al., 2020). Another study suggested the combination of old alternatives, such as rifampicin, with newer agents for critically ill patients infected with *S. maltophilia* (Savini et al., 2010). Rifampicin is known for its anti-biofilm activity and synergistic effect with several antibiotics targeting Gram-positive bacteria (Tang et al., 2013; Yan et al., 2018).

Regarding microbial intra-kingdom interactions, the antimicrobial susceptibility of different bacterial species growing inside polymicrobial biofilms has been investigated (Pompilio et al., 2015; Cendra et al., 2019; Rodríguez-Sevilla et al., 2019). Cross-kingdom interactions impact on antimicrobial susceptibility is still poorly studied, but some authors have described the antimicrobial susceptibility of *Candida albicans* yeast in polymicrobial biofilm with *Staphylococcus aureus* (Harriott and Noverr, 2009; Kong et al., 2016; Rogiers et al., 2018) or *Cutibacterium acnes* (Bernard et al., 2018). To our knowledge, only (Manavathu et al., 2014; Manavathu and Vazquez, 2015) described the antimicrobial susceptibility of an *in vitro* filamentous fungal (*A. fumigatus*) and bacterial (*Pseudomonas aeruginosa*) biofilm. In that model, *A. fumigatus* had the same antifungal susceptibility in mono- and polymicrobial biofilms. Regarding the susceptibility of *P. aeruginosa*, cefepime and imipenem were significantly less efficient in the polymicrobial biofilm than in the monomicrobial biofilm.

We previously showed that *S. maltophilia* inhibited *A. fumigatus* growth and modified hyphae development in a polymicrobial biofilm (Melloul et al., 2016), with strain-dependent manner (Melloul et al., 2018). The aim of the present study was to investigate the *in vitro* antimicrobial response of *A. fumigatus* and *S. maltophilia* in our polymicrobial biofilm in comparison with monomicrobial biofilm.

MATERIALS AND METHODS

Strains and Standardization of Inocula

Aspergillus fumigatus (Af) ATCC 13073-GFP (Wasylnka and Moore, 2002) expressing a constitutive Green Fluorescent Protein (AF_REF), and *S. maltophilia* (Sm) ATCC 13637 (SM_REF) were the clinical reference strains used in this study. Two other clinical strains obtained from sputa of CF patients, named AF_CF and SM_CF, were used. AF_CF is *A. fumigatus sensu stricto* as identified by molecular technique using sequence analysis of beta-tubulin gene as previously described (Loeffert et al., 2017). The genomic phylogeny of Sm was recently updated (Vinuesa et al., 2018) and *S. maltophilia* complex was defined, including Sm *sensu stricto* and several related genospecies. SM_CF is *S. maltophilia sensu stricto* through whole genome sequencing and phylogenomic analysis (Mercier-Darty et al., 2020). Both SM_REF and SM_CF belong to genogroup 6 (Hauben et al., 1999; Mercier-Darty et al., 2020), and no

Abbreviations: Af, *A. fumigatus;* Sm, *S. maltophilia;* AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; LVX, levofloxacin; RFN, rifampicin.

major difference in resistance genes was found. Af strains were cultured on 2% malt agar containing 0.05% chloramphenicol at 37°C for 5 days. The fungal suspensions were prepared as previously described to obtain an inoculum of 10^5 conidia/mL in 3-(N-morpholino) propanesulfonic acid (MOPS) - buffered RPMI 1640 [pH 7.0] with 2% glucose (G) + 10 % fetal bovine serum (FBS) (Sigma-Aldrich, France) (Melloul et al., 2016). Sm strains were streaked out on Luria-Bertani (LB) agar plate at 37°C for 24 h. The bacterial suspensions were also prepared as previously reported to obtain an inoculum of 10^6 bacteria/mL (Melloul et al., 2016). These inocula were used to test antimicrobial susceptibilities of planktonic cells and biofilms.

Fungal, Bacterial, and Polymicrobial Biofilm Formation

The *in vitro* biofilm formation in the 96-well plates (Thermo Fisher Scientific Inc, France) was adapted from the protocol previously described (Melloul et al., 2016). Briefly, 50 μL of the fungal (10^5 conidia/mL) or bacterial (10^6 bacteria/mL) inoculum was added to 50 μL of MOPS–RPMI (2 % G) + 10 % FBS to form Af or Sm monomicrobial biofilm. The Af-Sm polymicrobial biofilm was produced by simultaneous inoculation of 50 μL of each inoculum per well. The tested microbial associations were AF_REF + SM_REF and AF_CF + SM_CF. Plates were incubated at 37°C in static condition for 24 h to obtain mature biofilms (biofilm-embedded cells), then washed twice with PBS to remove planktonic cells.

Antimicrobial Agents

Pure antimicrobial powders were obtained from Sigma-Aldrich, France. The antifungal stock solutions of amphotericin B (AMB), itraconazole (ITC), and voriconazole (VRC) were prepared at 10 mg/mL in dimethylsulfoxide (DMSO). The antibiotic stock solutions of levofloxacin (LVX) and rifampicin (RFN) were prepared at 6.4 mg/mL in sterile distilled water and DMSO, respectively. Stock solutions were kept at −20°C until used. Working solutions were then adjusted in MOPS–RPMI (2% G).

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Planktonic Cells

The MICs of AMB, ITC, and VRC were determined by the EUCAST reference microdilution broth technique (Arendrup et al., 2020). The MICs of LVX and RFN were determined following the recommendations from the International Standards Organization (ISO, 2019). MIC was defined as the lowest concentration of drug required for complete growth inhibition with a visual endpoint for Af and a spectrophotometric endpoint for Sm using a microplate reader set at 550 nm (Multiskan FC®, Thermo Fisher Scientific Inc, France). To further compare planktonic and biofilm-embedded cells susceptibilities, the culture conditions of planktonic cells were adjusted to 10^5 conidia/mL or 10^6 bacteria/mL and were prepared in MOPS–RPMI (2% G) + 10% FBS medium. In such conditions, the minimum inhibitory concentration (MIC_b) was determined after 24 h of culture using same endpoints as above (**Figure 1A**).

MBC was evaluated and defined as the lowest concentration of antibiotic required to reduce Sm CFU by 99.9% as compared with the initial inoculum. MBC was determined by plating 200 μL from each well that showed no visible growth on cation-adjusted Mueller-Hinton (CAMH) (Sigma-Aldrich, France) agar plates incubated at 37°C for 48 h, and CFUs were enumerated. Each experiment was performed in triplicate.

Determination of Minimum Biofilm Inhibitory Concentration (MBIC) and Viability of *S. maltophilia* Cells Extracted From Biofilm in Formation

To determine the effects of antimicrobial agents on Af and Sm biofilm formation, 50 μL of the fungal (10^5 conidia/mL) or bacterial (10^6 bacteria/mL) inoculum was mixed with 50 μL of the antimicrobial agent (2X final concentration) into wells of the 96-well plates and incubated at 37°C in static condition for 24 h. Then, the supernatant was removed, and plates were washed twice with PBS. The final concentration range of the antimicrobial agent was 0.06–8 μg/mL. The lowest concentration of drug required to inhibit at least 90% of the biofilm formation (MBIC) was assessed by the crystal violet (CV) staining method (biomass measurement), and by the 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5carboxanilide (XTT) reduction method (metabolic activity measurement) (**Figure 1B**). Wells were stained with 200 μL of CV (0.02% for Af, 0.1% for Sm) for 30 min at room temperature, then washed thrice with PBS before adding 200 μL of 30% acetic acid for 10 min. The XTT reduction method was used according to Pierce et al. (2008) with minor modifications. Briefly, a final solution containing 0.5 mg/mL of XTT (Invitrogen, France) + 50 μM (Af) or 10 μM (Sm) of menadione (Merck, Germany) was added into wells and plates were incubated in the dark at 37°C for 2 h. The optical density values of blank wells were subtracted from the test wells. Each test was run in triplicates and three independent experiments were performed.

The viability of adherent bacterial cells was checked on agar plates. The MBIC based on CFU enumeration ($MBIC_{CFU}$) was defined as the lowest concentration of antibiotic required to reduce Sm cell number by 90% on biofilm in formation. Following antibiotic exposure of Sm inoculum, wells were washed, and adherent cells were scraped with swab and plated on LB agar for 24 h at 37°C. All assays were performed in triplicate and repeated three times.

Determination of Minimum Biofilm Eradication Concentration (MBEC) and Viability of *S. maltophilia* Cells Extracted From Mature Biofilm

Monomicrobial (Af or Sm) mature biofilms were exposed to a range of concentrations of drugs (100 μL) at 37°C for 24 h. The highest concentrations were 256 μg/mL for antifungal and 32 μg/mL for antibacterial agents. For each experiment, some biofilms were not treated with drugs (untreated biofilms).

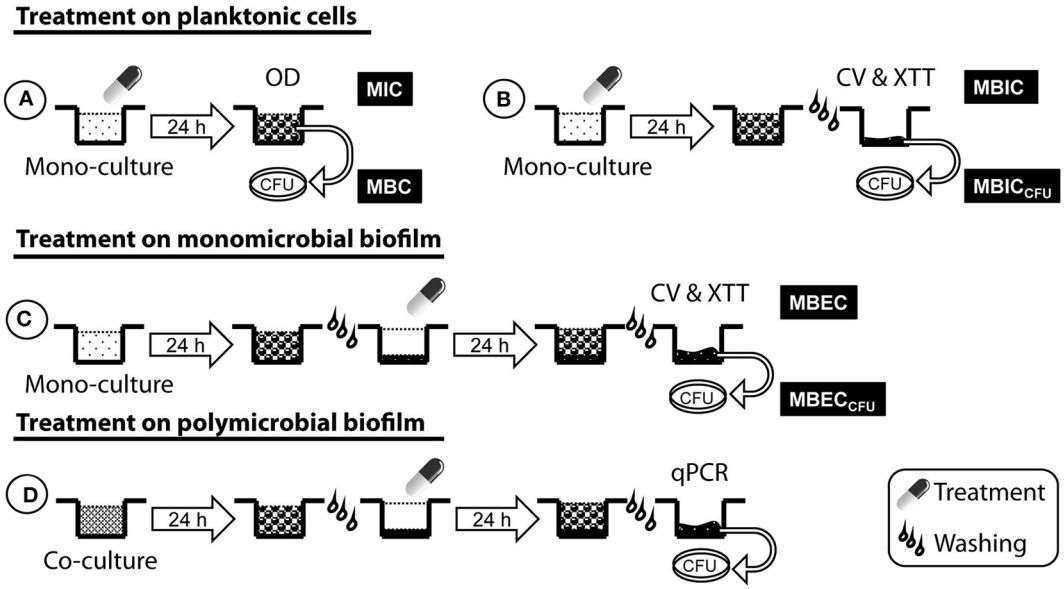

FIGURE 1 | Antimicrobial susceptibility testing of planktonic cells and biofilm forms. **(A,B)** Mono-cultures with conidia or bacteria were simultaneously inoculated with antimicrobial agents for 24 h. Then, the optical density (OD) was measured to obtain the MIC (in planktonic cells), and the wells were washed before crystal violet (CV) and XTT analyses to determine the drug concentration that inhibited the biofilm formation (MBIC). **(C,D)** Mono- and co-cultures were incubated for 24 h to obtain mature mono- and polymicrobial biofilms, and then treated with antimicrobial agents for another 24 h. **(C)** CV and XTT analyses on monomicrobial biofilm enabled determining the drug concentration that eradicated the mature biofilm (MBEC). The viability of Sm following antibacterial treatment was assessed on planktonic cells (MBC), adherent cells of biofilm in formation (MBIC$_{CFU}$), and mature biofilm (MBEC$_{CFU}$). **(D)** Antimicrobial susceptibility of mono- and polymicrobial biofilms was compared using qPCR and viable plate count.

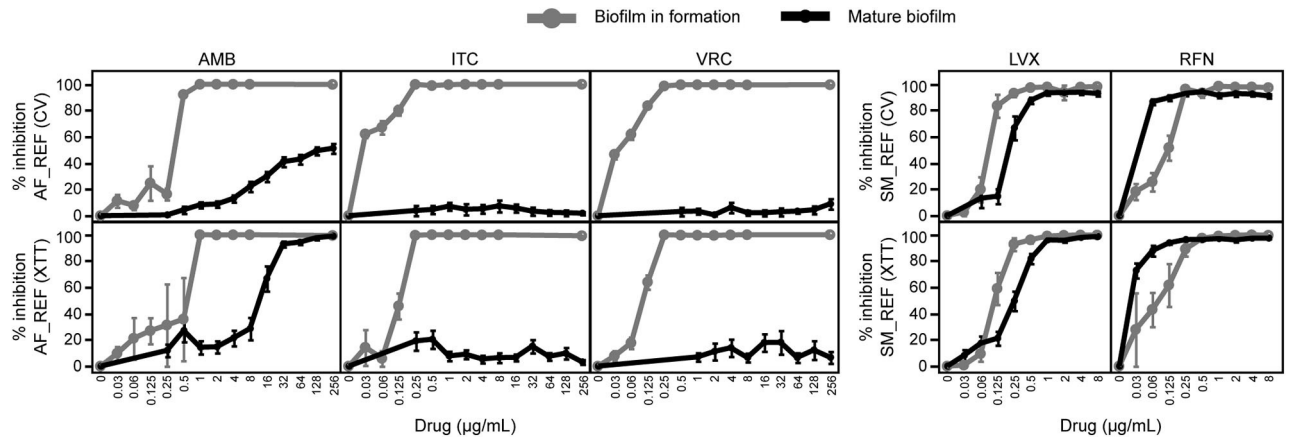

FIGURE 2 | Susceptibilities of biofilms in formation and mature biofilms to drugs. The biomasses and the metabolic activities of biofilms were measured by CV and XTT methods, respectively. Results were expressed in percentages of AF_REF or SM_REF inhibition after antimicrobial treatment compared with untreated controls. AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; LVX, levofloxacin; RFN, rifampicin.

Following washing, the susceptibility testing was performed using CV and XTT (**Figure 1C**) as described above. Both methods helped determine the MBEC, which was defined as the lowest concentration of drug required to eradicate at least 90% of mature biofilm.

The MBEC based on CFU enumeration (MBEC$_{CFU}$) was evaluated and defined as the lowest concentration of antibiotic required to reduce Sm cell number by 90% on mature biofilm. Following antibiotic treatment of Sm biofilm, wells were washed, and adherent cells were scraped with swab and plated on LB

TABLE 1 | Efficacy of drugs to inhibit growth of planktonic cells and to reduce metabolic activity of biofilm cells.

Strain	Drug	MIC	MIC_b	$MBIC_{XTT}$	$MBEC_{XTT}$
AF_REF	AMB	2	1	1	32
	ITC	0.50	0.25	0.25	>256
	VRC	0.50	0.25	0.25	>256
AF_CF	AMB	2	1	1	8
	ITC	0.50	0.25	0.25	>256
	VRC	0.50	0.25	0.25	>256
SM_REF	LVX	0.125	0.125	0.25	1
	RFN	1	0.25	0.25	0.125
SM_CF	LVX	0.50	0.50	1	2
	RFN	4	2	2	2

MIC_b represented MIC assessed with adapted culture conditions. Concentrations are expressed in μg/mL. AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; LVX, levofloxacin; RFN, rifampicin; MIC, minimum inhibitory concentration; MBIC, minimum biofilm inhibitory concentration based on XTT readout; MBEC, minimum biofilm eradication concentration based on XTT readout.

agar for 24 h at 37°C. All assays were performed in triplicate and repeated three times.

Determination of Polymicrobial Biofilm Susceptibility by Quantitative PCR

Polymicrobial (Af-Sm) mature biofilms were exposed to a range of concentrations of AMB or LVX at 37°C for 24 h. Two-drug combination experiments were performed with AMB at 64 μg/mL and LVX or RFN at 4 μg/mL. The quantity of Af and Sm DNAs was investigated in the drug-treated or drug-free biofilms by qPCR (**Figure 1D**). Following washing, biofilms were frozen at −20°C for 24 h. After thawing, biofilms were covered with 250 μL of tissue lysis buffer (ATL, Qiagen GmbH, Germany) and prepared as previously described (Melloul et al., 2016). DNA extraction using QIAamp DNA Mini Kit (Qiagen GmbH, Germany) was performed and the qPCR test was conducted following the protocol previously described (Melloul et al., 2016). Data were analyzed using LightCycler software V3.5 and results were expressed in conidial equivalent (CE) or bacterial equivalent (BE) in comparison with a standard curve plotted on DNA samples extracted from co-inoculated solutions with different concentrations of conidia (1–10^8 conidia) and bacteria (10–10^9 bacteria). Results obtained from treated biofilms were expressed in percentage of biomass inhibition compared with untreated biofilms. Each testing condition was performed in duplicate for three independent experiments.

Effect of *A. fumigatus* on *S. maltophilia* Biofilm Susceptibility

Comparative Analysis of Viable Bacterial Counts in Monomicrobial and Polymicrobial Biofilms

The effect of Af biomass on the susceptibility of Sm was assessed by a comparative analysis of viable bacterial counts extracted from mono- and polymicrobial biofilms (**Figure 1D**). One strain association (AF_REF + SM_REF) was used, and the response of SM_REF to LVX (1, 4, and 32 μg/mL) or AMB (64 μg/mL) + LVX (4 μg/mL) was assessed. Biofilms were thoroughly scraped with 200 μL of PBS and collected into tubes, and that was repeated twice for vigorous agitation using MagNA Lyser Instrument (Roche, France). Serial 10-fold dilutions up to 10^{-5} in PBS were performed and 100 μL from each dilution was plated on LB agar supplemented with 16 μg/mL of ITC to prevent Af growth. The number of CFUs was determined after 24 h of incubation at 37°C. Results were expressed in percentage of survival compared with untreated biofilms. All assays were repeated three times for three independent experiments.

Effect of Fungal Matrix Degradation on *S. maltophilia* Biofilm Susceptibility

To investigate the effect of AF_REF on the response of SM_REF to LVX, bacterial viability was assessed after enzymatic pretreatment intended to degrade the fungal biofilm extracellular matrix (ECM). The enzymatic degradation protocol was based on a previous research (De Brucker et al., 2015). For such, following Sm and Af-Sm biofilm formation, samples were washed and covered with MOPS–RPMI (2% G) containing 50 μg/mL proteinase K (Qiagen GmbH, Germany) for 2 h at 37°C or with MOPS–RPMI (2% G) as control. Then, biofilms were exposed to LVX at 1 μg/mL for 24 h at 37°C. Bacterial viability was determined using viable plate count as described above. The experiment was performed in triplicate and repeated three times.

Confocal Laser Scanning Microscopy (CLSM) Observations

For microscopic analyses, biofilms were developed on Lab-Tek™ slides (Thermo Fisher Scientific Inc, France) in a damp chamber. After 16 h of incubation, wells were washed with PBS. AF_REF, which expresses GFP, was visualized with FITC filter. AF_CF was visualized after Calcofluor-white staining (Invitrogen, France) using DAPI filter. Phenotypic modifications of Af in polymicrobial biofilm were investigated and compared with Af phenotype in monomicrobial biofilm by CLSM. Images of biofilms were obtained by Zeiss LSM 510 META confocal (Zeiss, Germany).

Transmission Electron Microscopy (TEM) Observations

Effects of LVX (8 μg/mL) on polymicrobial biofilms were investigated by TEM. Biofilms were prepared as previously described (Melloul et al., 2016). Briefly, biofilms were first fixed with 2.5% glutaraldehyde-cacodylate (pH 6.5) and then with 2% osmium tetroxide buffer. The fixed samples were dehydrated using a graded ethanol series and embedded in EPON resin for at least 72 h. Ultra-fine sections were cut via ultramicrotome (Leica EM UC7), gently collected on grids, and stained with lead-citrate and uranyl-acetate solutions before observation under TEM (JEOL 100 CX II instrument, Japan).

Data Analyses

Linear regression and Spearman's rank correlation helped determine the relationship between CV and XTT results. Data

failed the normality test (Shapiro–Wilk), hence the use of non-parametric tests. Comparisons of responses of mono- and polymicrobial biofilms to antimicrobial concentrations were performed using multiple linear regressions. Pairwise comparisons relied on Wilcoxon test. Statistical analyses were conducted using JMP 14.0 software. $P \leq 0.05$ were considered statistically significant.

RESULTS

Comparison of Colorimetric Methods

The effects of various drug concentrations on AF_REF and SM_REF biofilms depending on the used colorimetric method (i.e., CV for biomass measurement or XTT for metabolic activity measurement) are shown in **Figure 2**. The linear regression and Spearman's rank correlation showed that percentages of inhibition evaluated by CV and XTT methods gave consistent results for antimicrobial susceptibility testing of biofilms in formation ($R^2 = 0.83$, $p < 0.0001$; Spearman's $\rho = 0.8816$, $p < 0.0001$) and mature biofilms ($R^2 = 0.89$, $p < 0.0001$; Spearman's $\rho = 0.8562$, $p < 0.0001$). Therefore, the effects of antimicrobial agents on the other strains (AF_CF and SM_CF) were evaluated only by the XTT reduction method.

Effect of Antimicrobial Agents on Planktonic Cells and Biofilm in Formation

MIC_b helped to compare planktonic and biofilm-embedded cells susceptibilities. The susceptibility values of planktonic Af and Sm strains are listed in **Table 1**, which displays the mode for each condition amongst the three performed replicates. MIC_b values of the three antifungals were one dilution lower than MIC values, and this was not considered as a major discrepancy. MIC and MIC_b of LVX were similar, but showed discrepancy of up to two dilutions for RFN, indicating that Sm strains tended to be more susceptible to RFN in our culture conditions.

AF_REF and AF_CF were equally susceptible to AMB, ITC, and VRC (MIC_bs 1, 0.25, and 0.25 μg/mL, respectively). SM_REF and SM_CF were both susceptible to LVX (MIC_bs $\leq$ 0.50 μg/mL), and MIC_b of RFN for SM_CF (2 μg/mL) was 8-fold higher than that for SM_REF (0.25 μg/mL).

Overall, the susceptibility of planktonic cells (MIC_b) measured by turbidity was similar to the susceptibility of biofilm in formation ($MBIC_{XTT}$) measured by XTT (**Table 1**).

Decrease of Antimicrobial Susceptibility on Monomicrobial Mature Biofilm Compared With Planktonic Cells and Biofilm in Formation

Antifungal and antibacterial agents exhibited concentration-dependent activities against AF_REF and SM_REF biofilms (**Figure 2**). The lowest drug concentrations required to reduce metabolic activity of biofilm in formation ($MBIC_{XTT}$) and mature biofilm ($MBEC_{XTT}$) are shown in **Table 1**. AMB exhibited a greater effect on biofilm formation of Af since $MBIC_{XTT}$s (1 μg/mL) were 8 and 32-fold lower than $MBEC_{XTT}$s for AF_CF and AF_REF, respectively. ITC and VRC were efficient to inhibit biofilm formation of both Af strains ($MBIC_{XTT}$s = 0.25 μg/mL), but not to eradicate mature biofilms ($MBEC_{XTT}$s > 256 μg/mL). Only AMB was efficient on mature biofilms with a slightly greater effect on AF_CF ($MBEC_{XTT}$ = 8 μg/mL) compared with AF_REF ($MBEC_{XTT}$ = 32 μg/mL). The $MBIC_{XTT}$s of LVX (0.25 and 1 μg/mL for SM_REF and SM_CF, respectively) were lower than $MBEC_{XTT}$s (1 and 2 μg/mL). LVX exhibited a slightly greater effect to reduce metabolic activity of Sm biofilms in formation than Sm mature biofilms. For RFN, $MBIC_{XTT}$s, and $MBEC_{XTT}$s were similar, indicating that RFN had a similar effect on biofilm formation and mature biofilm according to the XTT results.

TABLE 2 | Efficacy of drugs to reduce cell numbers on planktonic cultures and biofilm forms of *S. maltophilia*.

Strain	Drug	MBC	$MBIC_{CFU}$	$MBEC_{CFU}$
SM_REF	LVX	0.25	0.25	8
	RFN	32	0.25	16
SM_CF	LVX	1	4	32
	RFN	>32	16	>32

Concentrations are expressed in μg/mL. LVX, levofloxacin; RFN, rifampicin; MBC, minimum bactericidal concentration; $MBIC_{CFU}$, minimum biofilm inhibitory concentration based on CFU enumeration; $MBEC_{CFU}$, minimum biofilm eradication concentration based on CFU enumeration.

The viability of antibiotic-treated Sm cells in planktonic cultures (MBC), biofilm in formation ($MBIC_{CFU}$), and mature biofilm ($MBEC_{CFU}$) is shown in **Table 2**. The MBC of LVX was similar to its MIC_b, whereas the MBC of RFN was at least 16-fold higher than MIC_b for both Sm strains (**Tables 1**, **2**). LVX reached its bactericidal effect on Sm strains at lower concentrations than RFN.

The efficacy of LVX to reduce cell numbers on planktonic cultures (MBC) and biofilm in formation ($MBIC_{CFU}$) was higher than on mature biofilm ($MBEC_{CFU}$), for both Sm strains (**Table 2**). LVX exhibited a slightly greater effect on SM_REF ($MBEC_{CFU}$ = 8 μg/mL) compared with SM_CF ($MBEC_{CFU}$ = 32 μg/mL). The MBC of RFN was similar to its $MBEC_{CFU}$ for both strains, but the $MBEC_{CFU}$ was up to 64-fold higher than $MBIC_{CFU}$. Thus, RFN only seems to reduce the number of adherent Sm cell by inhibiting the biofilm formation.

Fungal Growth Inhibition and Fungal Phenotype Modification in the Presence of *S. maltophilia*

In AF_REF + SM_REF biofilm, the fungal growth was significantly reduced ($p < 0.0001$) but not the bacterial growth ($p = 0.0520$), in comparison with the corresponding monomicrobial biofilms (**Figures 3A,B**). We obtained the same trend for CF strains: AF_CF in mono- vs. polymicrobial biofilm ($p < 0.0001$) and SM_CF in mono- vs. polymicrobial biofilm ($p = 0.1706$; **Figures 3A,B**). The fungal growth

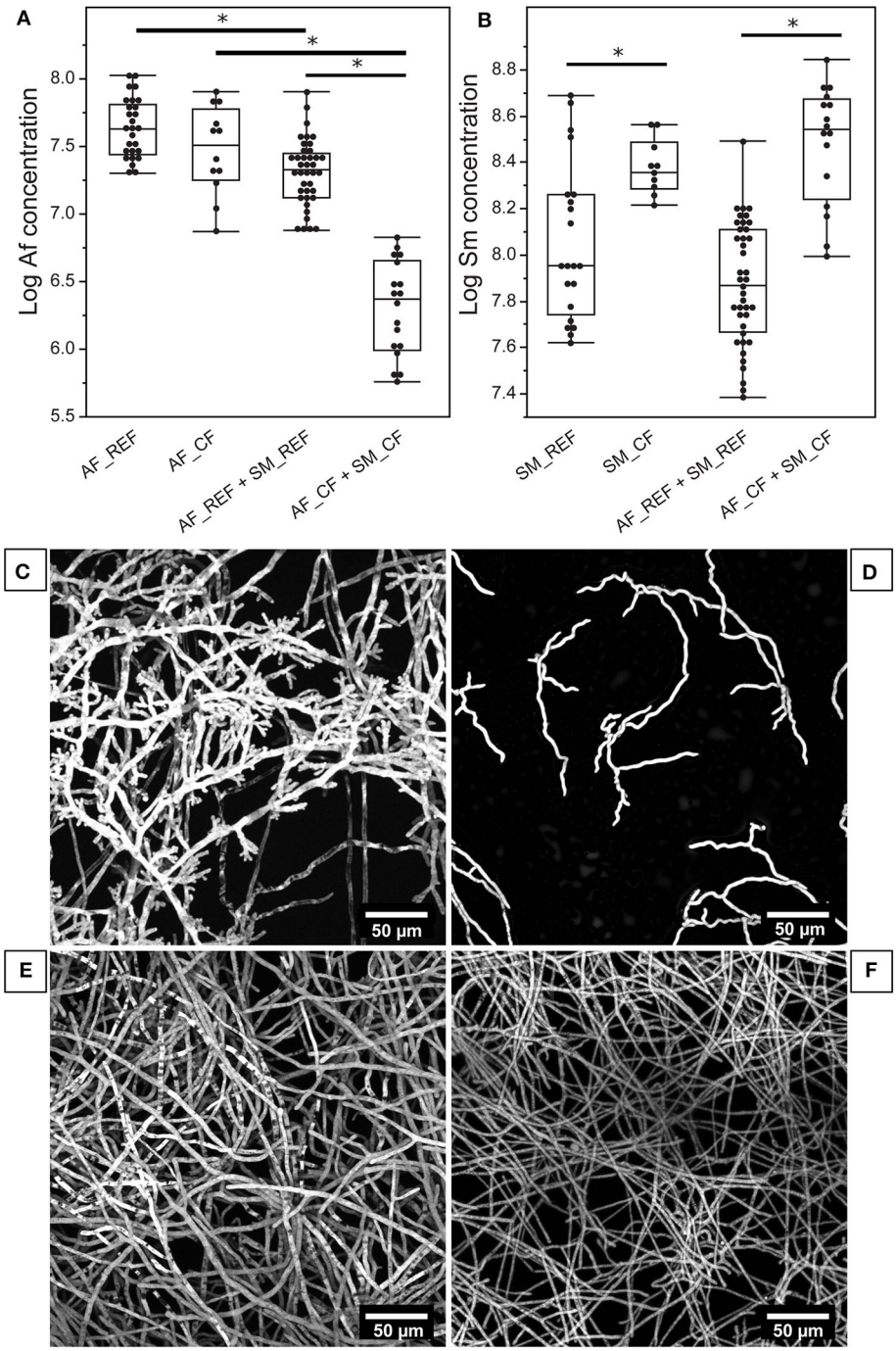

FIGURE 3 | Quantification of fungal and bacterial concentrations in biofilms and phenotype modifications of *A. fumigatus* in the presence of *S. maltophilia*. **(A,B)** Assessment of Af or Sm growth in mono- and polymicrobial biofilms after 24 h of culture by qPCR. *$p < 0.05$. **(C)** AF_REF phenotype in polymicrobial biofilm with SM_REF. **(D)** AF_CF phenotype in polymicrobial biofilm with SM_CF. **(E)** AF_REF phenotype in monomicrobial biofilm. **(F)** AF_CF phenotype in monomicrobial biofilm. Af, *A. fumigatus*; Sm, *S. maltophilia*.

inhibition was higher for the association of CF strains than for REF strains. Specifically, the growth of AF_REF and AF_CF in polymicrobial biofilms was respectively reduced by 2 and 10 compared with that in fungal biofilms. This difference can be attributed to the difference of Sm fitness (**Figure 3B**). SM_CF grew significantly faster than SM_REF in polymicrobial biofilm ($p < 0.0001$) and thus induced a larger inhibition of fungal growth. Moreover, the fungal phenotype

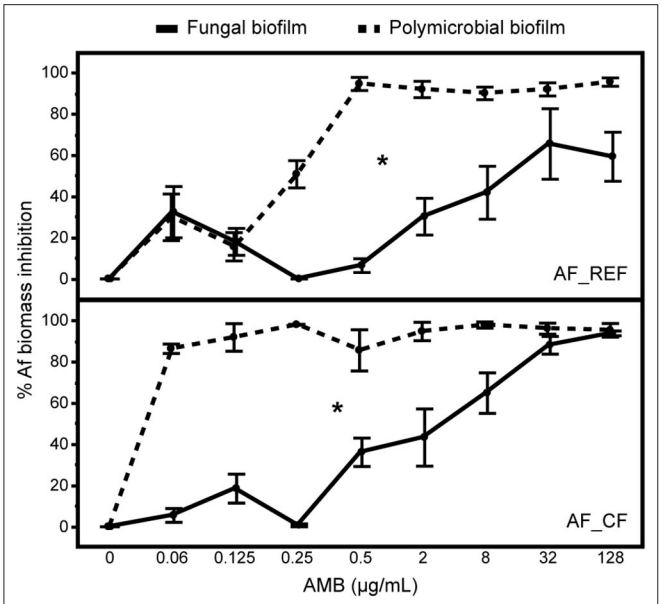

FIGURE 4 | Effects of amphotericin B on *A. fumigatus* in fungal and polymicrobial biofilms. Assessment of AMB activity on AF_REF or AF_CF in fungal (solid line) and polymicrobial (broken line) biofilms by qPCR. Percentages of biomass inhibition were calculated based on untreated biofilms results. *$p < 0.05$; Af, *A. fumigatus*; AMB, amphotericin B.

was modified and showed highly branched hyphae in the presence of bacteria for both associations (**Figures 3C,D**) in comparison with the corresponding Af monomicrobial biofilm (**Figures 3E,F**).

Modification of *A. fumigatus* Susceptibility to AMB in Polymicrobial Biofilm

Since no ITC and VRC activities on Af monomicrobial biofilms were found (**Table 1**), we focused on AMB activity for the following experiments. Af susceptibility to AMB in mono- and polymicrobial biofilms was tested for both strain associations: AF_REF + SM_REF and AF_CF + SM_CF. The percentage of fungal biomass inhibition measured in AMB-treated biofilms was significantly higher in polymicrobial biofilms for both Af strains (multiple linear regressions, biofilm * AMB concentration effect: $p < 0.001$; **Figure 4**); i.e., the fungus was more susceptible to AMB in the presence of Sm. The AMB concentrations required to obtain 90% of AF_REF or AF_CF biomass inhibition were at least 32 µg/mL in fungal biofilm and 0.5 or ≤ 0.06 µg/mL in polymicrobial biofilm. The difference in Af susceptibility between fungal and polymicrobial biofilms was observed from 0.25 and ≤ 0.06 µg/mL of AMB for AF_REF and AF_CF, respectively. Overall, Af susceptibility to AMB in polymicrobial biofilm was at least 64-fold higher compared with fungal biofilm (**Figure 4**), whereas the fungal growth was reduced by 2 times with SM_REF and 10 times with SM_CF (**Figure 3A**).

Modification of *S. maltophilia* Susceptibility to LVX in Polymicrobial Biofilm

The susceptibility of Sm in bacterial and polymicrobial biofilms was compared in response to LVX. **Figure 5A** results showed that LVX had a greater effect on SM_REF in bacterial biofilm than in polymicrobial biofilm (multiple linear regressions, $p < 0.0001$). In the bacterial biofilm, the inhibition level raised gradually and proportionally with the increase in LVX concentration until 90% at 1 µg/mL. This result was consistent with that of $MBEC_{XTT}$ (1 µg/mL, **Table 1**). Concerning SM_REF in polymicrobial biofilm, LVX had a limited effect (20% inhibition, regardless of LVX concentration) (**Figure 5A**). In addition to these results, the bacterial survival was assessed by subculturing the LVX-treated biofilms (**Figure 5B**). The results showed a significantly higher survival rate of SM_REF in polymicrobial biofilm than in bacterial biofilm following LVX treatment at 1 µg/mL ($p = 0.0003$), 4 µg/mL ($p = 0.0002$), and 32 µg/mL ($p = 0.0025$). In the bacterial biofilm, 2% (~10^6 bacteria/mL) and 0.001% (10^3 bacteria/mL) of survival rates were recorded following exposure to 1 and 32 µg/mL of LVX, respectively. In contrast, in the polymicrobial biofilm, almost 40 and 6% of Sm were still alive after exposure to 1 and 32 µg/mL of LVX, respectively. For SM_CF, qPCR results showed a similar antibacterial effect of LVX on the bacterial and polymicrobial biofilms (multiple linear regressions, $p = 0.8550$; **Figure 6**). Regardless of the LVX concentration used, the presence of AF_CF had no effect on SM_CF susceptibility to LVX, while the presence of AF_REF decreased the susceptibility of SM_REF to LVX. This difference was probably due to the higher inhibition of Af growth exhibited in the association of CF strains (**Figure 3A**). In addition, TEM experiments were performed to visualize effects of LVX (8 µg/mL) on polymicrobial biofilms. SM_REF cells grown with AF_REF did not show any signs of severe damage after LVX treatment (**Figures 7A,B**), whereas SM_CF cells appeared broken and emptied of their contents in LVX-treated polymicrobial biofilm (**Figures 7C,D**).

Decrease of *S. maltophilia* Susceptibility to LVX in Polymicrobial Biofilm Is Related to the *A. fumigatus* Biomass

Since the fungal biomass in the AF_CF + SM_CF biofilm was lower than in the AF_REF + SM_REF biofilm (**Figure 3A**), we hypothesized that the fungal biomass was responsible for the significant decrease of SM_REF susceptibility to LVX observed in polymicrobial biofilm (**Figure 5A**), in contrast to SM_CF (**Figure 6**). To test this hypothesis, we carried out experiments using different concentrations of AF_CF (10^5 or 10^6 conidia/mL) for the same SM_CF concentration (10^6 bacteria/mL) in order to reduce the fungal growth inhibition caused by the bacteria. There was a bigger fungal biomass (+ 0.5 log CE/mL, data not shown) in the polymicrobial biofilm formed with 10^6 conidia/mL than that observed in the polymicrobial biofilm with 10^5 conidia/mL. In the bacterial biofilm, 4 µg/mL of LVX was enough to achieve 90% SM_CF inhibition, which was not the case in the polymicrobial biofilm (performed with 10^6 conidia/mL) where 16 µg/mL of

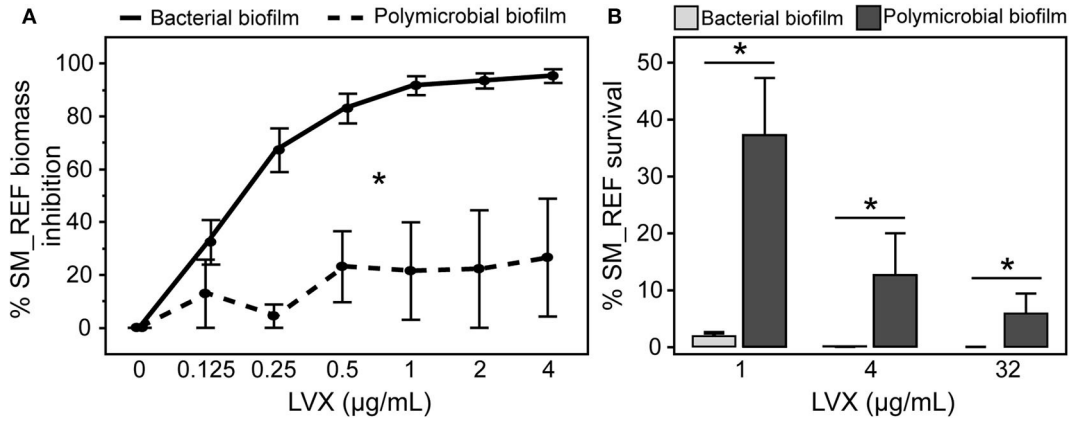

FIGURE 5 | Effects of levofloxacin on SM_REF in bacterial and polymicrobial biofilms. **(A)** Assessment of LVX activity on SM_REF in bacterial (solid line) and polymicrobial (broken line) biofilms by qPCR. **(B)** Percentages of SM_REF survival following LVX (1, 4, and 32 µg/mL) treatment in bacterial (light gray) and polymicrobial (dark gray) biofilms using viable plate count. *$p < 0.05$; LVX, levofloxacin.

LVX could not exceed 50% inhibition. The presence of higher AF_CF biomass helped to protect SM_CF from LVX (multiple linear regressions, $p = 0.0121$; **Figure 6**).

Role of the Fungal ECM in *S. maltophilia* Protection From LVX

Fungal ECM may have a role in decreasing Sm susceptibility to LVX in polymicrobial biofilm. To support this hypothesis, Sm and Af-Sm biofilms were pretreated with proteinase K to degrade proteins of ECM before analyzing the bacterial survival following LVX treatment at 1 µg/mL. Proteinase K did not affect the SM_REF response to LVX in bacterial biofilm ($p = 0.8563$; **Figure 8**). However, a significant decrease of SM_REF survival following LVX treatment was observed in polymicrobial biofilm pretreated with Proteinase K compared with unpretreated biofilm ($p = 0.0333$; **Figure 8**), suggesting that AF_REF ECM is involved in the protection of SM_REF from LVX.

Antifungal and Antibacterial Combination Strategies to Treat Polymicrobial Biofilm

Antifungal-antibacterial combinations were tested on Af-Sm biofilm in order to impair both pathogens and to provide more evidence that Af plays a role in protecting Sm from the effects of antibiotics. Bacterial and polymicrobial biofilms of REF strains were exposed to AMB at 64 µg/mL combined with LVX or RFN at 4 µg/mL, and to each drug alone.

In polymicrobial biofilm, the susceptibility of AF_REF to AMB alone or in combination with antibiotics did not differ (AMB vs. AMB + LVX: $p = 0.2027$; AMB vs. AMB + RFN: $p = 0.3801$) (data not shown). These results suggested that the antibiotics did not affect the antifungal efficacy of AMB on Af in polymicrobial biofilm.

When the polymicrobial biofilm was treated with AMB (64 µg/mL), the growth of SM_REF was significantly increased compared with untreated biofilms ($p < 0.0001$, data not shown).

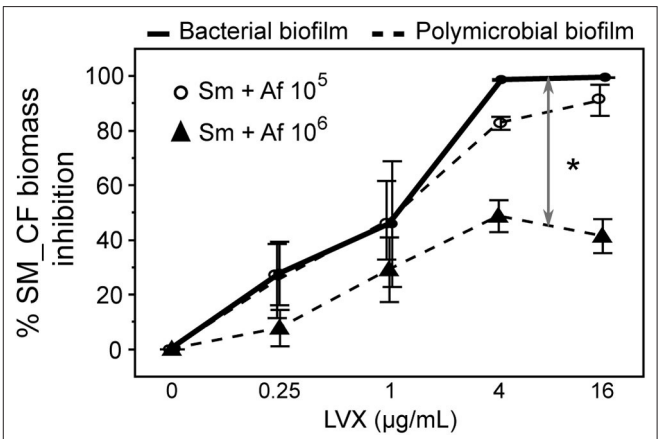

FIGURE 6 | Effects of levofloxacin on SM_CF in bacterial and polymicrobial biofilms. Susceptibility of SM_CF to LVX was assessed in bacterial (solid line) and polymicrobial biofilms (broken lines) with an initial AF_CF inoculum of 10^5 conidia/mL (circle) or 10^6 conidia/mL (triangle) by qPCR. *$p < 0.05$; LVX, levofloxacin.

SM_REF biomass was ~5 × 10^7 BE/mL in the untreated polymicrobial biofilm vs. 3 × 10^8 BE/mL in the AMB-treated. For such, the results of the two-drug combination were expressed in percentages of growth inhibition and survival compared with AMB-treated biofilm. The qPCR analysis demonstrated that AMB in combination with LVX or RFN significantly improved the antibacterial effect against Sm in polymicrobial biofilm in comparison with LVX or RFN alone (LVX vs. AMB + LVX: $p < 0.0001$; RFN vs. AMB + RFN: $p < 0.0001$; **Figure 9A**). Remarkably, the susceptibility of Sm in polymicrobial biofilm to antifungal-antibacterial combination was not significantly different from the susceptibility of Sm in bacterial biofilm to each antibacterial alone (Sm-LVX vs. Af-Sm-AMB + LVX: $p = 0.8668$; Sm-RFN vs. Af-Sm-AMB

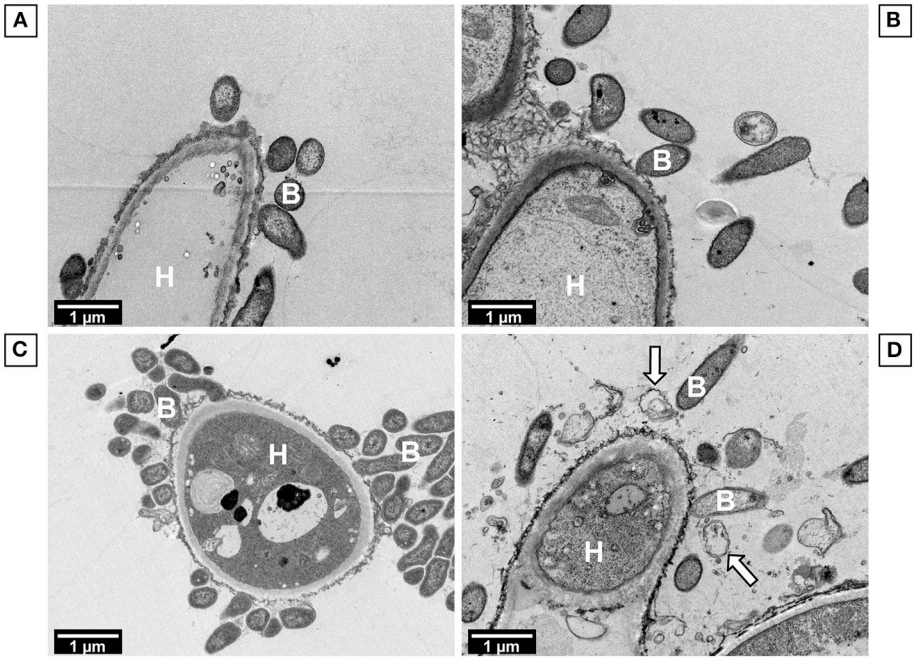

FIGURE 7 | TEM observations of *A. fumigatus-S. maltophilia* biofilms exposed to levofloxacin. **(A)** AF_REF + SM_REF untreated biofilm. **(B)** AF_REF + SM_REF biofilm treated with 8 µg/mL LVX. **(C)** AF_CF + SM_CF untreated biofilm. **(D)** AF_CF + SM_CF biofilm treated with 8 µg/mL LVX. H, hypha; B, bacteria. The white arrows show damages to bacterial cells.

+ RFN: $p = 0.3963$; **Figure 9A**). AMB + LVX combination was significantly more efficient against Sm in polymicrobial biofilm (90% of Sm growth inhibition) than AMB + RFN (75% of Sm growth inhibition; $p = 0.0002$). Therefore, survival experiments were performed with AMB in combination with LVX (**Figure 9B**). A higher reduction of Sm survival in polymicrobial biofilm following AMB + LVX treatment compared with LVX alone ($p = 0.0006$) was obtained by viable plate count. Moreover, AMB + LVX combination against Sm in polymicrobial biofilm was as efficient as LVX alone against Sm in bacterial biofilm ($p = 0.1096$; **Figure 9B**). These results suggest that the inhibition of Af with AMB prompted Sm susceptibility to antibiotics.

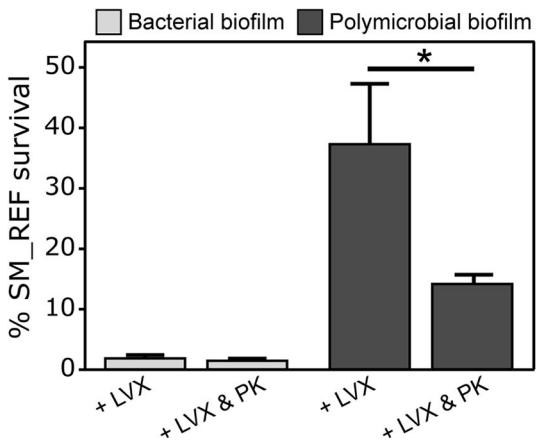

FIGURE 8 | Effects of *A. fumigatus* matrix on *S. maltophilia* biofilm susceptibility to levofloxacin. Bacterial and polymicrobial biofilms were treated with or without proteinase K (50 µg/mL) for 2 h before LVX treatment at 1 µg/mL. Percentages of SM_REF survival was assessed using viable plate count. *$p < 0.05$; LVX, levofloxacin; PK, proteinase K.

DISCUSSION

Managing cross-kingdoms polymicrobial infections, especially of biofilm-forming microbes, remains an outstanding challenge to overcome resistance to a wide range of clinical antimicrobial agents. Conventional treatment of infectious diseases relies on standard susceptibility testing of planktonic cells, which does not take into account microbial interactions. For such, this approach becomes unsuitable against polymicrobial biofilm with sessile cells embedded in ECM. We previously showed *A. fumigatus-S. maltophilia* interactions in polymicrobial biofilm (Melloul et al., 2016, 2018), and from there we sought to determine whether the antimicrobial response of both pathogens would be modified in their polymicrobial biofilm.

The present study highlights the modulated antimicrobial response of a filamentous fungal-bacterial biofilm. We showed that *A. fumigatus* monomicrobial biofilms were susceptible to amphotericin B, but not to itraconazole, and voriconazole. Amphotericin B susceptibility of *A. fumigatus* increased when

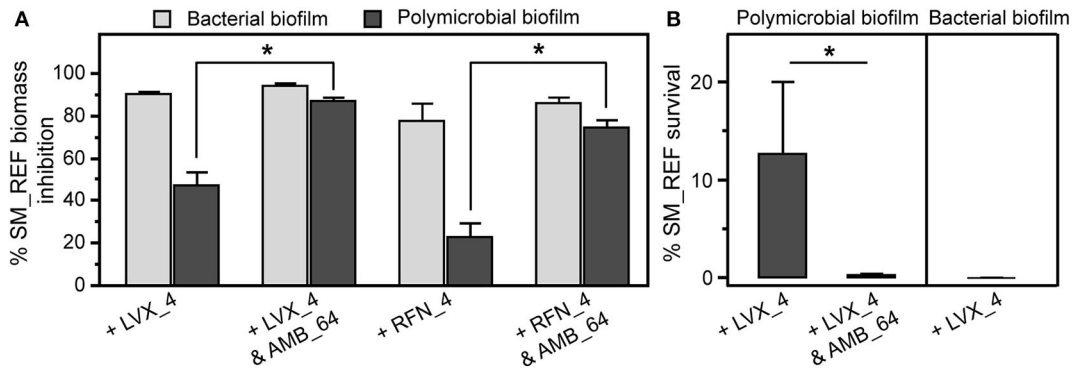

FIGURE 9 | Effects of antifungal-antibacterial combination on *A. fumigatus-S. maltophilia* biofilm. **(A)** Susceptibility of SM_REF in bacterial (light gray) and polymicrobial (dark gray) biofilms to LVX and RFN alone or in two-drug combination with AMB assessed by qPCR. **(B)** Percentages of SM_REF survival following LVX alone or in two-drug combination with AMB treatment in bacterial and polymicrobial biofilms using viable plate count. *$p < 0.05$; AMB_64, amphotericin B (64 μg/mL); LVX_4, levofloxacin (4 μg/mL); RFN_4, rifampicin (4 μg/mL).

it was embedded in polymicrobial biofilm with *S. maltophilia*. Levofloxacin and rifampicin were efficient in inhibiting *S. maltophilia* monomicrobial biofilms, but much higher concentrations were needed to eradicate it. *S. maltophilia* susceptibility to levofloxacin decreased in the polymicrobial biofilm and was fungal biomass-dependent. The inhibited effect of *S. maltophilia* on *A. fumigatus* growth was more pronounced for the association of CF strains than the reference strains. The combination of amphotericin B with levofloxacin or rifampicin was efficient to impair both pathogens in the polymicrobial biofilm.

Antifungal susceptibility of *A. fumigatus* biofilms was assessed by the XTT reduction method, already used several years ago (Mowat et al., 2007; Seidler et al., 2008). We focused on viability test to assess antibacterial susceptibility of *S. maltophilia* biofilms, which have been used in previous research (Di Bonaventura et al., 2004; Pompilio et al., 2016). The antibiosis effect of *S. maltophilia* on *A. fumigatus* growth was revealed by qPCR. This technique was also used to compare the antimicrobial susceptibility of species in mono- and polymicrobial biofilms.

Our results revealed clear differences in antifungal susceptibilities between biofilms in formation and mature biofilms of *A. fumigatus* (i.e., $MBIC_{XTT}$ vs. $MBEC_{XTT}$, **Table 1**). The formation of *A. fumigatus* biofilm was prevented by the three antifungal agents ($MBIC_{XTT}$s $\leq$ 1 μg/mL). *A. fumigatus* mature biofilm was inhibited by amphotericin B ($MBEC_{XTT}$ range = 8–32 μg/mL), but not by the two azoles ($MBEC_{XTT}$s > 256 μg/mL), as already shown in the work of Mowat et al. (2007). These results suggest that azoles could be useful in preventing biofilm formation rather than in treating mature biofilm. To date, the reasons for the decreased susceptibility of *A. fumigatus* biofilm to drugs have not been fully elucidated (Latgé and Chamilos, 2019).

Considering the results with azoles, we focused on amphotericin B activity on *A. fumigatus* in polymicrobial biofilms with *S. maltophilia* and we found an increase susceptibility compared with their corresponding *A. fumigatus* monomicrobial biofilms (**Figure 4**). A possible explanation is the modification of fungal phenotype due to the presence of bacteria. In our model, the modification of *A. fumigatus* cell wall by *S. maltophilia* (Melloul et al., 2018) resembles the one induced by caspofungin or dirhamnolipids (diRhls) secreted by *P. aeruginosa* which specifically inhibit the fungal 1,3-glucan synthase activity (Briard et al., 2017). We suppose that some diRhls-like molecules secreted by *S. maltophilia* would modify *A. fumigatus* phenotype similarly to echinocandins. This could justify the impairment of *A. fumigatus* response to amphotericin B, as showed for clinical *Aspergillus* spp. strains treated with a combination of amphotericin B and echinocandins (Panackal et al., 2014). Iron could be another important parameter in this interaction between *S. maltophilia* and *A. fumigatus*. The role of two siderophores secreted by *P. aeruginosa* (pyoverdine and pyochelin) involved in the reduction of *A. fumigatus* growth has been analyzed (Sass et al., 2018; Briard et al., 2019) and the production of catecholate siderophores from *S. maltophilia* clinical strains has been shown (García et al., 2012; Nas and Cianciotto, 2017). In our polymicrobial model, we can hypothesize that siderophores secreted by *S. maltophilia* could deprive *A. fumigatus* from iron. In turn, iron deficiency could increase *A. fumigatus* susceptibility, as reported by Zarember et al. (2009) who showed that iron deprivation gave better *A. fumigatus* response to amphotericin B treatment.

The viability of antibiotic-treated *S. maltophilia* in planktonic cultures (MBC), biofilm in formation ($MBIC_{CFU}$), and mature biofilm ($MBEC_{CFU}$) is shown in **Table 2**. Results showed a reduced levofloxacin susceptibility of biofilm-embedded bacteria. This finding is consistent with the results of previous studies (Passerini de Rossi et al., 2009; Pompilio et al., 2016). The *in vitro* activity of other fluoroquinolones against biofilm-embedded *S. maltophilia* cells has already been reported (Di Bonaventura et al., 2004; Passerini de Rossi et al., 2009; Wu et al., 2013; Wang et al., 2016). However, no study analyzed their effect on *S. maltophilia* in polymicrobial biofilms with a filamentous fungus. Our study demonstrated that *S.*

maltophilia response to levofloxacin was impacted by the presence of *A. fumigatus*. Manavathu et al. (2014) documented a decrease of bacterial susceptibility in *A. fumigatus-P. aeruginosa* polymicrobial biofilm with no explanation of the underlying mechanism. Our work demonstrated that levofloxacin effect on *S. maltophilia* in polymicrobial biofilms, of both associations of strains, decreased and was fungal biomass-dependent. The network of *A. fumigatus* hyphae could protect *S. maltophilia* from levofloxacin. The higher fitness of SM_CF, in comparison with SM_REF, could account for the larger antibiosis effect of the CF bacterial strain on the CF fungal growth in polymicrobial biofilm. Alike, in our latest study, we showed that *S. maltophilia* antibiosis on *A. fumigatus* was dependent on the bacterial fitness (Melloul et al., 2018). The fungal biomass of AF_CF + SM_CF biofilm was insufficient to protect the bacteria from levofloxacin, but upon increasing the fungal biomass (using a larger initial inoculum), the polymicrobial biofilm provided SM_CF with a better protection from levofloxacin (**Figure 6**). We further suggested that *A. fumigatus* ECM could prevent drug diffusion by acting as a physical barrier and enhance *S. maltophilia* tolerance to levofloxacin. This hypothesis was partially validated using proteinase K pretreatment to damage the ECM structure of the polymicrobial biofilm, similarly to what De Brucker et al. (2015) did for *C. albicans* and *Escherichia coli* to obtain a lower bacterial tolerance to ofloxacin. Moreover, matrix components released by *A. fumigatus* could alter the physiology of *S. maltophilia* by restricting penetration of nutrients or oxygen into the aggregates. Indeed, Stewart et al. (2015) showed that a low oxygen level seems to be the primary mechanism for tolerance of biofilms to quinolones. Further investigations are warranted to identify the fungal ECM components, which could promote antibacterial protection of *S. maltophilia* in *A. fumigatus-S. maltophilia* biofilm.

Some studies put forwards the use of rifampicin to treat *S. maltophilia* infections (Savini et al., 2010; Betts et al., 2014), but to our knowledge, we are the first to explore its activity against *S. maltophilia* biofilm. Our experiments needed high doses of rifampicin to eradicate planktonic cells (MBC, **Table 2**) and mature biofilms ($MBEC_{CFU}$, **Table 2**) of both *S. maltophilia* strains. Rifampicin was only efficient to eradicate the SM_REF cells of biofilm in formation ($MBIC_{CFU}$, **Table 2**).

To date, few studies explored *in vitro* effects of antifungal agents in combination with antibacterial agents against polymicrobial biofilms. We demonstrated that levofloxacin or rifampicin combined with a high dose of amphotericin B was significantly more efficient to eradicate *S. maltophilia* in polymicrobial biofilm than the antibiotic alone (**Figure 9**). This result corroborates the protective effect of *A. fumigatus* on *S. maltophilia* in polymicrobial biofilm, since once the fungus was destroyed by amphotericin B, the antibiotic had a stronger effect on the bacteria.

Most CF patients with *A. fumigatus* infection are put on azole therapies though their efficacy is discussed (Burgel et al., 2016). In the same way, the success of management of *S. maltophilia* infections in CF patients remains unclear (Amin and Waters, 2016). Finally, treatment failure could be attributed in some cases to the *in vivo* presence of biofilm forms, as it was proposed for *A. fumigatus* (Ramage et al., 2011). Our *in vitro* results demonstrated that the microbial interactions between *A. fumigatus* and *S. maltophilia* mutually modulate their responses to antimicrobial agents. This could be particularly relevant in CF patients where lungs can be characterized by decreased oxygen pressure. Also, these findings add a toll on therapeutic decision-making, since the microbial interactions and the biofilm-forming ability are not usually taken into account to design treatment options.

In conclusion, microbial interactions within polymicrobial biofilms can modulate the antimicrobial response of pathogens. In polymicrobial biofilms, *S. maltophilia* increased the antifungal susceptibility of *A. fumigatus* to amphotericin B, whereas *A. fumigatus* protected *S. maltophilia* from levofloxacin. Further work analyzing the underlying mechanisms of antimicrobial combinations on polymicrobial biofilms would be valuable in the future.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR'S NOTE

This work was presented in part at the 6th European Congress on Biofilms (EUROBIOFILMS) (Glasgow, Scotland, September 2019).

AUTHOR CONTRIBUTIONS

EM and FB conceived the design of the study. EM supervised the experiments. LR performed the experiments and the statistical analysis. LR and EM designed the figures. LR, EM, and FB participated in results analysis. P-LW and ED helped in the interpretation of antibacterial and antifungal activities, respectively. J-WD and GR analyzed *S. maltophilia* strains. LR, EM, JG, and FB drafted the manuscript. All authors read and approved the final manuscript.

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Synthesis of the Hydroxamate Siderophore N^{α}-Methylcoprogen B in *Scedosporium apiospermum* Is Mediated by *sidD* Ortholog and Is Required for Virulence

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Scedosporium species rank second among the filamentous fungi capable to colonize chronically the respiratory tract of patients with cystic fibrosis (CF). Nevertheless, there is little information on the mechanisms underpinning their virulence. Iron acquisition is critical for the growth and pathogenesis of many bacterial and fungal genera that chronically inhabit the CF lungs. In a previous study, we showed the presence in the genome of *Scedosporium apiospermum* of several genes relevant for iron uptake, notably SAPIO_CDS2806, an ortholog of *sidD*, which drives the synthesis of the extracellular hydroxamate-type siderophore fusarinine C (FsC) and its derivative triacetylfusarinine C (TAFC) in *Aspergillus fumigatus*. Here, we demonstrate that *Scedosporium apiospermum sidD* gene is required for production of an excreted siderophore, namely, N^{α}-methylcoprogen B, which also belongs to the hydroxamate family. Blockage of the synthesis of N^{α}-methylcoprogen B by disruption of the *sidD* gene resulted in the lack of fungal growth under iron limiting conditions. Still, growth of *ΔsidD* mutants could be restored by supplementation of the culture medium with a culture filtrate from the parent strain, but not from the mutants. Furthermore, the use of xenosiderophores as the sole source of iron revealed that *S. apiospermum* can acquire the iron using the hydroxamate siderophores ferrichrome or ferrioxamine, i.e., independently of N^{α}-methylcoprogen B production. Conversely, N^{α}-methylcoprogen B is mandatory for iron acquisition from pyoverdine, a mixed catecholate-hydroxamate siderophore. Finally, the deletion of *sidD* resulted in the loss of virulence in a murine model of scedosporiosis. Our findings demonstrate that *S. apiospermum sidD* gene drives the synthesis of a unique extracellular, hydroxamate-type iron chelator, which is essential for fungal growth and virulence. This compound scavenges iron from pyoverdine, which might explain why

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S. apiospermum and *Pseudomonas aeruginosa* are rarely found simultaneously in the CF lungs.

Keywords: *Scedosporium*, iron uptake, extracellular siderophore, N^{α}-methyl coprogen B, virulence factor, xenosiderophores, cystic fibrosis

INTRODUCTION

During the past few decades, opportunistic fungal pathogens from the genus *Scedosporium* have been increasingly recognized as the cause of potentially life-threatening infections in immunocompromised patients (Ramirez-Garcia et al., 2018). Likewise, a high occurrence was observed among patients with other underlying conditions such as cystic fibrosis (CF). These molds indeed represent the second most common filamentous fungi inhabiting the CF airways, after *Aspergillus fumigatus*. Nevertheless, there is only little information about the critical virulence determinants driving *Scedosporium* persistence, infection, and morbidity in the CF context.

Like bacteria, the survival of fungi is dependent upon their ability to acquire metals that function as cofactors of almost one-third of their proteins (Waldron and Robinson, 2009). Among these metals, iron is essential for nearly all living organisms due to its crucial role in many enzymes and metabolic processes. In the human host, the amount of available free iron is meager (10^{-24} M) (Caza and Kronstad, 2013) because of (i) the poor solubility of the metal ion in its highest oxidation state (Fe^{3+}), which is the predominant form of iron in aerobic environments at physiological pH; and (ii) its sequestration by host proteins such as ferritin, transferrin, and hemoglobin. Consequently, pathogenic organisms had evolved sophisticated mechanisms to ensure adequate iron supply for cellular processes. Siderophore production is thought to be the primary mechanism used for iron uptake in *Aspergillus* species as they cannot acquire iron directly from heme, ferritin, or transferrin. For instance, Schrettl et al. (2004) demonstrated that the non-siderophore reductive iron assimilation (RIA) system, presenting high affinity for ferric iron, was dispensable for the establishment of infection in a murine model of invasive aspergillosis, while disruption of the genes involved in siderophore biosynthesis resulted in a dramatic reduction of growth and virulence (Schrettl et al., 2004; Schrettl et al., 2007; Yasmin et al., 2012).

Siderophores are non-ribosomal peptides (NRPs), which have been classified as catecholate, carboxylate, hydroxamate, and mixed types, according to the functional group(s) involved in iron chelation. Most fungi produce hydroxamate-type siderophores using acylated N^5-hydroxy-L-ornithine as building blocks (Haas, 2014). In these siderophores bearing the functional group RC(O)N(OH)R'—with R and R' as organic residues and CO as a carbonyl group—the building blocks bind to ferric ions as bidentate ligands through their oxygen atoms. To further increase their affinity for Fe^{3+}, the vast majority of fungal siderophores include three hydroxamate moieties linked covalently by peptide or ester bonds to form hexadentate complexes, satisfying the six-coordinate octahedral geometry preferred for ferric ions. These structures exhibit high dissociation constants ranging from 10^{-22} to 10^{-32} M (Winkelmann, 2007), which corresponds to an affinity that surpasses that of all other biologically relevant iron ligands. For example, *Aspergillus fumigatus* secretes two hexadentate hydroxamate siderophores, namely, fusarinine C (FsC) and triacetylfusarinine C (TAFC), which have a higher affinity for iron than transferrin so that the fungus can obtain iron directly from the host protein (Hissen et al., 2004).

In a previous study, we demonstrated that the *Scedosporium apiospermum* genome comprises several genes orthologous to those required for siderophore production in *A. fumigatus* (Le Govic et al., 2018), notably the *NPS6* ortholog of *A. fumigatus sidD* gene encoding a nonribosomal peptide synthetase (NRPS), which is involved in the last step of FsC synthesis (Schrettl et al., 2007). In the fungal kingdom, *sidD* orthologs were described to be responsible not only for the synthesis of fusarinine- but also of coprogen-derived siderophores. Bioinformatic investigations showed that *S. apiospermum sidD* gene (SAPIO_CDS2806) encodes a putative NRPS whose architecture resembles that of coprogen- or fusarinine-type siderophore-producing NRPSs (Le Govic et al., 2019). Accordingly, phylogenetic analysis revealed that the protein encoded by *S. apiospermum sidD* belongs to the NPS6/SidD family, which gathers NRPS members driving the biosynthesis of extracellular siderophores, including coprogen and fusarinine NRPSs (Le Govic et al., 2019). However, none of the NRPS *in silico* analysis tools was able to predict the nature of the substrates of *S. apiospermum* SidD. The aims of this study were, therefore, (i) to determine if *S. apiospermum sidD* is responsible for the biosynthesis of an extracellular siderophore, (ii) to identify the compound produced, and (iii) to assess the importance of this compound in fungal growth and virulence.

MATERIALS AND METHODS

Strains and Culture Conditions

The *S. apiospermum* wild-type (WT) strain used in this study, deposited at the BCCM/IHEM culture collection (Brussels, Belgium) under the accession number IHEM 14462, was isolated in 1998 from a sputum sample from a CF patient in Tours, France. As described below, a non-homologous end-joining-deficient strain (*Δku70*) was obtained from this WT strain, and was subsequently used to generate *sidD* disruptants.

Strains were maintained by regular passages on Potato Dextrose Agar (PDA) plates supplemented with 0.5% chloramphenicol. For cultivation of the *Δku70* parent strain and the *ΔsidD* disruptants, phleomycin (20 μg/ml) and hygromycin B (50 μg/ml) were also added to the culture medium, respectively, in order to maintain the selection pressure.

Genomic DNA Extraction

Fresh mycelia were collected from 9-day-old cultures grown on PDA plates. After grinding in liquid nitrogen and addition of a 10 mM Tris-HCl lysis buffer (pH 8) supplemented with 1 mM EDTA, 2% Triton X100, 1% SDS, and 0.1 M NaCl, the total genomic DNA was extracted by the addition of an equal volume of phenol:chloroform:isopropanol (25:24:1; Sigma-Aldrich, Saint-Louis, Mi) and chloroform/isoamyl alcohol (24:1; Sigma-Aldrich), and then precipitated by the addition of 2 volumes of 100% ethanol. After washing with 70% ethanol and digestion of RNA with 0.2 mg/mL RNase A, DNA was quantified on a Qubit® 2.0 Fluorometer (Invitrogen, Cergy Pontoise, France) and integrity was checked by 1% agarose gel electrophoresis. Genomic DNA was stored in TE buffer at 4°C.

Plasmid Construction

Two constructions were prepared to produce the double mutant deficient for the genes encoding the Ku70 subunit and the NRPS SidD.

For prior disruption of the *KU70* gene, transforming DNA was obtained by PCR amplification of a fragment containing the whole *KU70* coding sequence of *S. apiospermum* (SAPIO_CDS7374), together with its upstream (1169 bp) and downstream (1986 pb) flanking sequences. The primers used for PCR (SaKU70-F-BamHI and SaKU70-R-ClaI; **Supplementary Table 1**) contained BamHI and ClaI restriction sites. This allowed to clone the PCR product into plasmid pBluescript II KS(+) (Agilent, Les Ulis, France) at the corresponding restriction sites, which led to plasmid pPV221. Then the ORF corresponding to *KU70* within pPV221 was interrupted and substituted in part by a SbfI/StuI fragment from the pAN8-1 plasmid [1479914] containing the phleomycin resistance gene (*BLE*), leading to plasmid pPV229. Transforming DNA (10 µg) was released from pPV229 by digestion with SpeI and ClaI, and integrated at the *KU70* locus as described below.

For disruption of *sidD* gene, the cassette was obtained after cloning the flanking regions within plasmid pPV189, which harbors the hygromycin B resistance gene (*HPH*) as a selection marker. The 5' and 3' flanking regions of *sidD* gene were obtained from DNA from the wild-type strain by PCR amplification using primers SaSidD-5'UTR-F-ClaI and SaSidD-5'UTR-R-HindIII, and primers SaSidD-3'UTR-F-NotI and SaSidD-3'UTR-R-BstXI, respectively (**Supplementary Table 1**). Amplified fragments were digested with primer specific restriction enzymes, i.e., ClaI and HindIII for the 5' upstream PCR product, and NotI and BstXI for the 3' downstream PCR product and finally introduced sequentially in the corresponding sites of the plasmid pPV189 to yield pYLG108.

Fungal Transformation

The transformation was achieved on protoplasts obtained from 24-h-old germ tubes as described by Turgeon et al. (2010) and Liu and Friesen (2012) with 5 µg of DNA. In brief, germ tubes were first collected by filtration on 20-µm pore size Miracloth® membranes (Merck, Darmstadt, Germany) and incubated at 37°C for 3.5 h under constant shaking (120 rpm) in OM/glucanex solution [1.2 M $MgSO_4$, 10 mM Na_2HPO_4 (pH 5.8), and 12.5 g/L glucanex]. Protoplasts were recovered by centrifugation in a Tris-HCl buffer (10 mM) containing 1.2 M sorbitol to maintain the osmotic pressure, and then stored at 4°C in the same buffer supplemented with 10 mM $CaCl_2$. The cassette was integrated in protoplasts by heat shock in the presence of polyethylene glycol (PEG). Afterwards protoplasts were poured onto soft agar medium (1 M sucrose, 0.2% yeast extract, 0.2% casaminoacids, and 1.28% molten agar), which was covered 16 h later with the same culture medium supplemented with 20 µg/mL phleomycin or 50 µg/mL hygromycin B, according to the resistance gene used in the disruption cassette. Cultures were incubated for 3 days at 37°C, and transformants capable to grow in the presence of phleomycin or hygromycin B were selected.

Mutants were maintained on PDA supplemented with 0.5% chloramphenicol and 20 µg/mL phleomycin for the *Δku70* mutant or with 0.5% chloramphenicol and 50 µg/mL hygromycin B for the double mutant *Δku70*/*ΔsidD*.

Validation of Gene Disruption

Monospore isolates of transformants growing in the presence of 20 µg/mL phleomycin or 50 µg/mL hygromycin B were subcultured, and their genotype was analyzed by Southern blot as previously described (Pateau et al., 2018) to confirm the integration of the disruption cassette at the target locus. Genomic DNA was extracted and digested overnight with the appropriate restriction enzyme (EcoRI for analysis of the *Δku70* mutant, and SacI for the double mutant). After separation of the digested genomic DNA by agarose gel electrophoresis, gels were incubated successively in 0.25 N HCl, 1.5 M NaCl/0.5 M NaOH, and finally 0.5 M Tris-HCl (pH 7.5)/1.5 M NaCl. DNA fragments were then transferred on nylon membranes (Amersham Hybond™-N+, GE Healthcare). After crosslinking for 3 min under UV light, gels were incubated overnight at 55°C in the presence of an appropriate probe. The probe was either a double SpeI/StuI digest of pPV229 corresponding to *KU70* upstream region for validation of the *Δku70* mutant (**Figure 1A**) or a PCR product obtained using SaSidD-3'UTR-F-NotI as forward primer and SaSidD-3'UTR-R-BstXI as the reverse primer (**Supplementary Table 1** and **Figure 1C**) for validation of the *Δku70*/*ΔsidD* double mutant. Each probe was labeled with Illustra™ Shrimp alkaline phosphatase (GE Healthcare life sciences, Chicago, Il) according to the manufacturer's recommendations. Finally, alkaline phosphatase was revealed by the addition of its substrate, and the membrane was imaged by chemiluminescence (LAS4000 GE Healthcare).

Siderophore Detection and Characterization

Siderophore Production

Characterization of siderophores was performed on lyophilized filtrates obtained from *S. apiospermum* WT and *ΔsidD* null-mutant (M1, M2, and M3) strains. At first, conidia were harvested from colonies by aseptically scraping the plates as previously described (Le Govic et al., 2018). Approximately 2×10^7 conidia of each strain were inoculated into 50 ml of Yeast Nitrogen Base medium (0.69% YNB w/o amino acids; ForMedium, Norfolk, UK) supplemented with 2% glucose and 36 µM $FeSO_4$. WT strain was cultured with and without the addition of the iron chelator bathophenantroline

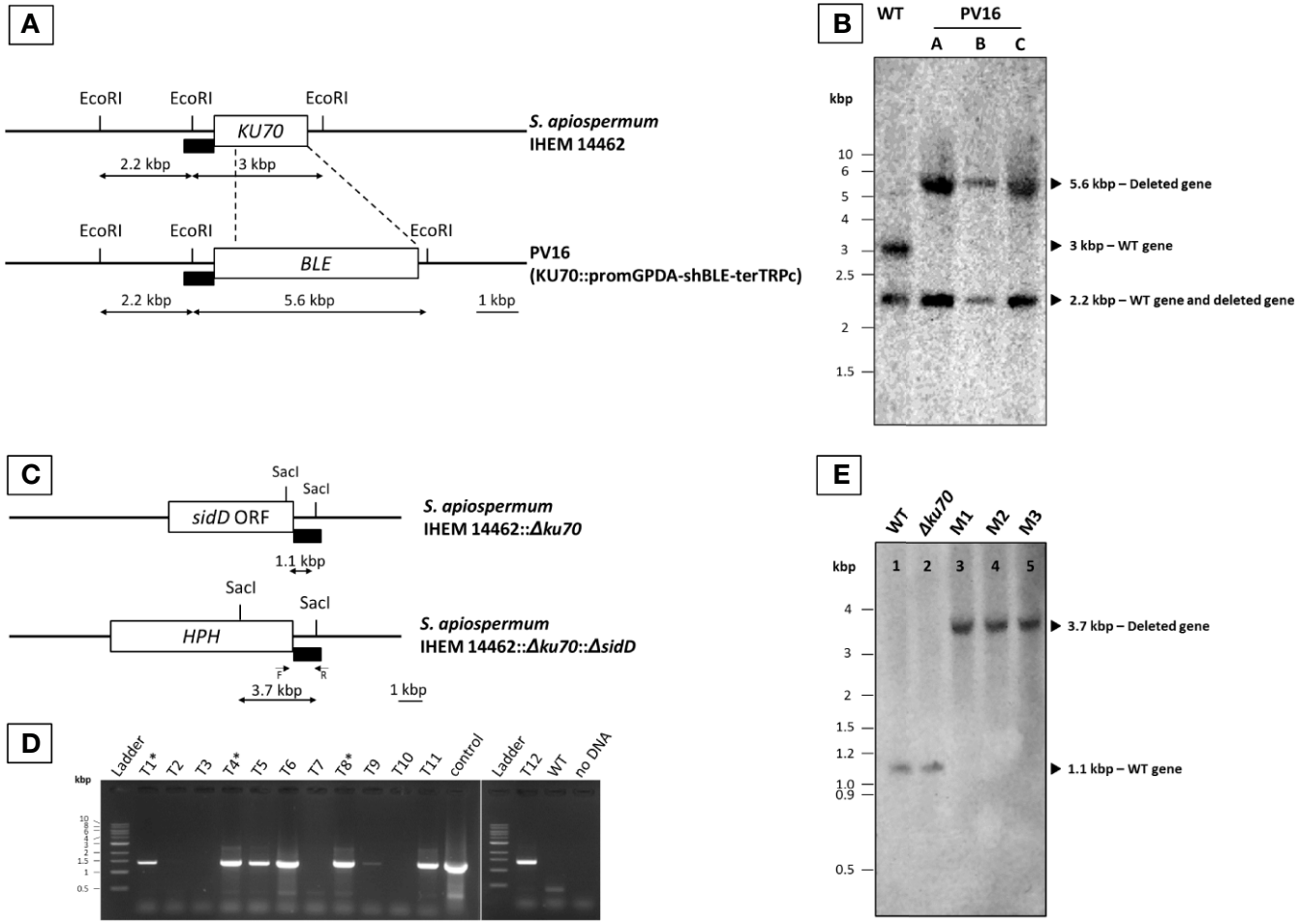

FIGURE 1 | Generation of *S. apiospermum Δku70* and *Δku70/ΔsidD* mutants. **(A)** Restriction map of *S. apiospermum KU70* locus. EcoRI restriction sites are indicated, and the box indicates the hybridization site of the probe (a SpeI/StuI fragment of pPV229 corresponding to the upstream region of *KU70*). The size of the expected fragments for each mutant is indicated by arrows. **(B)** Southern blot analysis of the *KU70* deletion strains. Genomic DNA of the wild-type strain IHEM 14462 (WT) and the *Δku70* mutants (*Δku70*-A, B, and C) was digested by EcoRI. The 3-kbp band corresponding to the *KU70* wild-type locus was not detected for strains *Δku70*-A, B, and C, in which the expected 5.6-kbp band demonstrated the correct disruption of the *KU70* gene by the phleomycin resistance gene. **(C)** Restriction map of *S. apiospermum sidD* locus and strategy of construction of the disruption cassette. SacI restriction sites are indicated, and the black box indicates the hybridization site of the probe (downstream region of the *sidD* gene). The size of the expected fragments is indicated by arrows. F and R correspond to the primers used for PCR verification of hygromycin-resistant transformants. **(D)** PCR amplification of twelve randomly selected hygromycin-resistant transformants. Positive amplification indicates an integration of the deletion cassette within the *S. apiospermum* genome, but cannot differentiate between correct gene replacement and ectopic integration of the cassette. The PCR-positive transformants depicted with a symbol (*) are those selected for further analysis (after that noted M1, M2, and M3). Control: plasmid pYL108, including the *sidD* disruption cassette. **(E)** Southern blot analysis of the *sidD* deletion strains. Chromosomal DNA of the wild-type strain IHEM 14462 (lane 1; WT), its *Δku70* null mutant derivative (lane 2; *Δku70*), and of three *ΔsidD* mutant strains (lanes 3–5; M1 to M3) was digested by SacI and probed with a hybridization probe corresponding to a ~1 kbp fragment of the *S. apiospermum sidD* 3'-flanking region. The expected signals were 1.1 kbp for the WT and parent strains and 3.7 kbp for the *ΔsidD* mutants.

disulfonate (BPS, 100 μM; Sigma-Aldrich, Saint-Quentin Fallavier, France), while the three independent mutants were grown in YNB without BPS. After 48 h at 37°C under agitation (120 rpm), the whole culture flasks were filtered through Miracloth® mesh filter to remove hyphae. The filtrates were then clarified by successive passages through 0.45-μm and 0.22-μm-pore size membranes (Dominique Dutscher, Brumath, France), split into two parts (~25 ml), and finally lyophilized.

Siderophore Extraction and Purification

Aliquots of each lyophilized sample (20 mg) were resuspended in 1 ml ultra-pure water (UHPLC-MS grade, Honeywell, Germany). Before matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance (MALDI FT-ICR) mass spectrometry (MS) analysis, all samples (100 μl) were desalted by solid-phase extraction with a Sep-Pak C18 cartridge (Waters, Prague, Czech Republic). Briefly, polar contaminants were washed out with 200 μl of water followed by the extraction of siderophores using 400 μl of methanol. Extracts were evaporated under vacuum (2 h, 35°C), and solubilized in 100 μl of 50% acetonitrile (ACN).

MALDI FT-ICR MS Analysis

Two microliters of the prepared solutions were spotted on a ground steel MALDI plate, dried and covered by 1 μl of α-cyano-4-hydroxycinnamic acid (CHCA; 10 mg/ml in 50% ACN/0.1% trifluoroacetic acid) matrix. MALDI MS analyses were performed using the Solarix FT-ICR 12T (Bruker Daltonics, USA) mass spectrometer. All measurements were acquired in positive ion mode in a 150–1500 *m/z* mass range after an external calibration against Pepmix II (Bruker Daltonics) and clusters of CHCA with a mass accuracy better than 2 ppm. To increase ion intensity, the continuous accumulation of selected ions (CASI) mode with a quadrupole-narrowing window in the 500–1000 mass range was used. Desorption/ionization of siderophores was performed using SmartBeam II laser (laser power of 40%, 200 shots, 2 kHz), and instrument parameters was tuned to optimal absolute ion signal intensity. Mass spectra of selected ions were collected at a 1 Da isolation width and 20–25 V collision energy. Final spectra represented an average of 16 or 32 acquired scans. Data were processed using the DataAnalysis software (v.4.1, Bruker Daltonics) and siderophores were annotated in CycloBranch (v.2.0.8) against our databases (Pluháček et al., 2016; Novák et al., 2020).

Cultural Features

All strains were grown on PDA plates containing 100 μM BPS and supplemented with ~25 ml lyophilized culture filtrates from the WT strain, the *Δku70* parent strain, or the mutants. The ability of *S. apiospermum* to assimilate iron from xenosiderophores was also assessed by using 20 μM of iron-saturated ferrichrome, ferrioxamine, or pyoverdine (Sigma-Aldrich) as the sole source of iron. Plates were point-inoculated and then incubated for seven days at 37°C.

Virulence Assay

Virulence of the *S. apiospermum* strains was tested in a murine model of disseminated scedosporiosis. For this purpose, 4-week-old male OF-1 mice (Charles River, Criffa S.A. Barcelona, Spain) weighing 30 g were used. Animals were immunosuppressed 1 day prior infection by intraperitoneal (i.p.) single dose of cyclophosphamide at 200 mg/kg together with intravenous (i.v.) fluorouracil dose at 150 mg/kg. To determine the optimal inoculum size, groups of 5 animals were inoculated i.v. in the lateral vein of the tail with 0.2 ml of a conidial suspension resulting in 2×10^2, 2×10^3, 2×10^4, or 2×10^5 CFU/animal. For the comparison virulence study between WT and knockout strains, inocula consisting on 2×10^3 CFU/animal of each fungal strain were injected i.v. *via* the lateral tail vein in groups of 14 mice (9 for survival and 5 for fungal burden studies) randomly established. To prevent bacterial infections, mice received ceftazidime subcutaneously (5 mg/kg/day). Mortality was recorded twice daily for 20 days. On day 6 post-infection, mice from the fungal burden groups, as well as surviving animals at the end of the experiment, were euthanized for tissue burden determination. Brain, lungs, and kidneys were removed aseptically, weighed and mechanically homogenized in 1 ml of sterile saline. Homogenates were 10-fold diluted in sterile saline, and the dilutions were placed onto PDA agar and incubated at 30°C for determination of the fungal load (expressed in CFU per g of tissue). All animal care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee (protocol number 8248).

RESULTS

Disruption of the *sidD* Gene in *S. apiospermum*

Because of the high frequency of non-homologous recombination events in *S. apiospermum*, all our attempts to produce a *ΔsidD* mutant from the wild-type strain failed (data not shown). Therefore, we first produced a mutant strain deficient for the non-homologous end joining (NHEJ) by disruption of the *KU70* gene encoding one of its protein subunits. Almost 20 colonies growing in the presence of 20 μg/ml phleomycin were obtained after transformation of the WT strain IHEM 14462 with the *KU70* deletion cassette. The genotype of monospore isolates obtained from these transformants (*Δku70*) was verified by Southern blot. As illustrated in **Figure 1B**, a band of the expected size (5.6 kbp) was visualized for PV16-A, B, and C monospore isolates after hybridization with the probe, instead of the 3-kbp band observed for the WT strain, thus demonstrating the successful disruption of the *KU70* gene.

The *sidD* gene was then disrupted in the *Δku70* mutant by introducing the *HPH* resistance gene at the *sidD* locus. A total of twelve clones were randomly selected based on their hygromycin resistance phenotype. Transformant stability was assessed by two successive subcultures on PDA supplemented with 50 μg/ml hygromycin B. All transformants showed resistance to hygromycin B, suggesting that the *HPH* gene was stably maintained in their genome. Genomic DNA of the WT strain and all hygromycin-resistant transformants was then extracted

for molecular characterization. PCR using the primer pair Hph-F and SaSidD-3'UTR-R-BstXI (see primers in **Supplementary Table 1**) led to the amplification of a DNA fragment of the expected size (1,300 bp) for seven out of the 12 transformants (**Figure 1D**). Three PCR-positive transformants (M1, M2, and M3) were randomly selected and further purified by a round of single-spore isolation and two successive subcultures. Finally, southern blot analysis revealed a single band of 1.1 kbp for the WT strain as well as for the *Δku70* parent strain. In contrast, a 3.7-kbp band was evidenced for the three transformants M1, M2, and M3, as expected for a correct *sidD* gene disruption event (**Figures 1C, E**).

Siderophore Synthesis in *S. apiospermum*

The *S. apiospermum* WT strain and *ΔsidD* mutants were examined for the production of siderophores by the high-resolution MALDI MS analysis. This analysis was performed on lyophilized filtrates obtained from the WT strain and *ΔsidD* mutants (M1, M2, and M3) grown in minimal YNB medium under iron-sufficient and/or iron-depleted conditions.

Analysis of the extracellular siderophores performed on culture filtrate from the WT strain revealed one possible candidate against the siderophore/secondary metabolites database, namely, N^{α}-methylcoprogen B ($C_{34}H_{56}N_6O_{12}$) both in desferri- (*m/z* 741.403, $[M+H]^+$) and ferri- (*m/z* 794.314, $(M+Fe-2H)^+$) forms (**Figure 2A**). Characterization of these ions was further confirmed by their tandem MS fragmentation patterns (**Figures 2C, D**) compared with the literature (Antelo et al., 2006; Bertrand et al., 2009). Besides, the alkali ion metal attachments were common in desferri- or ferriforms (**Supplementary Video 1**). CycloBranch annotated $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, $[M+Fe-2H]^+$, $[M+Fe+Na-3H]^+$, or $[M+Fe+K-3H]^+$ ions, some of them also accompanied with less abundant isotopes (^{54}Fe, ^{41}K). Interestingly, the intensity of the 741.403 compound (protonated desferri-N^{α}-methylcoprogen B) did not increase in the culture filtrate from WT grown in the presence of BPS. The most likely explanation is that N^{α}-methylcoprogen B is able to extract iron from BPS, which therefore does not create a massive iron starvation. Conversely, another signal at *m/z* 424.05 increased, which could be related to BPS. A compound at *m/z* 742.406 was also seen in the culture filtrate of the WT strain, belonging to the isotopic structure (A+1 ion) of the desferri-N^{α}-methylcoprogen compound as the first isotope, which differs in neutron number regarding the monoisotopic mass 741.403. Of note, N^{α}-methylcoprogen B was absent in all *ΔsidD* mutants, indicating that *sidD* gene is essential for its synthesis (**Figure 2B**). Moreover, other siderophores like FsC or TAFC, as well as dimerumic acid, were not found in any of the samples.

The impact of *sidD* deletion was then investigated by cultivating the fungus on PDA plates containing BPS and

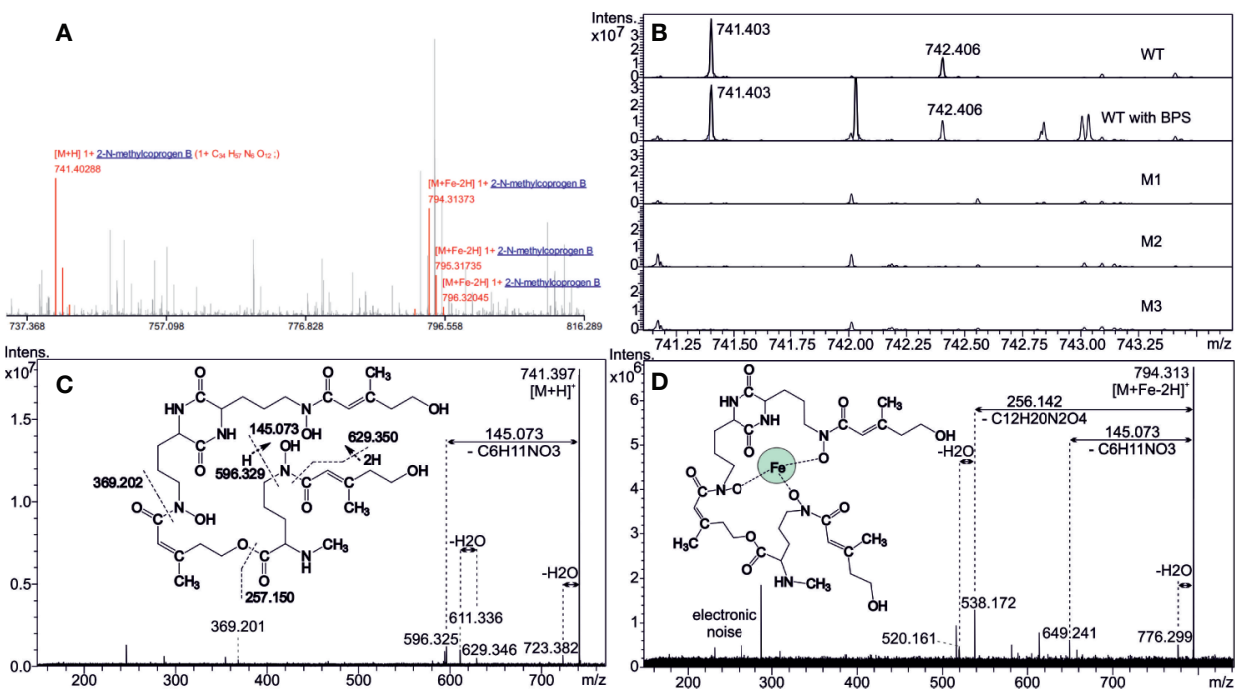

FIGURE 2 | Detection of extracellular siderophores. **(A)** Matrix-assisted laser desorption/ionization with Fourier transform ion cyclotron resonance (MALDI FT-ICR) mass spectrum annotated by CycloBranch. Both iron-free and iron-bound N^{α}-methylcoprogen B derivatives were matched against our in-house compound library (2 ppm accuracy). **(B)** Comparison of the mutual absolute abundance for 741.403 ion (iron-free N^{α}-methylcoprogen B) in spectra of WT and all the mutants. Note the inherent high mass resolving power of the FT-ICR instrument. **(C, D)** MALDI MS/MS showing the fragmentation patterns of protonated N^{α}-methylcoprogen B in desferri- (*m/z* 741) and ferri- (*m/z* 794) forms at 20 and 25 eV collisional energies, respectively. Hydrogen transfers have taken place in some fragmentation steps, and intrinsic cleavage sites, in either amide or ester bonds, are indicated in **Figure 2C**.

supplemented with culture filtrates from either the WT strain or the mutants. The disruption of *sidD* resulted in the absence of growth under iron-restricted conditions (**Figures 3A, B**). In contrast, growth could be restored by supplementation of the culture medium (PDA + BPS) with a culture filtrate from the WT strain (**Figure 3C**). Conversely, incorporation of lyophilized filtrates from the mutants in the culture medium did not rescue hyphal development (**Figure 3D**), corroborating the biochemical analyses, i.e., abrogation of extracellular siderophore biosynthesis following inactivation of *sidD*. Furthermore, iron supplementation alone (20 μM) partially reversed the inhibitory effect of BPS on hyphal development, with mutant growth oriented towards the WT strain and the *Δku70* parent strain, suggesting siderophore piracy (**Figure 3E**).

Finally, the ability of *S. apiospermum* to assimilate iron from xenosiderophores was assessed by using Fe(III)-saturated ferrichrome, ferrioxamine, or pyoverdine (20 μM each) as the sole source of iron (**Figure 4**). Supplementation of PDA medium with iron-saturated ferrichrome or ferrioxamine led to normal growth of all strains, indicating their direct assimilation by the fungus independently of siderophore production (**Figures 4C, D**). On the other hand, pyoverdine fully reversed the inhibitory effect of BPS on the WT and parent strains only, while the mutants exhibited growth directed towards the WT or their parent strains, which suggests siderophore spoliation (**Figures 4E, F**). To ensure that iron uptake by *S. apiospermum* from pyoverdine is mediated by its siderophore, the WT strain, the *Δku70* parent strain, and the mutants were cultivated separately. In these experiments, pyoverdine supported the growth of the WT and parent strains only (**Figures 4G, H**), confirming that N^{α}-methylcoprogen B is essential for iron acquisition from pyoverdine in *S. apiospermum*.

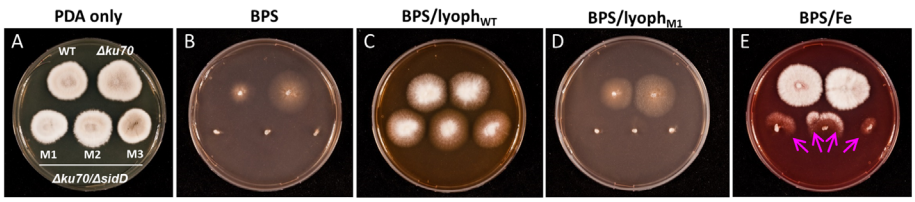

FIGURE 3 | Impact of *sidD* deletion upon *S. apiospermum* growth. **(A)** Colonial growth phenotypes on PDA after seven days of incubation at 37°C. **(B–D)** SidD deficiency results in the lack of growth in the presence of 100 μM BPS, which can be restored by the addition to the culture medium of lyophilized culture filtrate (initial volume 25 ml) from the WT strain but not from mutant strains. **(E)** Iron supplementation alone (20 μM) partially reversed the effect of BPS, with mutant growth directed towards the WT and parent strains, suggesting siderophore piracy (arrows).

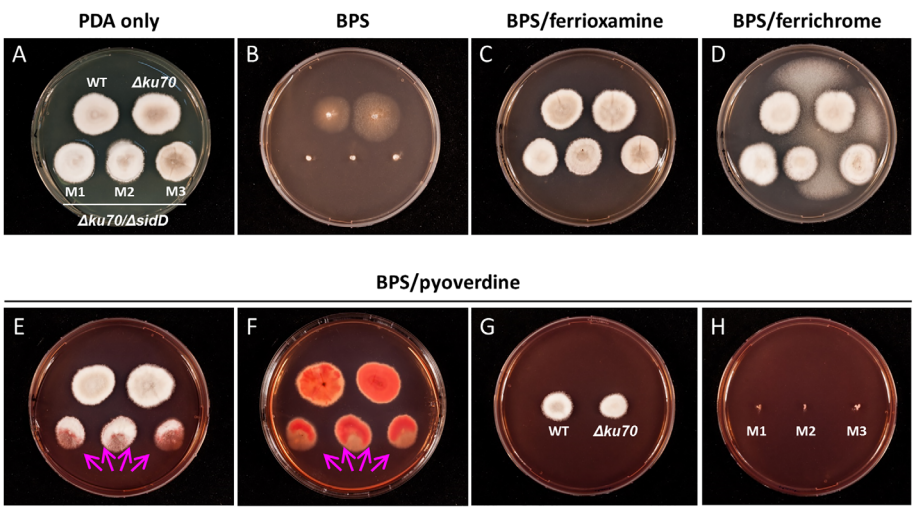

FIGURE 4 | The ability of *S. apiospermum* to assimilate iron from xenosiderophores. **(A, B)** Colony growth phenotypes on PDA ± BPS after seven days of incubation at 37°C. **(C, D)** Ferrioxamine and ferrichrome (20 μM each) fully rescued the growth of all strains in the presence of 100 μM BPS. The fungus growing between *Scedosporium* colonies corresponds to contamination by *Aspergillus* sp. **(E, F)** Iron-bound pyoverdine (20 μM) fully restored growth for the WT and parent strains, while *ΔsidD* mutant strains only displayed oriented growth (arrows). Reddish pigmentation observed at the reverse of the colonies indicates the accumulation of iron. **(G, H)** Separated cultivation of WT, parent, and mutant strains, suggesting that N^{α}-methylcoprogen B is mandatory for iron acquisition from pyoverdine.

sidD Deficiency Attenuates the Virulence of *S. apiospermum* in a Neutropenic Murine Model of Disseminated Scedosporiosis

To determine whether *sidD* plays a role in fungal pathogenesis, we compared the WT strain, the *Δku70* parent strain, and the *ΔsidD* mutants regarding their virulence in a neutropenic mouse model of disseminated scedosporiosis. In a preliminary study, four different inocula of *S. apiospermum* WT strain IHEM 14462 were assayed (2×10^2, 2×10^3, 2×10^4, or 2×10^5 CFU/animal) to establish the inoculum that can cause acute infection. In the inoculum escalation study no animal infected with the higher inocula, i.e., 2×10^5 or 2×10^4 CFU/animal; survived to the infection with a mean survival time (MST) of 3 and 4 days, respectively. All animals except one (20%) challenged with 2×10^3 CFU succumbed to the infection (MST = 6), while only 40% of animals receiving 2×10^2 CFU died (data not shown). Therefore, the 2×10^3 CFU inoculum load was used for challenging immunosuppressed mice with the different strains included in this study. In the virulence comparison study, infection by WT strain caused a slightly higher mortality (MST = 5 days with all mice dying 7 days post-infection) than observed in the inoculum-size study ($P = 0.35$). A significant difference in survival was seen between mice infected with the WT strain and those challenged with the *Δku70* mutant ($P = 0.006$) (**Figure 5A**). Nevertheless, disruption of *sidD* gene resulted in a marked reduction in virulence. Animals challenged with the *ΔsiD* mutants survived significantly longer than those infected with the WT strain ($P < 0.0001$) or with their *Δku70* parent strain ($P \leq 0.001$). All animals infected with the *ΔsiD* mutants (isolates M2 and M3), except one infected with isolate M1, survived to the experiment.

Due to the high mortality reached, only one animal from the WT group was used for CFU determination on day 6. In consequence data from two animals euthanatized 5 days post-infection were included (**Figure 5B**). No CFU were recovered from organs of animals infected with *ΔsiD* mutants, while animals inoculated with the WT or *Δku70* strains showed high fungal load in kidneys ($\log_{10}$ mean ± SD, 4.69 ± 0.11 and 4.55 ± 0.17 CFUs/g, respectively). No colonies were recovered from brain or lungs. At the end of the experimental time (day 20 post-infection), no fungal elements were isolated from any organs of *ΔsiD*–infected animals (data not shown).

DISCUSSION

Genome inspection of *S. apiospermum* strain IHEM 14462 previously evidenced several genes putatively involved in siderophore biosynthesis (Le Govic et al., 2018), notably an ortholog of *sidD*, which encodes an NRPS that is known to drive the synthesis of the hydroxamate-type siderophore fusarinine C in *A. fumigatus* (Schrettl et al., 2007). Like other siderophore-producing fungi, notably *Aspergillus* spp., the *S. apiospermum sidD* gene is clustered with five other genes implicated in siderophore biosynthesis (*sidI*, *sidF*, and *sidL* orthologs) and transport (one *sitT* and one *mir* orthologs) (Le Govic et al., 2018). Additionally, the transcriptomic analysis supported the role of *S. apiospermum sidD* gene in iron metabolism, since it was induced during iron starvation and conversely repressed in iron-repleted conditions (Le Govic et al., 2018). However, although the structural organization of *S. apiospermum* SidD resembles that of *Aspergillus* species, none of the bioinformatics tools were able to predict the exact nature of the compound produced (Le Govic et al., 2019). Our first aim was to investigate the possible role of *S. apiospermum sidD* in the production of a secreted siderophore and, if applicable, to determine the exact nature of the compound synthesized.

Previously, Pateau et al. (2018) prepared from *S. aurantiacum* defective strains for a cytosolic Cu, Zn-superoxide dismutase

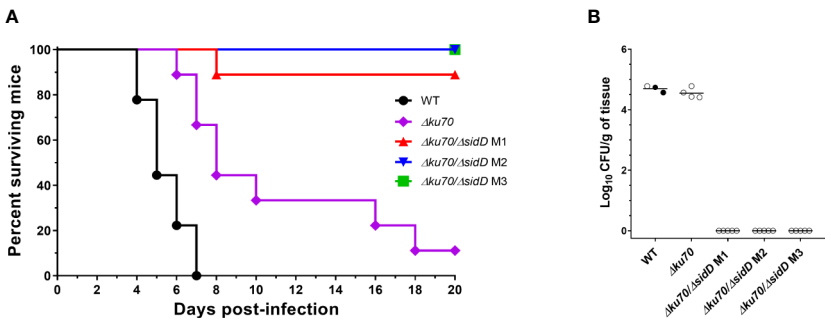

FIGURE 5 | SidD deficiency attenuates the virulence of *S. apiospermum* in a murine model of disseminated scedosporiosis. **(A)** Survival of cyclophosphamide/fluorocytosine-treated mice infected with the indicated *S. apiospermum* strains (2×10^3 CFU/animal). Data were obtained from a total of 9 mice infected per strain. For visibility, the percent surviving was indicated at day 18 for the animals inoculated with the *sidD* mutant strain M2. Statistical differences for mouse survival were calculated using the Log-Rank test. **(B)** Tissue burden in kidneys of cyclophosphamide/fluorocytosine-treated mice infected with the indicated strains, as revealed by determination of the fungal load (in CFUs/g) 6 days post-infection (n = 5). Dark dots correspond to animals euthanatized 5 days post-infection. Horizontal lines indicate median values.

(SODC) and showed a surprisingly high frequency of homologous recombination of 30%. Other results from our laboratory suggested a markedly lower frequency in *S. apiospermum*, similar to those reported in the literature for other filamentous fungi (i.e., <3%) (Meyer, 2008). All our attempts to obtain defective strains for *sidD* gene in the wild-type background failed, resulting exclusively in ectopic recombination events. Thus, we first focused our attention on the generation of a strain deficient for the NHEJ system by deletion of the *KU70* gene. This strategy is the gold standard to increase the frequency of homologous recombination in filamentous fungi (Krappmann, 2007) and was necessary to obtain a *ΔsidD* mutant in *S. apiospermum*. In our study, we have been limited by the genetic tools available for *S. apiospermum* and particularly by the limited number of resistance markers that can be applied to *Scedosporium* species. Indeed, these fungi are resistant to many antifungals. Currently, only two drugs are effective, phleomycin, which was used for engineering of the *Δku70* strain, and hygromycin B, which was used for selection of the defective strain *Δku70*/*ΔsidD*. Complementation was not possible since no other selection markers were available. Further genetic tools should be developed to allow the production of multiple mutants and complementation in *Scedosporium* species.

Mass spectrometry analysis performed on culture filtrates from the WT strain yielded two specific signature ions, corresponding to N^{α}-methylcoprogen B in its ferri- and desferri- forms, respectively. N^{α}-methylcoprogen B is a linear, hexadentate, hydroxamate-type siderophore. This compound was absent in the three *ΔsidD* mutants studied, demonstrating that *sidD* gene is required for its synthesis. Other siderophores like fusarinines or ferrichromes were not found in any sample. Besides N^{α}-methylcoprogen B, Bertrand et al. (2009) found that *S. apiospermum* also produces a dihydroxamate compound called dimerumic acid. Still, its involvement in iron homeostasis is controversial since it is both described as a degradation product of coprogen and as a natural product in other molds (Donzelli and Krasnoff, 2016). Moreover, the production of dimerumic acid in *S. apiospermum* may be strain-dependent since it was not detected in 8 out of 10 strains tested (including strain IHEM 14462), whereas N^{α}-methylcoprogen B was found in the culture supernatant for all the strains studied (Bertrand et al., 2009). Interestingly, the *in vivo* production of N^{α}-methylcoprogen B was evidenced from sputum samples of CF patients colonized by a *Scedosporium* species (Bertrand et al., 2010). Besides, *Scedosporium* spp. were found to be the greatest siderophore producers *in vitro* compared with other CF fungal colonizers like *Aspergillus* species and *Exophiala dermatitidis* (Bertrand et al., 2010).

Siderophore-assisted acquisition of iron seems mandatory for *Scedosporium* survival, since the disruption of *sidD* resulted in the total lack of growth under iron-restricted conditions. At the same time, the incorporation of lyophilized filtrates from WT strain (thus containing N^{α}-methylcoprogen B) within the culture medium restored the phenotype. The lack of growth of the double mutants also highlights the almost absence of compensatory mechanisms for extracellular siderophore deficiency or at least the suboptimal role of RIA when the fungus is cultivated under iron-restricted conditions. Indeed, we previously showed that *S. apiospermum* possesses three RIA related genes which are overexpressed during iron carency, but their implication in fungal adaptation seems marginal in these conditions. Nonetheless, the RIA system might explain the WT-like growth of the *sidD* mutants when iron is not limiting. In addition to self-produced N^{α}-methylcoprogen B, *S. apiospermum* is able to appropriate iron through the acquisition of siderophore produced by other microorganisms (xenosiderophores), underlining its capacity for adaptation to changing environments and enhanced niche colonization. Our experiments revealed different patterns in xenosiderophore utilization by *S. apiospermum*, i.e., a direct acquisition from the hydroxamate-type siderophores ferrioxamine and ferrichrome, which probably utilizes the same membrane transporters, and an indirect acquisition from the mixed catechol-hydroxamate siderophore pyoverdine, which necessitates the presence of N^{α}-methylcoprogen B. From a pathophysiological point of view, the latter observation might explain why prior colonization of the CF airways by *Scedosporium* species prevents the establishment of *P. aeruginosa* (Blyth et al., 2010). Other recent *in vitro* experiments found that co-cultures of *S. aurantiacum* and *P. aeruginosa* resulted in inhibition of scedosporial growth, raising the hypothesis of a siderophore-driven competition between the microorganisms for extracellular iron (Kaur et al., 2015; Chen et al., 2018; Homa et al., 2019). Nonetheless, this bacterial-fungal antagonism was not confirmed by Schwarz et al. (2017). These authors reported an increased rate of co-colonization with the mucoid phenotype of *P. aeruginosa* in *Scedosporium*-colonized CF patients. Interestingly, Sass et al. (2018) recently showed that *P. aeruginosa* was able to inhibit the growth of *A. fumigatus* through pyoverdine-mediated iron deprivation, since *A. fumigatus* is unable to utilize pyoverdine and, conversely, siderophore production by *A. fumigatus* was found to protect against the pyoverdine-mediated inhibition (Sass et al., 2019). Further epidemiological investigations, along with metabolomics studies of the lung microbiome, would help to understand better the bacterial-fungal interactions that occur within the CF bronchial mucus.

Our experiments showed that the abrogation of extracellular siderophore biosynthesis following inactivation of the NRPS SidD significantly decreased virulence of *S. apiospermum* in an immunocompromised murine model of disseminated scedosporiosis. Similarly, the deficiency of SidD/NPS6 orthologs caused a dramatic reduction of virulence in *A. fumigatus*, *Fusarium graminearum*, *Bipolaris maydis* (formerly *Cochliobolus heterostrophus*), *Bipolaris oryzae* (formerly *Cochliobolus miyabeanus*), and *Alternaria brassicicola*, demonstrating that siderophores are conserved virulence determinants of human, animal, and plant fungal pathogens (Oide et al., 2006; Schrettl et al., 2007). Likewise, Schrettl and coworkers (Schrettl et al., 2007) showed complementary, but different roles for extra- and intracellular siderophores during *A. fumigatus* infection, supporting extracellular siderophore

production as a therapeutic target. However, the contribution of individual iron metabolism-regulating mechanisms in virulence dramatically varies according to a pathogen. For instance, the siderophores synthesized by the phytopathogenic basidiomycetes *Ustilago maydis* and *Microbotryum violaceum* are dispensable for virulence (Mei et al., 1993; Birch and Ruddat, 2005), while some animal pathogenic ascomycetes (e.g., *Candida albicans*) and basidiomycetes (e.g., *Cryptococcus neoformans*) do not produce siderophores. Moreover, it has been demonstrated that in *U. maydis* and *C. albicans*, RIA (but not siderophores) was crucial for virulence (Ramanan and Wang, 2000; Eichhorn et al., 2006).

Of note, the *Δku70* parent strain was associated with a slightly lower virulence compared with the WT strain. However, both strains were phenotypically undistinguishable in terms of growth rate and ability to exploit iron from various sources. These observations are in line with other studies in which the NHEJ pathway in several filamentous models was inactivated through the deletion of the *ku70* gene (Ninomiya et al., 2004; Nayak et al., 2006; Choquer et al., 2008; Hoff et al., 2010; Li et al., 2010; Qi et al., 2015; Gandía et al., 2016). The resulting *Δku70* strains were not reported to exhibit noticeable phenotypic differences with the WT strains, and they were further used as parent strains to delete genes of interest. Considering the lack of changes in growth features following deletion of the *ku70* gene, the slight difference observed in our experiments in virulence among the WT and *Δku70* parent strains is probably due to some other disturbances in the mutated strain.

The main limitation of our study was the inability to assess the role of siderophore production in the development of a pulmonary infection. Indeed, regular route of *Scedosporium* acquisition is through inhalation, being the infection located primarily in the lungs. In an immunocompromised host, the disease may disseminate through the bloodstream, affecting multiple organs including the central nervous system. Likewise, in patients with CF, *Scedosporium* species are among the most common filamentous fungi colonizing the airways, where they are mainly regarded as « by-standers »; however, this colonization of the airways may be the cause of an invasive pulmonary infection with subsequent hematogenous dissemination of the fungus in case of lung or heart/lung transplantation. Such invasive conditions are associated with a high letality rate despite treatment, which is predominantly due to the lack of effective antifungal therapy. Therefore, our main objective here was to assess the importance of siderophore production in *Scedosporium* invasiveness in order to demonstrate its potential as new fungal-specific drug target. Hence we used the gold standard model for such purpose. The fact that the *ΔsidD* mutants were unable to cause a disseminated infection demonstrates that SidD is essential for virulence when *S. apiospermum* spreads through the bloodstream; however, this does not mean it will be essential in other tissues—e.g., in the airways. This could explain the conflicting results reported for the interactions with *P. aeruginosa*. Unfortunately, there is currently no validated animal model to evaluate the virulence of *Scedosporium* species after nasal or tracheal inoculation of spores, nor their ability to colonize the airways in immunocompetent mice, especially in CFTR deficient immunocompetent mice. Nevertheless, as already mentioned, N^{α}-methylcoprogen B was detected from almost all sputum samples analyzed by Bertrand et al. (2010) from CF patients colonized by *Scedosporium* species.

Altogether, our results revealed that the *S. apiospermum sidD* gene drives the synthesis of a unique extracellular siderophore, N^{α}-methylcoprogen B, that was found to be essential for fungal growth and virulence. This compound seems important for iron acquisition from pyoverdine, which might explain the apparent antagonism between *S. apiospermum* and *P. aeruginosa* within the CF lung. Further studies including evaluation of cultural characteristics and virulence of *sidC* (intracellular siderophore NRPS gene) disruptants in murine models of pulmonary or disseminated scedosporiosis are also needed to delineate the respective roles of intra- and extracellular siderophores in pathogenicity and protection of the fungus against the host immune defenses. Likewise, this study opens the way for investigating other genes encoding putative virulence factors in *Scedosporium* spp.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/ **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Universitat Rovira i Virgili Animal Welfare and Ethics Committee (protocol number 8248).

AUTHOR CONTRIBUTIONS

YL, J-PB, and PV conceived and designed the study. YL and PV performed genetic engineering, cultural studies, and siderophore production experiments. DL collected mass spectrometry data. VH performed mass spectrometry data curation, formal analysis, review, and editing. JC and DD performed virulence assays and corresponding analysis. YL wrote the first draft of the manuscript. NP and SL contributed to the discussion and correction of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.587909/full#supplementary-material

SUPPLEMENTARY VIDEO 1 | CycloBranch real time performance indicating the dereplication process in *Scedosporium* extract

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Peptidorhamnomannans From *Scedosporium* and *Lomentospora* Species Display Microbicidal Activity Against Bacteria Commonly Present in Cystic Fibrosis Patients

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Scedosporium and *Lomentospora* species are filamentous fungi that cause a wide range of infections in humans. They are usually found in the lungs of cystic fibrosis (CF) patients and are the second most frequent fungal genus after *Aspergillus* species. Several studies have been recently performed in order to understand how fungi and bacteria interact in CF lungs, since both can be isolated simultaneously from patients. In this context, many bacterial molecules were shown to inhibit fungal growth, but little is known about how fungi could interfere in bacterial development in CF lungs. *Scedosporium* and *Lomentospora* species present peptidorhamnomannans (PRMs) in their cell wall that play crucial roles in fungal adhesion and interaction with host epithelial cells and the immune system. The present study aimed to analyze whether PRMs extracted from *Lomentospora prolificans*, *Scedosporium apiospermum*, *Scedosporium boydii*, and *Scedosporium aurantiacum* block bacterial growth and biofilm formation *in vitro*. PRM from *L. prolificans* and *S. boydii* displayed the best bactericidal effect against methicillin resistant *Staphylococcus aureus* (MRSA), *Burkholderia cepacia*, and *Escherichia coli*, but not *Pseudomonas aeruginosa*, all of which are the most frequently found bacteria in CF lungs. In addition, biofilm formation was inhibited in all bacteria tested using PRMs at minimal inhibitory concentration (MIC). These results suggest that PRMs from the *Scedosporium* and *Lomentospora* surface seem to play an important role in *Scedosporium* colonization in CF patients, helping to clarify how these pathogens interact to each other in CF lungs.

Keywords: cystic fibrosis, peptidorhamnomannan, *Scedosporium*, bacteria, interaction

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INTRODUCTION

Cystic Fibrosis (CF) is an autosomal recessive disease originated from a mutation in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-regulated epithelial chloride channel (Cohen and Prince, 2012). It results in deficient mucociliary functions, leading to the presence of thicker mucus especially in bronchia and pancreas and, consequently, patients suffer of digestive and respiratory problems (Delhaes et al., 2012). The occurrence of CF is estimated to be one in 3,000 or 4,000 births, and one in 25–30 Caucasians carries the mutation in CFTR gene. In the USA about 1,000 people are diagnosed every year, with CF (Sanders and Fink, 2016).

Sticky bronchial mucus is the most challenging problem in CF patients, because it facilitates the occurrence of airway infections and neutrophilic inflammation that are mostly responsible for morbidity and death in these patients (Stoltz et al., 2015). For these reasons, chronic pulmonary infections are frequent and extensively studied. Several pathogens including bacteria and fungi are commonly associated with CF lung colonization. Bacterial pathogens are the most common cause of CF lung infections, in which *Staphylococcus aureus* and *Haemophilus influenza* are frequent in children and *Burkholderia cepacia* complex and *Pseudomonas aeruginosa* are found especially in adults (Ciofu et al., 2013; Vandeplassche et al., 2019). However, fungal colonization is usually found in the lungs of CF patients, although its dynamics are less clear (Middleton et al., 2013). In this context, the most frequent fungi are *Aspergillus*, *Penicillium*, *Scedosporium*, and *Candida* species (Ziesing et al., 2016; Engel et al., 2019).

For these reasons, polymicrobial colonization is a well-known situation found in CF lungs and the interaction between bacteria and fungi is a field of extensive studies in order to better understand the complexity and dynamics of polymicrobial relationship in this context (Delhaes et al., 2012). A variety of studies demonstrate that bacteria produce molecules known to inhibit fungal growth. Phenazine and other secreted molecules, for instance, are produced by *P. aeruginosa* and inhibit *Aspergillus fumigatus*, *Candida albicans*, and *Scedosporium* species (Gibson et al., 2009; Mowat et al., 2010; Kaur et al., 2015; Chen et al., 2018; Briard et al., 2019). On the other hand, little is known about how fungi can affect bacterial growth in CF conditions. *C. albicans* inhibits *P. aeruginosa* growth by producing farnesol, a quorum-sensing molecule which plays a role in fungal morphogenesis (Semighini et al., 2006).

In the context of fungal molecules that could influence bacterial growth, the present work studied peptidorhamnomannans (PRMs), glycoconjugates commonly exposed on *Scedosporium* and *Lomentospora* cell wall that play important roles in cell adhesion and interaction with host immune system, leading to the release of inflammatory cytokines by phagocytic cells (Lopes et al., 2011). PRMs have been already described and characterized in *Scedosporium boydii*, *Scedosporium apiospermum*, and *Lomentospora prolificans* (Pinto et al., 2001; Lopes et al., 2010; Xisto et al., 2015), as well as in *Scedosporium aurantiacum* (de Meirelles et al., 2020). The PRMs of these four species possess similar epitopes as well as distinct species-specific oligosaccharide chains. For these reasons, in the present work PRMs extracted and purified from these four species were tested for their effect on bacterial species relevant to CF, such as *P. aeruginosa*, *B. cepacia*, methicillin-resistant *S. aureus*, and *Escherichia coli*.

MATERIAL AND METHODS

Microorganisms and Culture Conditions

B. cepacia (American Type Culture Collection ATCC 25416), *E. coli* (ATCC 11229), Methicillin Resistant *S. aureus*—MRSA (ATCC 9393) and *P. aeruginosa* (ATCC 27853) were studied in this work. Strains were maintained in Luria-Bertani (LB) broth medium (peptone 10 g/l, yeast extract 5 g/l and NaCl 5 g/l) under refrigeration at 4°C. For recent overnight cultures, an aliquot of each strain was spread on LB agar and incubated for 24 h at 37°C, and bacterial suspensions were prepared from scrapings.

L. prolificans (FMR3569 strain), *S. apiospermum* (RK107-0417 strain) and *S. aurantiacum* (IHEM21147 strain) were supplied by Dr. J. Guarro, Unitat de Microbiologia, Facultat de Medicina e Institut d`Estudis Avançats, Réus, Spain. *S. boydii* (HLPB strain) was supplied by Dr. Bodo Wanke, Hospital Evandro Chagas, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil. Strains were maintained at room temperature on Sabouraud (SAB; 2% glucose, 1% peptone, 0.5% yeast extract) agar slants as stock culture. Mycelia were obtained by growing cells in SAB liquid culture medium for seven days at room temperature with shaking.

Extraction and Purification of Peptidorhamnomannan (PRM)

The crude glycoprotein was extracted from *L. prolificans*, *S. apiospermum*, *S. boydii*, and *S. aurantiacum* with 0.05M phosphate buffer, pH 7.2, at 100°C for 2 h. After filtration, the solution was dialyzed, evaporated to a small volume, and lyophilized. Peptidorhamnomannans were purified by hexadecyltrimethylammonium bromide (Cetavlon, Merck, Darmstadt, Germany) fractionation, according to Barreto-Bergter et al. (2008).

Determination of MIC and MBC

Minimum inhibitory concentration (MIC) of the PRM isolated from *L. prolificans*, *S. apiospermum*, *S. boydii*, and *S. aurantiacum* was determined through broth microdilution method in LB broth against bacteria, according to Vieira et al. (2018). MIC was defined as the lowest concentration capable of inhibiting 50% of bacterial growth (MIC%). Tests were performed on 96-well microplates. PRM at final concentrations ranging from 500 µg/ml to 1.95 µg/ml in LB broth medium were added to each well (50 µl/well). From recent overnight cultures, 50 µl of bacterial suspensions with turbidity equivalent to 0.5 tube of the McFarland scale (1.5 x 10^8 CFU/ml) were added in each well and incubated at 37°C for 24 h. Streptomycin/penicillin was used

as reference compounds (8–0.015 μg/ml) and cultures in LB broth without antibiotics were used as control. After determining MIC values, MBC (minimum bactericidal concentration) was determined by subculturing an aliquot of 10 μl from each well that showed complete growth inhibition in LB agar medium without addition of PRM and evaluation of bacterial growth (Vieira et al., 2018). After 24 h, MBC values were defined as the lowest concentration of PRM able to kill bacteria.

Biofilm Assay

Inhibition of Biofilm Formation

For biofilm formation, 50 μl of *B. cepacia* (ATCC 25416) and MRSA (ATCC9393) bacterial solutions (prepared as described in MIC section) were added in a 96-well plate and mixed with 1/4, 1/2 and 1 MIC of each PRM. After 24 h of incubation at 37°C, the formed biofilm was gently washed with PBS to remove planktonic cells, air-dried for 10 min and stained for 10 min with 0.5% crystal violet (total biofilm biomass) or 1% safranin (biofilm matrix). The staining solutions were discarded and biofilms were gently rinsed twice with sterile distilled water. Crystal violet impregnated in the biofilm was dissolved in 200 μl of ethanol (95%, v/v), and the colored solution was read at an absorbance of 595 nm using a spectrophotometer (Spectra MAX 340 Tunable; Molecular Devices Ltd., San Jose, CA, USA). Safranin was dissolved in water (100 μl), and the absorbance read at 492 nm.

Inhibition of Preformed Biofilm

The *B. cepacia* and MRSA biofilms were prepared as described in the previous section, and after incubation for 24 h at 37°C, the supernatant was removed and 1/2, 1 and 2 MICs of each PRM were added to the formed biofilm. After 24 h at 37°C, the biofilm was gently washed with PBS and crystal violet and safranin staining were performed as in the previous section.

Effect of PRM on ROS Production and Membrane Potential and Integrity

In order to evaluate the effect of fungal PRMs on oxidative stress and membrane integrity and potential, *B. cepacia* cells were grown in LB broth in the presence of ¼ MIC of all four PRMs for 24 h at 37°C. Furthermore, fluorescence staining was performed using DCFDA (2',7'-dichlorodihydrofluorescein diacetate), Nile Red and JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'tetraethylbenzimidazolylcarbocyanine iodide) (both from Sigma-Aldrich, MO, USA), according to Ullrich et al. (2003); Pereira et al. (2007) and Van Acker et al. (2016), with modifications.

Staining with DCFDA was performed at a final concentration of 10 μM in PBS for 45 min at room temperature in the dark. After washing with PBS, fluorescence (λ excitation = 485 nm, λ emission = 535 nm) was measured using a SpectraMax plate reader (Van Acker et al., 2016).

Nile Red staining at a final concentration of 8 μg/ml was done for 45 min at room temperature in the dark. After washing with PBS, fluorescence (λ excitation = 550 nm, λ emission = 635 nm) was measured as above (Pereira et al., 2007).

JC-1 stain was used at 2.5 μg/ml in PBS 0.01 M, pH 7.2, for 45 min at 37°C in the dark. After washing with PBS, fluorescence (λ excitation = 515 nm, λ emission = 529 nm (green) and 590 nm (red) was measured as above (Ullrich et al., 2003). The membrane potential was determined by the red/green fluorescence intensity ratio.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA). A variance two-way ANOVA was performed using Tukey's and Bonferroni's comparisons tests to evaluate biofilm formation and inhibition of preformed biofilm.

RESULTS

Minimum Inhibitory and Bactericidal Concentrations (MIC and MBC) of PRMs

MIC and MBC values were determined for PRMs isolated from *L. prolificans*, *S. apiospermum*, *S. boydii*, and *S. aurantiacum* against *B. cepacia*, *E. coli*, MRSA, and *P. aeruginosa* (**Table 1**). *L. prolificans* PRM showed considerable inhibitory activity against *B. cepacia* and MRSA, with MIC of 31.3 and 15.6 μg/ml, respectively. However, for *E. coli* MIC was 500 μg/ml. *S. apiospermum* PRM present MIC of 500 μg/ml for *B. cepacia* and MRSA, and >500 μg/ml for *E. coli*. *S. boydii* PRM displayed MIC of 125 μg/ml for *B. cepacia*, 125 μg/ml for MRSA and >500 μg/ml for *E. coli*. *S. aurantiacum* PRM showed MIC at 125 μg/ml for *B. cepacia* and MRSA, and >500 μg/ml for *E. coli*. All PRMs tested displayed MIC at >500 μg/ml for *P. aeruginosa*.

TABLE 1 | Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of fungal PRMs isolated from *L. prolificans*, *S. apiospermum*, *S. boydii* and *S. aurantiacum* tested against *B. cepacia*, *E. coli*, MRSA and *P. aeruginosa*.

Bacteria	PRM (μg/ml)							
	L. prolificans		*S. apiospermum*		*S. boydii*		*S. aurantiacum*	
	MIC_{50}	MBC	MIC_{50}	MBC	MIC_{50}	MBC	MIC_{50}	MBC
B. cepacia	31.3	31.3	500	>500	125	500	125	> 500
E. coli	500	> 500	> 500	> 500	> 500	> 500	> 500	> 500
MRSA	15.6	31.3	500	>500	125	250	125	> 500
P. aeruginosa	>500	>500	>500	>500	>500	>500	> 500	> 500

MIC50, minimal concentration that inhibits 50% of growth; MBC, minimal bactericidal concentration; MRSA, methicillin resistant S. aureus.

Regarding MBC (**Table 1**), *L. prolificans* PRM was found to be bactericidal at 31.3 μg/ml for *B. cepacia* and MRSA, at 500 μg/ml for *E. coli*, but was not able to kill *P. aeruginosa*. *S. boydii* PRM displayed MBC at 500 μg/ml for *B. cepacia* and 250 μg/ml for MRSA, and was not able to kill either *E. coli* or *P. aeruginosa*. *S. apiospermum* and *S. aurantiacum* PRMs did not show bactericidal activity for any bacterium tested. Supplementary Material shows bacteria incubated with different concentrations of all PRMs. After 24h at 37°C, aliquots were spotted on LB agar in the absence of any PRM, in order to determine MBCs shown in **Table 1**.

These results indicate that *L. prolificans* PRM was most active against bacteria compared to the other PRMs, whereas *S. apiospermum* PRM was the less potent molecule to inhibit bacterial proliferation. In addition, *B. cepacia* and MRSA were the most susceptible species when incubated with all four PRMs.

Inhibition of Biofilm Formation

Since *B. cepacia* and MRSA were the species most susceptible to fungal PRMs, we chose these two species to evaluate whether fungal PRMs could also affect bacterial biofilm formation. In this context, the total formed biomass and the extracellular matrix were evaluated. When *B. cepacia* was analyzed, *L. prolificans* and *S. apiospermum* PRMs could inhibit biomass formation at MIC and ½ MIC (**Figure 1A**). However, PRM isolated from *S. boydii* and *S. aurantiacum* showed a significant inhibition of *B. cepacia* biomass formation at MIC, ½ MIC and also ¼ MIC (**Figure 1A**). Biofilm matrix was reduced by all PRMs at 1 MIC, ½ MIC and ¼ MIC (**Figure 1B**).

Regarding biofilm formation by MRSA, similar results were observed for biomass (**Figure 1C**). However, extracellular matrix was reduced at MIC and ½ MIC by *L. prolificans* and *S. apiospermum* PRMs, and at all three concentrations used for *S. boydii* and *S. aurantiacum* PRMs (**Figure 1D**).

These data suggest that besides growth inhibition observed in MIC and MBC experiments, biofilm formation is also decreased when bacteria are grown in the presence of fungal PRM.

Inhibition of Preformed Bacterial Biofilm

Due to the inhibitory potential of fungal PRMs on bacterial growth and biofilm formation, we decided to check whether these four PRMs could also be active against preformed biofilms. *L. prolificans* PRM reduced preformed biomass and extracellular matrix of *B. cepacia* at MIC and ½ MIC, whereas PRMs isolated from *S. apiospermum*, *S. boydii* and *S. aurantiacum* showed similar results at all concentrations tested (**Figures 2A, B**). When preformed MRSA biofilm was evaluated, all four PRMs decreased biomass matrix at all concentrations used (**Figures 2C, D**), except for *L. prolificans* PRM at ¼ MIC, which was not able to significantly reduce MRSA matrix (**Figure 2D**).

These results suggest that fungal PRMs affect not only bacterial growth and the process of biofilm formation, but they affect also preformed bacterial biofilms.

PRMs Effect on ROS Production, Membrane Potential, and Integrity

In order to understand the effects of PRMs on bacteria, oxidative stress was evaluated in bacterial cells, as well as the integrity and the potential of the membrane. To perform these analyses,

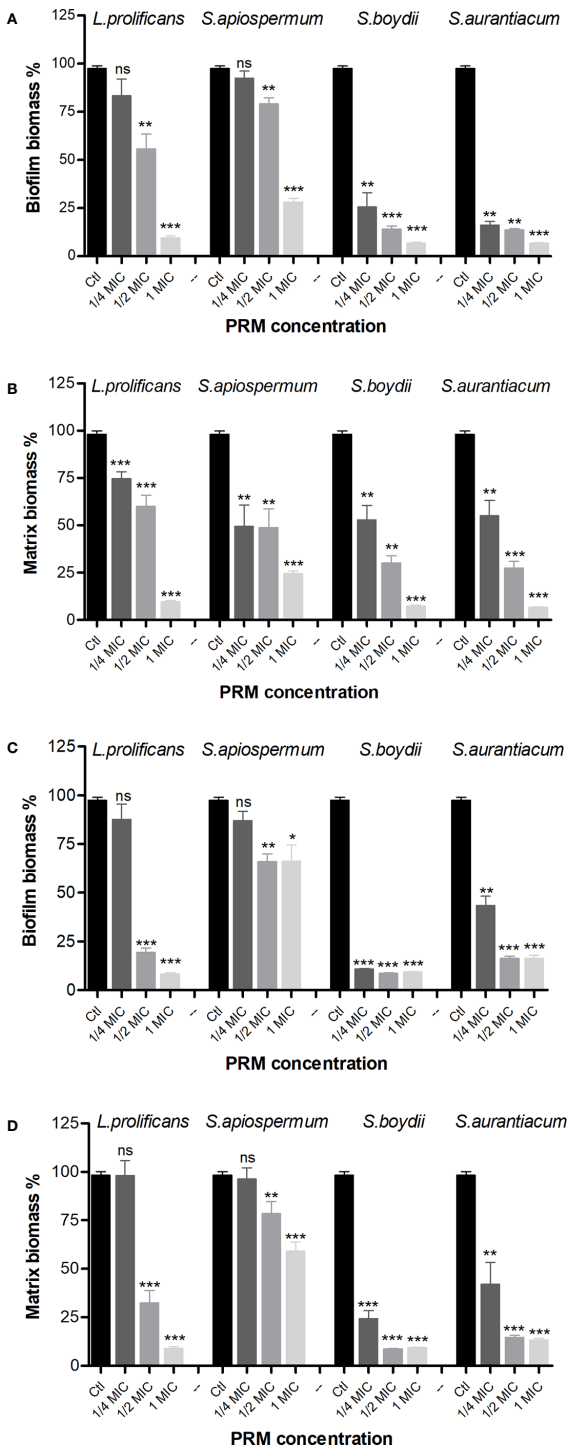

FIGURE 1 | Biofilm formation of *B cepacia* **(A, B)** and MRSA **(C, D)** in the presence of PRMs isolated from *L. prolificans*, *S. apiospermum*, *S. boydii* and *S. aurantiacum*. **(A, C)** represents the evaluation of biofilm growth. **(B, D)** show extracellular matrix. Ctl, control without addition of PRM. MIC, ¼ MIC and ½ MIC were based on the values shown in **Table 1**. The values represent the mean ± S.D. of three independent experiments performed in triplicate. Asterisks denote values statistically different from control. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. ns, no significant.

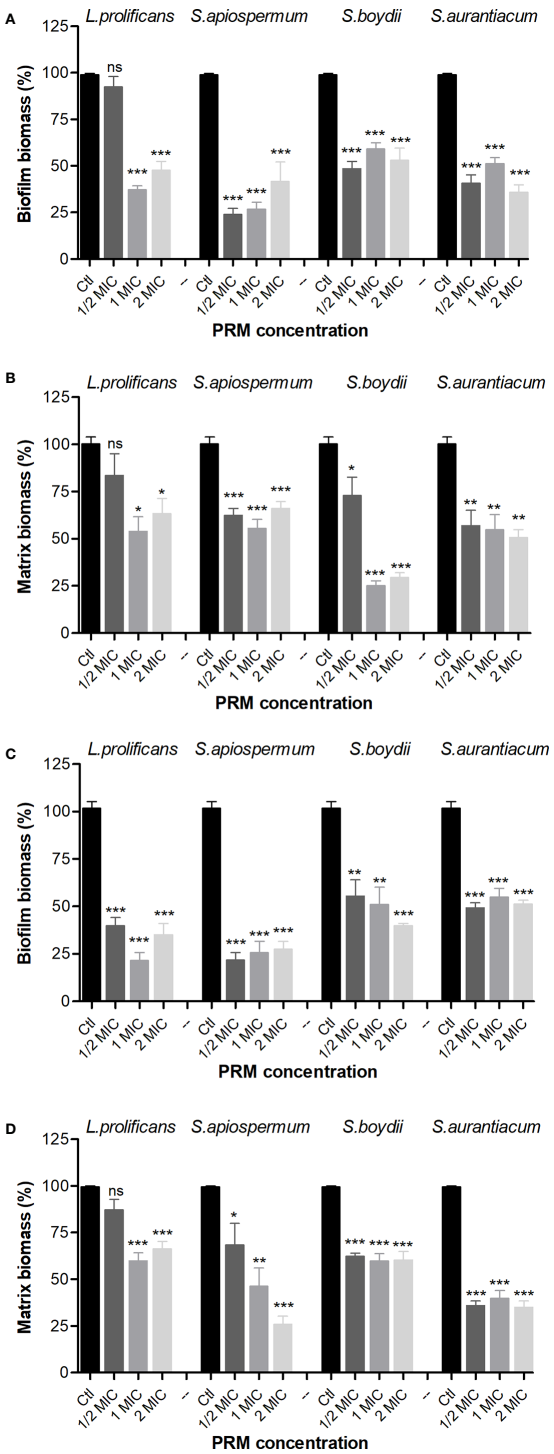

FIGURE 2 | Effect of PRMs isolated from *L. prolificans*, *S. apiospermum*, *S. boydii* and *S. aurantiacum* on preformed biofilm of *B cepacia* **(A, B)** and MRSA **(C, D)**. **(A, C)** represent the evaluation of biofilm formation. **(B, D)** show extracellular matrix. Ctl, control without addition of PRM. MIC, ¼ MIC and ½ MIC were based on the values shown in **Table 1**. The values represent the mean ± S.D. of three independent experiments performed in triplicate. Asterisks denote values statistically different from control. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. ns, no significant.

B. cepacia was chosen as a representative model due to its relevance in CF patients with worse prognosis.

The production of reactive oxygen species (ROS), which provoke oxidative stress, was measured using DCFDA staining. DCFDA is oxidized by ROS inside the cells to form fluorescent 2', 7'-dichlorofluorescein (DCF). In this context, *L. prolificans* PRM was the only molecule able to increase ROS production, indicating induction of oxidative stress in *B. cepacia* (**Figure 3A**). The other PRMs did not influence ROS production when compared to the control.

Disruption of membrane integrity is a mechanism by which some compounds cause cell killing. For this reason, we used Nile Red staining that binds to polyhydroxyalkanoates (PHAs), common prokaryotic storage compounds of carbon and energy present in intracellular lipid droplets (Jendrossek, 2009). *S. boydii* PRM decreased Nile Red fluorescent intensity, suggesting that it affects lipid integrity in *B. cepacia*, whereas the other PRMs did not alter bacterial lipids when compared to the control (**Figure 3B**).

Reduction of energy generation as consequence of cell killing could be measured by evaluating the membrane potential using JC-1 staining. All fungal PRMs tested caused a decrease in membrane potential compared to the control (untreated cells), indicating that PRMs induce a lower membrane polarization in *B. cepacia* (**Figure 3C**).

DISCUSSION

Cystic fibrosis patients exhibit a reduced clearance of mucus from the lungs which leads to chronic infections caused by bacteria and fungus, being the primary cause of mortality (Lipuma, 2010; Cohen and Prince, 2012). The study of polymicrobial infections in this environment is not only important for understanding the pathogenesis but also for providing insights for the development of new antimicrobial drugs, since the relationships between these microorganisms in the context of CF are frequently antagonistic and therefore may reveal new approaches for pathogen inhibition (Peleg et al., 2010).

Bacteria play a major part in lungs of CF patients. They are usually the first microorganisms to colonize this site and are the cause of a common exacerbated inflammatory response observed in the respiratory tract. Guss et al. (2011) identified eight bacterial phyla, including more than 60 genera, in lungs from CF patient, therefore evidencing the diversity of this microbial community. However, as previously mentioned, there are some more prevalent species colonizing the pulmonary tract in CF, such as *P. aeruginosa*, *S. aureus*, and *B. cepacia*. The presence of *B. cepacia* is considered a threat to CF patients, due to its high patient-to-patient transmissibility and antibiotic resistance, as well as a poorer prognosis of the disease outcome (Kenna et al., 2017). A recent study showed that *E. coli* was recovered from the sputum of up to 25% of patients with CF, mainly from those with poor nutritional status and lung function, although this did not predict clinical decline (Edwards et al., 2020). In our study, *P. aeruginosa*, *S. aureus*, *B. cepacia*, and *E. coli* were used as

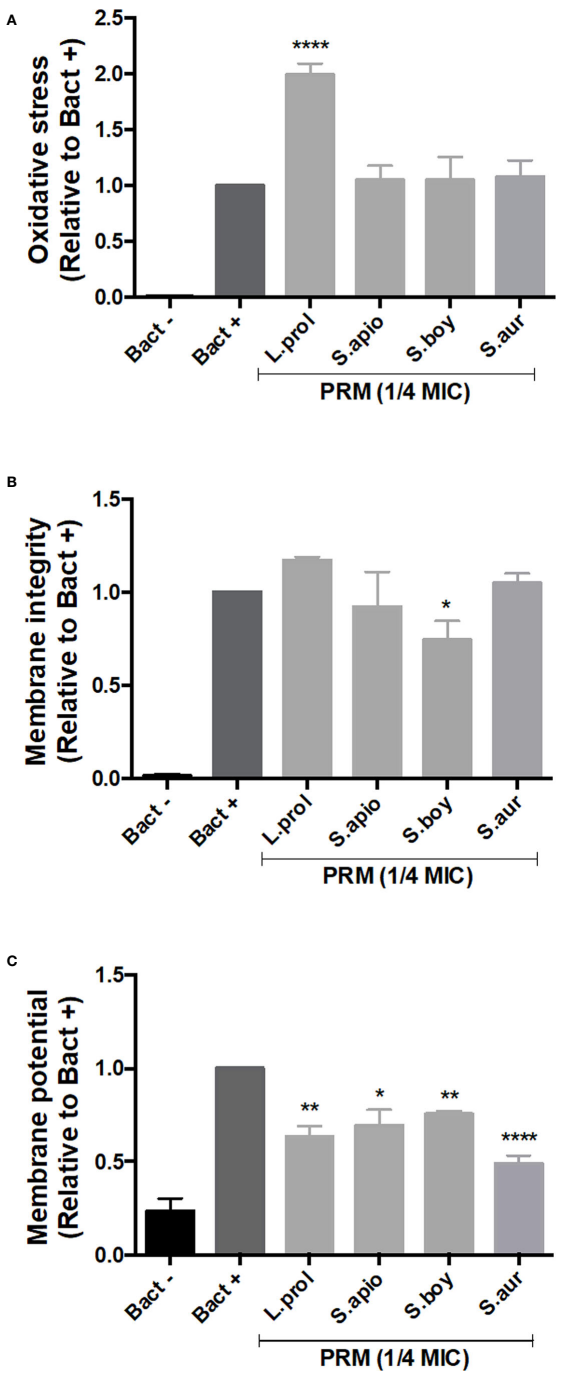

FIGURE 3 | Effect of PRMs isolated from *L. prolificans*, *S. apiospermum*, *S. boydii* and *S. aurantiacum* on oxidative stress **(A)**, membrane integrity **(B)** and membrane potential **(C)** of *B cepacia*. Oxidative stress, membrane integrity and potential were measured by DCFDA, Nile Red and JC-1 staining, respectively. Bact-, unstained control. Bact+, stained cells in the absence of PRM. L. prol, *L prolificans*. S. apio, *S. apiospermum*. S. boy, *S. boydii*. S. aur, *S. aurantiacum*. ¼ MIC was based on the values shown in **Table 1**. The values represent the mean ± S.D. of three independent experiments performed in triplicate. Asterisks denote values statistically different from control. *$p < 0.05$; **$p < 0.01$; ****$p < 0.001$.

representative species for CF to evaluate the bacterial inhibitory effect of fungal PRMs isolated from *L. prolificans*, *S. boydii*, *S. apiospermum*, and *S. aurantiacum*.

Bacteria are the main cause of CF lung infections, but fungal species are also able to chronically colonize the respiratory tract of these patients. It is known that fungi from the genus *Scedosporium* are the third most prevalent in CF patients after *Aspergillus* and *Penicillium*. *S. apiospermum* is the most frequent (28.6%), followed by *S. boydii* (19.3%), *S. aurantiacum* (10.0%), and *L. prolificans* (3.6%) (Engel et al., 2019). Other studies described *Scedosporium* species as the second most prevalent filamentous fungi in CF patients (Pihet et al., 2009; Sudfeld et al., 2010).

Considering the polymicrobial pattern of CF lung infections, it is relevant to study the bacteria-fungi interactions and the molecules produced by fungi that could be involved in these interactions. In this context, the present work used PRMs isolated from *L. prolificans* (Barreto-Bergter et al., 2008), *S. boydii* (Pinto et al., 2005), *S. apiospermum* (Barreto-Bergter et al., 2011), and *S. aurantiacum* (de Meirelles et al., 2020). These glycoconjugates are exposed on fungal surfaces, since they are recognized by antibodies anti-PRM (Lopes et al., 2010; Xisto et al., 2015). Cell culture supernatants of *Scedosporium* and *Lomentospora* species for the presence of PRM were not examined in this manuscript. *In vitro* studies show that variable amounts of galactomannan (GM), a component of the cell wall of several *Aspergillus* species as well of a diverse range of fungi, are released during early logarithmic growth (Latgé et al., 1994). *In vivo* it is known that galactomannans can be detected in the bronchoalveolar lavage of patients with invasive aspergillosis, released from *A. fumigatus* cell wall during infections (Sarfati et al., 1995; He et al., 2012; Teering et al., 2014). Further studies, not only focused on the characterization of extracellular PRM from *Scedosporium* and *Lomentospora* species but also on carbohydrate-containing molecules isolated from cell wall or secreted into the cell culture supernatant of these species cultivated in synthetic cystic fibrosis sputum medium (SCFM) (Han et al., 2017), that mimics the environment in the CF lungs species will improve the knowledge of this complex bacteria-fungus interaction in CF.

In addition, PRMs of *S. boydii* and *S. apiospermum* are involved in the adhesion to an epithelial cell line and in intracellular survival within macrophages, respectively (Pinto et al., 2004; Lopes et al., 2010). PRMs isolated from *S. boydii* and *L. prolificans* also play a role in host immune stimulation, increasing the release of pro-inflammatory cytokines such as TNF-α (Figueiredo et al., 2010; Xisto et al., 2015).

Taken together, all data presented in our study demonstrated that PRMs possess antibacterial and anti-biofilm effects. Regarding the inhibition of bacterial growth, PRM from *L. prolificans* was the most effective, followed by those from *S. aurantiacum*, *S. boydii*, and *S. apiospermum*, against *B. cepacia* and MRSA, which were the most susceptible bacteria. *P. aeruginosa* and *E. coli* seemed to be resistant to all PRM. Considering the anti-biofilm effect, PRM from *S. boydii* and *S. aurantiacum* showed to be the most effective, because ¼ MIC was necessary to observe *B. cepacia* and MRSA biofilm reduction.

Although *S. apiospermum* is described as the most common *Scedosporium* species identified in lungs of CF patients, MIC and MBC results obtained in this work showed that its PRM was the less potent molecule against bacteria, whereas *L. prolificans* PRM, which is the less frequent fungi of *Scedosporium/Lomentospora* group isolated from CF patients, was the most active against bacteria. In addition, all PRMs presented high MICs against *P. aeruginosa* and *E. coli*. Rhamnose-containing molecules, such as the rhamnolipid biosurfactant isolated from a sponge associated marine fungus *Aspergillus* spp has been described with antimicrobial activity against *C. albicans* and some Gram-negative bacteria (Kiran et al., 2009).

Variation in the antibacterial potential among PRMs could be due to differences in their oligosaccharide chains. PRMs isolated from the *Scedosporium* and *Lomentospora* species used in this work share similar epitopes, such as the α-Rhap-(1→3) α-Manp-(1→2)- α-Manp (Lopes et al., 2011; de Meirelles et al., 2020). On the other hand, structural differences are observed among these PRMs, which could result in changes in the antimicrobial activity. *L. prolificans* PRM, for instance, contains a pentasaccharide lacking the β-Galp side-chain (Barreto-Bergter et al., 2008) and a high proportion of 2-*O*-substituted Rhap units, which are absent in *S. boydii* and *S. apiospermum* PRMs (Pinto et al., 2005; Barreto-Bergter et al., 2008; Barreto-Bergter et al., 2011). Nevertheless, more studies are needed in order to clarify the reason why *L. prolificans* PRM present a higher activity against the bacteria tested when compared to the other PRMs.

Due to the relevance of biofilms in infection, the influence of PRM on bacterial biofilms was also evaluated. All four PRMs inhibit biofilm formation and are active against preformed biofilms of *B. cepacia* and MRSA at MIC values. Extracellular matrix was also reduced by PRMs, indicating that bacterial biofilms were weakened in the presence of PRM. Several glycoconjugates and polysaccharides have been already described as potent inhibitors of bacterial biofilms. C-fucosylpeptide and galactosylated peptide dendrimers have been shown to inhibit biofilm formation and to disperse preformed biofilm by interfering with *P. aeruginosa* LecA and B, a lectin molecule responsible for bacterial adherence on host tissues (Kadam et al., 2011; Reymond et al., 2013). Human and plant oligosaccharides, such as galactooligosaccharides, have already been known to block bacterial adhesion to surfaces, decreasing biofilm formation of *E. coli*, *Burkholderia pseudomallei* and other bacteria (Thomas and Brooks, 2004; Shoaf et al., 2006; Lane et al., 2010; Quintero et al., 2011; Rendueles et al., 2013). Regarding fungal molecules, chitosan, a deacetylated derivative of chitin found on the fungal cell wall is recognized as a potent antibacterial agent and inhibits growth and biofilm formation by different bacteria, such as *S. aureus* and *E. coli* (Asli et al., 2017). For other fungal glycoconjugates, such as the rhamnolipid biosurfactant of *Aspergillus* spp., antimicrobial activity against *Streptococcus* spp., *Micrococcus luteus*, and *Enterococcus faecalis* has also been reported (Kiran et al., 2009).

In order to evaluate some possible mechanisms involved in bacterial death, we evaluated oxidative stress, membrane integrity and membrane potential in *B. cepacia* treated with PRMs using DCFDA, Nile Red and JC-1 staining, respectively. *L. prolificans* PRM increased ROS production when compared to control. PRMs isolated from *S. boydii*, *S. apiospermum* and *S. aurantiacum* did not cause any effect, suggesting that structural differences among PRMs might be related to ROS production by *B. cepacia*. On the other hand, a decrease of the membrane potential was observed in *B. cepacia* in the presence of all PRMs used. Membrane integrity was only affected when bacterial cells were treated with *S. boydii* PRM. Membrane alteration detected by Nile Red is well known in bacteria treated with penicillin, which leads to disorganized cell surface (Pereira et al., 2007). However, few information about ROS induction, membrane disorganization and changes in membrane potential caused by glycoconjugates and polysaccharides is described in the literature.

The knowledge of how fungi can influence bacterial growth in the polymicrobial colonization of lungs from CF patients is still scarce. Several studies have been performed in order to understand how bacteria, especially *P. aeruginosa*, inhibit competitors in CF airways (Gibson et al., 2009; Mowat et al., 2010; Delhaes et al., 2012; Kaur et al., 2015; Chen et al., 2018; Briard et al., 2019). However, studies on the mechanisms by which fungal molecules inhibit bacteria are rare. Therefore, the present work aimed to evaluate the effect of the main glycoconjugate found on the *Scedosporium* and *Lomentospora* cell wall on bacterial growth. PRMs from *Scedosporium* and *Lomentospora* species were able to kill bacteria associated to lungs of CF patients and interfere with both, bacterial biofilm formation and preformed biofilms. The mechanisms involved in *B. cepacia* death are different among the PRMs tested in this work, and can be associated with structural differences in these PRMs. Since CF prognosis is hard for all patients and infections of the respiratory tract are the main cause of mortality in this population, studies that contribute to the understanding of the dynamics of bacteria-fungi interactions in the context of CF are crucial for developing better approaches to increase the patients' quality of life.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

EO, MX, RRP, and VR conceived, designed, and performed the experiments. EO, MX, RRP, VR, and EBB analyzed the experiments. MX, RRP, and EBB drafted the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.598823/full#supplementary-material

SUPPLEMENTARY MATERIAL 1 | Growth of *B. cepacia*, *E. coli*, MRSA and *P. aeruginosa* in the presence of different concentrations (0.98 – 500 μg/ml) of PRM isolated from *L. prolificans*, *S. apiospermum*, *S. boydii* and *S. aurantiacum*.

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Urban Life as Risk Factor for Aspergillosis

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Aspergillus fumigatus (*Af*) frequently colonizes the airways of patients with cystic fibrosis (CF) and can cause severe diseases, such as allergic bronchopulmonary aspergillosis, *Af* bronchitis or even *Af* pneumonia. However, risk factors, including environmental factors, for acquiring *Af* in the respiratory tract of patients with CF are rarely studied and described. The aim of this study was to investigate whether urban or rural life could affect colonization with *Af* in the respiratory tract of patients with CF. Due to privacy policy, registry data are usually not linked to patients´ home addresses. It is therefore very difficult to analyze the influence of the patient´s residential environment. This prospective questionnaire survey was carried out in 31 German CF centers in 2018. Only completed surveys, including a clearly assigned type of residential area were included. Statistical analysis was performed by chi-squared test and logistic regression models. A total of 1016 questionnaires were analyzed (Patients` age: 23 ± 13; 0–88 years; female gender: n=492; 48%). The majority of patients with CF live in large cities (n =314; 30.9%) or urban districts (n=461; 45.4%). Prevalence of 30.2% was found for *Af*, within the 12 months of investigation period. *Af* colonization was significantly associated with urban life (p=0.004). Urban live should be considered as possible new risk factor for colonization with *Af* in the respiratory tract of patients with CF. These new results may raise the awareness of the influence of environmental factors on patient outcomes and should be included in patient guidance and preventive measures.

Keywords: cystic fibrosis, aspergillosis, *Aspergillus fumigatus*, allergic bronchopulmonary aspergillosis, urban life, rural life, respiratory infection

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INTRODUCTION

The life-limiting autosomal recessive disorder Cystic fibrosis (CF) is characterized by abnormal viscous secretions in the lower airways leading to obstruction, inflammation, tissue damage and destruction of the lung (Elborn, 2016).In patients with CF, fungal colonization of the respiratory tract is frequently found (Pihet et al., 2009). *Aspergillus fumigatus* (*Af*), the most common filamentous fungus in CF patients with a prevalence of 30 %, is becoming more frequently isolated (Ziesing et al., 2016; Brandt et al., 2018; Schwarz et al., 2018; Hong et al., 2020). The airways are constantly exposed to fungal spores, yet which are only a part of the airway pathogens in

the environment. Hundreds of *Aspergillus* spores are inhaled every day, and due to their small size, they directly enter the small airways (Latge, 1999). In CF airways, these spores are able to persist and germinate, leading to immune responses with leukocyte infiltration and mucus accumulation (Singh et al., 2018). *Aspergillus* species are associated with negative patient outcome and can cause severe diseases, like allergic bronchopulmonary aspergillosis (ABPA) (Singh et al., 2018). About 1–15 % of the CF patients suffer from this serious *Aspergillus*-related disease (Stevens et al., 2003). ABPA is caused by hypersensitivity to allergens of *Af* (Janahi et al., 2017), is characterized by a variety of clinical and immunological responses (Stevens et al., 2003) and is associated with a negative effect on lung function (Kraemer et al., 2006). Until now, diagnosis of ABPA in patients with CF remains challenging (Janahi et al., 2017). In future, approximately 70 % of the world´s population will live in urban areas and modern urbanites will spend approximately 90 % of their times indoors (Rydin et al., 2012; Leung and Lee, 2016; Flies et al., 2019). If the patient's environment poses a potential risk to *Aspergillus* colonization, preventive measures could be identified. Limited work has been done examining *Af* colonization in CF patients regarding their type of residential area in Germany. As registry data of the patient are not linked with their home address, this questionnaire survey was conducted to gain evidence of an association between aspergillosis and urban or rural life.

MATERIALS AND METHODS

Design and Development

This questionnaire survey was conducted in German CF centers in 2018. The target population was people with CF independent of age and living in Germany. Participation was voluntary. Ethical aspects were considered and approval for the study was gained by the Ethics Committee of the Charité – Universitätsmedizin Berlin (EA2/057/18).

The questionnaire included several items, which are not documented in the medical data base German Cystic Fibrosis Registry. Therefore, beside patients' age and sex and their history of *Af* colonization and ABPA diagnosis within the last 12 months, the postal code of the place of residence, were asked for in the questionnaire. For a positive of *Af* colonization within the 12 month of observation period, at least one positive microbiological indication was required. The ABPA diagnosis was determined in every single CF center and was based on the minimal 2003 Cystic Fibrosis Foundation (CFF) consensus criteria (Stevens et al., 2003). No other *Af* associated phenotypes were considered. Besides *Af*, no other *Aspergillus* spp. were included. The questionnaire was sent to 94 CF centers in Germany in 2018 and data of 1,477 patients have been received from 31 CF centers. Postal code was correlated to rural and urban area with data from the German Federal Institute for Building, Urban and Spatial Research (BBSPR). The population density was correlated from the postal code with data from the German Statistic Federal Office (DESTATIS).

Statistical Analysis

Only completed questionnaires with distinct classification for the type of residential area and population density were included in final analysis. The residential area was classified as large city, urban district, rural district and sparsely populated rural area. Population density was graduated in high, medium and low. Means and standard deviations were calculated for metrical variables. Frequency and percentage were used for categorical variables. Associations of categorical variables were analyzed using Pearson´s chi-square tests. In addition, all parameters were considered within binary logistic regression analysis. For multiple binary logistic regression analysis (only for *Af* colonization), the following parameters have been adjusted: age (years) and residential area (large city, urban district, rural district and sparsely populated rural area). In this exploratory study the level of significance (α=0.05) was not adjusted for multiple testing. Statistics were calculated using IBM SPSS Statistics 24.

RESULTS

A total of 1,465 patients with CF participated and a total of 1,016 completed questionnaires were analyzed. Of the included patients, 48.4% were female (n = 492; male: n = 524; 51.6 %). The mean age was 23 ± 13 years (range: 0–88 years). The majority of CF patients live in large cities (n = 314; 30.9 %) and urban districts (n = 461; 45.4 %; **Table 1**).

Prevalence of 30.2 % (n = 307) was found for *Af* colonization within the 12 months of observation period (**Table 1**). *Af* colonization was significantly associated with urban life (**Tables 2**, **4**) including an increased prevalence of 36.6 % of *Af* colonization in large cities (**Table 2**). Binary logistic regression of *Af* colonization was performed including gender (male gender: p = 0.244), age (p < 0.001), different types of residential area (p = 0.022) and population densities (p = 0.918). Adjusted odds ratios for age and residential areas were shown in **Table 4**.

ABPA was documented in 16.2 % (n = 165) of the CF patients (**Table 1**), with a slightly pronounced documentation in large cities but without statistical significance compared to the other types of residential area (p = 0.054; **Table 2**). The individual associations between ABPA diagnosis and the investigated parameters were also examined using logistic regression (n = 1016; male gender: p = 0.913; residential area: p = 0.067; population density: p = 0.424). Odds ratio (1.021) and 95 % confidence interval (1.009–1.034) could only be determined for age (p = 0.001).

In addition to the residential area, population density was determined for analyzation. It shows that the majority of CF patients live in regions with a medium population density (n = 601; 59.2 %) and only 13.5 % (n = 137) in areas with high population density (**Table 1**). No statistical significance was calculated for *Af* (p = 0.357) nor ABPA (p = 0.871) documentation within the observation period in association with population density (**Table 3**).

TABLE 1 | Demographic baseline characteristics.

	All CF patients	CF patients < 18 years	CF patients ≥ 18 years	P value
	1016 (100 %)	395 (38.9 %)	621 (61.1%)	
Female sex, n (%)	492 (48.4%)	197 (49.9%)	295 (47.5%)	0.461
Age (years), mean ± SD (range)	23 ± 13 (0 - 88)	10 ± 5 (0–17)	31 ± 10 (18–88)	
Af colonization[a], n (%)	307 (30.2%)	73 (18.5%)	234 (37.7%)	<0.001
ABPA[a], n (%)	165 (16.2%)	30 (7.6%)	135 (21.7%)	<0.001
Type of residential area, n (%)				
Large city	314 (30.9 %)	91 (23.0%)	223 (35.9%)	<0.001
Urban district	461 (45.4 %)	186 (47.1%)	275 (44.3%)	0.401
Rural district	141 (13.9 %)	68 (17.2%)	73 (11.8%)	0.016
Sparsely populated rural area	100 (9.8 %)	50 (12.7%)	50 (8.0%)	0.018
Population density, n (%)				
High	137 (13.5%)	46 (11.6%)	91 (14.7%)	0.188
Medium	601 (59.2%)	213 (53.9%)	388 (62.5%)	0.007
Low	278 (27.4%)	136 (34.5%)	142 (22.8%)	<0.001

[a]*During 12 months observation period.*

TABLE 2 | *Af* colonization and ABPA diagnosis in different types of residential area for all included CF patients.

All included CF patients, n = 1016					
	Large city, n = 314	**Urban district, n = 461**	**Rural district, n = 141**	**Sparsely populated rural area, n = 100**	**P value**
Af colonization, n (%)	115 (36.6 %)	138 (29.9 %)	34 (24.1 %)	20 (20.0%)	0.004
ABPA, n (%)	64 (20.4%)	67 (14.5%)	16 (11.3%)	18 (18%)	0.054
CF patients >= 18 years, n = 395					
	Large city n = 91	**Urban district n = 186**	**Rural district n = 68**	**Sparsely populated rural area n = 50**	**P value**
Af colonization, n (%)	22 (24.2 %)	33 (17.7%)	8 (11.8 %)	10 (20.0%)	0.247
ABPA, n (%)	11 (12.1%)	6 (3.2%)	7 (10.3%)	6 (12.0%)	0.021
CF patients ≥ 18 years, n = 621					
	Large city n = 223	**Urban district n = 275**	**Rural district n = 73**	**Sparsely populated rural area n = 50**	**P value**
Af colonization, n (%)	93 (41.7 %)	105 (38.2 %)	26 (35.6 %)	10 (20.0%)	0.039
ABPA, n (%)	53 (23.8%)	61 (22.2%)	9 (12.3%)	12 (24.0%)	0.210

TABLE 3 | *Af* and ABPA documentation in relation to population density for all included CF patients.

All included CF patients, n = 1016				
	Population density			**P value**
	High, n = 137	**Medium, n = 601**	**Low, n = 278**	
Af colonization, n (%)	45 (32.8%)	187 (31.1%)	75 (27.0%)	0.357
ABPA, n (%)	21 (15.3%)	101 (16.8%)	43 (15.5%)	0.871
CF patients >= 18 years, n = 395				
	Population density			**P value**
	High n = 46	**Medium n = 213**	**Low n = 136**	
Af colonization, n (%)	10 (21.7%)	42 (19.7%)	21 (15.4%)	0.503
ABPA, n (%)	4 (8.7%)	9 (4.2%)	17 (12.5%)	0.017
CF patients ≥ 18 years, n = 621				
	Population density			**P value**
	High n = 91	**Medium n = 388**	**Low n = 142**	
Af colonization, n (%)	35 (38.5%)	145 (37.4%)	54 (38.0%)	0.977
ABPA, n (%)	17 (18.7%)	92 (23.7%)	26 (18.3%)	0.306

TABLE 4 | Adjusted odds ratios for age and residential areas of *Af* colonization.

All included CF patients, n = 1016			
	Odds ratio	**95% Confidence Interval**	**P value**
Age (years)	1.028	1.017 – 1.038	<0.001
Residential area	–	–	0.019
Large city (vs sparsely populated rural area)	2.114	1.220 – 3.661	0.008
Urban district (vs sparsely populated rural area)	1.724	1.008 – 2.949	0.047
Rural district (vs sparsely populated rural area)	1.265	0.672 – 2.383	0.466

Among the 1016 patients with CF included in this analysis, n = 395 (38.9 %) were less than 18 years of age. N = 197 (49.9 %) of them were female. Prevalence of 18.5 % (n = 73) was found for *Af*. ABPA was documented in 7.6 % (n = 30) in this cohort (**Table 1**). Both, *Af* colonization and ABPA diagnosis was significantly lower in CF patients <18 years compared to adult CF patients (p<0.001; **Table 1**). Due to small case numbers for CF patients < 18 years, examination of *Af* colonization and ABPA measures regarding the type of residential area (**Table 2**)

and population density (**Table 3**), the results have to be carefully interpreted.

N = 621 (61.1 %) CF patients were ≥ 18 years of age and 47.5 % (n = 295) of them were female. Prevalence for *Aspergillus* was 37.7 % (n = 234; **Table 1**) *Af* colonization was significantly associated with urban life in large cities (p = 0.039; **Table 2**). ABPA was documented in n = 135 (21.7%) of the adult CF patients (**Table 1**). No statistical significance was calculated for *Af* colonization (p = 0.977) nor ABPA (p = 0.306) in association with population density (**Table 3**).

DISCUSSION

This nationwide questionnaire survey explored the association of aspergillosis documentation in different types of residential area and various population densities. *Af* colonization is significantly associated with urban life in large cities (**Tables 2**, **4**). History of ABPA during the 12 months of observation period was not significantly associated with urban or rural life. ABPA tended to be elevated in large cities and sparsely populated rural areas (**Table 2**). In contrast to residential area types, population density measures showed no significance with regard to aspergillosis (except ABPA data for CF patients < 18 years; p = 0.017; **Table 3**). One could speculate that population density is not the decisive factor. This inconsistency may be related to different data origins (BBSPR vs. DESTATIS). In contrast to residential area types, population density was divided in only three categories (high, medium, low), not reflecting extreme values (e.g. large cities and sparsley populated rural area). Maybe other environmental parameters, better reflected by different residential area types, are more important, e.g. architecture, furnishing or exposure to air pollution including particulate matter. A French study previously described *Af* distribution: Urbanized areas and heterogeneous agricultural areas were mentioned to be positively related to *Af* (Rocchi et al., 2020). Particularly, large cities seem to have negative effects. But also, agricultural air pollutants contribute to human health problems (Aneja et al., 2009). Particulate air pollution was associated with an increased risk of pulmonary exacerbations in CF (Brugha et al., 2018), a decline in lung function (Goss et al., 2004) and can exert proinflammatory and immunomodulatory effects (Reinmuth-Selzle et al., 2017). Our findings may reflect that in urban as well as sparsely populated rural areas synergistic effects of more frequent *Af* acquisition and immunomodulatory effects of air pollution influence the CF patients *Af* outcome. It is postulated that, pollution exposure early in life might associate with an increased risk of early infection to certain microbes (Brugha et al., 2018). Furthermore, the exposure to environmental factors might be even more harmful in children than in adults, because of behavioural and physiological factors (Goldizen et al., 2016). This may partly explain the determined frequencies for ABPA in patients <18 years in the studied residential area types. In future, the majority of people will live in urban areas, spending most of their time indoors (Rydin et al., 2012; Leung and Lee, 2016; Flies et al., 2019). Many issues are connected with this progress and the influence of urban environment associated factors will increase. The association between urban environments and disease is complex and context specific. Urban environments pose potential health risks and offer potential health benefits, such as improved access to health care, education, employment, or infrastructure (Flies et al., 2019). These health care aspects could lead to possible bias in the present work. One could speculate, that more severely affected CF patients tend to move to larger cities to benefit from better medical care. Infections require inhalation of fungal spores from the environment (Harun et al., 2010). *Aspergillus* is among the most common life-threatening airborne opportunistic fungus found indoors (Rocchi et al., 2020). The presence of *Af* in CF sputum is associated with worse respiratory quality of life in CF (Hong et al., 2020) and chronic Af is associated with more frequent pulmonary exacerbation dependent hospitalizations (Hong et al., 2018). CF patient registry data has already been used for risk factor analysis and macrolides, inhaled antibiotics and inhaled corticosteroids were associated with persistent *Af* isolation (Hong et al., 2018). In contrast, this questionnaire survey focused on urban and rural areas or population density items. Therefore, the impact of simultaneous exposure to multiple contaminants (other fungi, bacteria) has not been observed. Some species showed differences in concentrations according to geographic locations and the distribution of fungal spores is not homogeneous in the atmosphere (Rocchi et al., 2020). Regional or geographical patterns as well as different outdoor parameters or indoor characteristics (thermo-isolation, ventilation, architecture) were not considered in this study. Furthermore, no information on the frequency of microbiological examinations in the individual CF centres was available and questionnaire associated bias have to be considered. The possible influence of contact with pets will be discussed in detail in a separate article of this thematic special issue (see "Frequent pet contact as risk factor for allergic bronchopulmonary aspergillosis in cystic fibrosis"). Due to the 12 months observation period, the importance of seasonality on *Af* distribution can be excluded. The aim of the study was to investigate whether urban or rural life could affect the appearance of a colonization with *Af* in the respiratory tract of patients with CF. There is increasing evidence that there are adverse effects associated with the environment in large cities. The understanding of potential risk factors for aspergillosis permits the implementation of patient guidance and preventive measures.

CONCLUSION

Urban live should be considered as possible risk factor for colonization with *Af* in the respiratory tract of patients with CF. These new results may raise the awareness of the influence of environmental factors on patient outcomes and should be included in patient guidance and preventive measures.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Charité—Universitätsmedizin Berlin. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

CG, PE, ST, UD, KN, and CS contributed to the conception and design of the study. UD and CG organized the database. KN and CG performed the statistical analysis. CG, PE, ST, UD, KN, and CS wrote the manuscript. All authors contributed to the article and approved the submitted version.

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The Lung Microbiome of Three Young Brazilian Patients With Cystic Fibrosis Colonized by Fungi

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Microbial communities infiltrate the respiratory tract of cystic fibrosis patients, where chronic colonization and infection lead to clinical decline. This report aims to provide an overview of the diversity of bacterial and fungal species from the airway secretion of three young CF patients with severe pulmonary disease. The bacterial and fungal microbiomes were investigated by culture isolation, metataxonomics, and metagenomics shotgun. Virulence factors and antibiotic resistance genes were also explored. *A. fumigatus* was isolated from cultures and identified in high incidence from patient sputum samples. *Candida albicans, Penicillium* sp., *Hanseniaspora* sp., *Torulaspora delbrueckii*, and *Talaromyces amestolkiae* were isolated sporadically. Metataxonomics and metagenomics detected fungal reads (*Saccharomyces cerevisiae*, *A. fumigatus*, and *Schizophyllum* sp.) in one sputum sample. The main pathogenic bacteria identified were *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Burkholderia cepacia* complex, and *Achromobacter xylosoxidans*. The canonical core CF microbiome is composed of species from the genera *Streptococcus, Neisseria, Rothia, Prevotella*, and *Haemophilus*. Thus, the airways of the three young CF patients presented dominant bacterial genera and interindividual variability in microbial community composition and diversity. Additionally, a wide diversity of virulence factors and antibiotic resistance genes were identified in the CF lung microbiomes, which may be linked to the clinical condition of the CF patients. Understanding the microbial community is crucial to improve therapy because it may have the opposite effect, restructuring the pathogenic microbiota. Future studies focusing on the influence of fungi on bacterial diversity and microbial interactions in CF microbiomes will be welcome to fulfill this huge gap of fungal influence on CF physiopathology.

Keywords: cystic fibrosis, lung microbiome, metagenomic, colonization, *Aspergillus fumigatus*, *Burkholderia*, lung microbiota

INTRODUCTION

Cystic fibrosis (CF) is the most common lethal autosomal recessive disorder in Caucasians and is characterized by a mutation in the cystic fibrosis transmembrane conductance regulator gene (CFTR) (Cutting, 2015; Lavelle et al., 2016). The codified CFTR protein is responsible for the transport of chloride ions over the apical membranes of epithelial cells from tissues such as airways, intestine, pancreas, kidneys, sweat glands, and male reproductive tract (Bell et al., 2020). In the sinopulmonary tract, the transport of chloride anions contributes to the hydration of the mucus, forming a physical barrier against microbial pathogens and particles, which can be expelled by expectoration or swallowed through the action of ciliary movements in the trachea (Patel et al., 2020). In addition, the CFTR protein also transports bicarbonate ions, controls the levels of acidity in fluid secretions, and interacts with other membrane proteins to maintain tight junctions. All these roles of CFTR protein allow mucociliary clearance (MCC), whose impairment leads to the production of more viscous mucus, predisposing patients to develop chronic infections of airways (Procianoy et al., 2020). As a result, acute episodes of lung infections are observed in CF, receiving the general designation of "pulmonary exacerbation" (De Koff et al., 2016).

To overcome the deficiency in MCC and to prevent microbial colonization in the respiratory tract, the prescription of inhaled and systemic antibiotics is the first line of treatment available (De Koff et al., 2016). Pulmonary microbial colonization is facilitated by the building up of mucus, which may be a nutrient source for anaerobic bacteria such as *Streptococcus, Prevotella*, and *Veilonella*, whose metabolism releases short-chain fatty acids that serve as substrates for *Pseudomonas aeruginosa*. These bacteria are recognized as keystone taxa because they facilitate the growth of canonical CF pathogens through cross-feeding pathways (Silveira et al., 2020; Flynn et al., 2020). However, despite the frequent detection of these taxa in CF patients, a very high inter-patient variability of the airway microbiome has been reported (Bacci et al., 2020).

The CF microbiome varies according to age and clinical conditions of each patient, and it is generally more diverse in the younger than in the elderly, with decreasing diversity directly correlated to the loss of pulmonary function (Cox et al., 2010; Coburn et al., 2015; Caverly and Lipuma, 2018). Studies have also pointed out fungi of the genera *Candida* and *Aspergillus* as members of CF microbiomes, mainly related to cases of pulmonary exacerbation (Bevivino et al., 2019; Soret et al., 2020). However, research and treatment have conventionally focused on bacterial pathogens with little attention to fungal species. The fungi have been sub-notified as agents of CF infections for several reasons: (i) they might be erroneously regarded as contaminants in laboratory cultures; (ii) there are usually limited resources to recover fungal species in routine analysis of clinical samples; (iii) there is a lack of robust clinical guidelines and laboratory tools to assure unambiguous taxonomic identification of fungi; and (iv) clinicians tend to get puzzled by laboratory reports on unfamiliar fungal genera, which leads to difficulties in evaluating their real significance concerning the clinical findings in CF cases (Martín-Gómez, 2020).

In the era of next-generation sequencing (NGS) technologies, knowledge about the microorganisms related to CF has greatly enhanced. Previously overlooked microorganisms have been revisited, and new paradigms emerged in the CF microbiome. Knowledge of microbial composition has been correlated with the degree of lung function and contributes to a more assertive clinical diagnosis (Coburn et al., 2015; Caverly and Lipuma, 2018; Quinn et al., 2019).

Taking into consideration the increased detection of fungal infections in patients with CF and the novel tools available to evaluate the microbiome, this study was carried out to describe the lung microbial diversity of three young CF patients who were culture-positive for filamentous fungi at different stages of the disease.

CASUISTIC AND METHODS

Participants and Sample Collection

Three young patients with CF were recruited at the 'Ambulatório Multidisciplinar de Fibrose Cística (AMFC)' at the 'Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo (HC-FMRP-USP)' during 2018. The patients were unequivocally diagnosed with CF, either by detection of two mutations in the CFTR gene or by an abnormally high result for the sweat chloride test (≥60 mmol/L). They were enrolled due to their high visit frequency to the hospital, severe lung disease and presented a history of fungal isolation in sputum and chronic infections by pathogenic bacteria. They were named patient A (male, 12 years old), patient B (female, 9 years old), and patient C (male, 17 years old). The patients also presented the loss of pulmonary function, according to the spirometric parameters (% of predicted forced expiratory volume in one second, FEV1%), with test results for patients A, B, and C of 39, 64, and 37%, respectively. During the study period, the patients received antibiotics for pulmonary exacerbation (**Supplementary Table S1**). The patients were followed up to seven months, with a collection of sputum samples during routine clinical appointments and/or during hospitalization due to pulmonary exacerbation. A total of twelve sputum samples were collected with an average interval of 44 days and were distributed as follows: five from patient A (PA1 to 5), three from patient B (PB1 to 3), and four from patient C (PC1 to 4). The sputum samples were collected by expectoration into a sterile cup after mouth rinsing with water to prevent excessive salivary contamination. To each sputum sample, an equal volume of sterile dithiothreitol (DTT) solution (DTT 50 μg ml^{-1} dissolved in phosphate-buffered saline plus 0.1% gelatin) was added. After 30 min, the samples were vortexed to homogenize and cultured for bacterial isolation by seeding on blood agar, chocolate agar, salt mannitol agar, MacConkey agar, and *Burkholderia cepacia* selective agar at the routine clinical laboratory of the HC-FMRP-USP, with incubation at 37°C, under anaerobiosis for 24 to 48 h. Fungal isolation was performed by plating on Sabouraud Dextrose Agar (SDA—Acumedia, Michigan, USA) with 50 μg ml^{-1} chloramphenicol and in SDA with 50 μg ml^{-1}

chloramphenicol plus 500 μg ml^{-1} cycloheximide. Seeded plates were incubated at both 25 and 37°C for up to three weeks. The remaining sputum samples were frozen at −80°C until DNA extraction for metagenomics analysis

Ethics Statement

The sputum samples from the three patients who volunteered for the study were collected at HC-FMRP-USP following the ethical guidelines of the hospital. This study was approved by the Ethics Committee of "Faculdade de Ciências Farmacêuticas de Ribeirão Preto da Universidade de São Paulo" (FCFRP-USP), under protocol number 2.492.043, in accordance with the HC-FMRP-USP as a co-participating institution. The parents of the patients signed a term of Written Informed Consent to permit the participation of the teenagers in this study.

Bacterial Identification and Molecular Identification of Fungal Isolates

The bacterial isolates were biochemically identified by VITEK®2 (bioMérieux, France) at HC-FMRP-USP. Fungal colonies were identified by conventional methods and DNA sequencing of the internal transcribed spacer (ITS) region. *Aspergillus fumigatus* was additionally identified by the sequencing of calmodulin (cmd)- and beta-tubulin (*benA*)-encoding genes. Briefly, fungal DNA was extracted as previously described (Tonani et al., 2018) and used for PCR amplification with Phusion High Fidelity DNA Polymerase (New England BioLabs Inc.) with the primers ITS1 and ITS4 for ITS (White et al., 1990); *cmd5* and *cmd6* for calmodulin (Hong et al., 2005); and *bt2a* and *bt2b* for β-tubulin (Glass and Donaldson, 1995). PCR-purified products were sequenced with the same primers in the ABI3730 DNA Analyzer (Applied Biosystems). Each DNA sequence was analyzed with ChromasPro Software (ChromasPro1.7.6, Technelysium Pty. Ltd., Tewantin QLD, Australia) and tested against publicly available DNA sequences in the NIH genetic sequence database (Altschul et al., 1990).

Metagenomic DNA Extraction

The total DNA from sputum samples was extracted using the commercial Quick-DNA Fungal/Bacterial Miniprep kit (Zymo Research, USA) according to the instructions of the manufacturer. DNA quality and quantity were measured by a Qubit® 3.0 fluorometer (Thermo Fisher Scientific, USA) following standard methods provided by the manufacturer.

Preparation of Samples for Metataxonomic Analysis

To assemble the community taxonomic composition of bacteria and fungi, the 16S *rRNA* V3–V4 and ITS2 (Internal Transcribed Spacer) regions were amplified from aliquots of 1.0 μl from the whole genomic DNA sample extract.

The 16S *rRNA* V3–V4 regions were amplified by a first PCR employing the universal primers: forward 5′–TCGTCGGC AGCGTCAGATGTGTATAAGA GACAGCCTACGG GNGGCWGCAG–3′, and reverse GTCTCGTGGGCTCG GAGATGTGTGTGTATAAGAGACAGGACTACHVGGGT ATCTAATCC–3′. The reaction media of each PCR contained 10 μl of GoTaq® Colorless Master Mix 2× (Promega, USA), 1.0 μl of metagenomic DNA and 0.3 μM of forward and reverse primers. The final volume of 20.0 μl was achieved by completing the reactional volume with ultrapure water.

The PCR conditions were characterized by a first denaturation step at 94°C/3 min followed by 25 cycles of denaturation at 94°C/30 s, annealing at 55°C/30 s, extension at 72°C/30 s, and a final extension step at 72°C/5 min.

To evaluate the quality and to confirm the amplification, agarose gel 2% (w/v) electrophoresis stained with UniSafe Dye 0.003% (v/v) was performed. The average amplicon size obtained for each sample was 500 bp.

Regarding the ITS2 amplification run for fungi, the forward 86-F 5′-TCGTCGGCAGCGTCAGATGTGTATAAGA GACAGGTGAATCATCGAATCTTTGAA-3′ and reverse ITS4R 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAG ACAGTC CTCCGCTTATTGATATGC-3′ were used. The PCR components were the same as described above for 16S *rRNA* V3–V4 region amplification, although the PCR conditions to amplify ITS regions were slightly different: initially, a denaturation cycle at 95°C for 5 min was performed, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 40 s, extension at 72°C for 1 min, and a final extension step at 72°C for 5 min. All PCRs were conducted on a Veriti™ Thermal Cycler (Applied Biosystems, USA).

The integrity checking of the generated amplicons was performed by agarose gel electrophoresis 2% (m/v) stained with UniSafe Dye 0.003% (v/v). The average amplicon size obtained for each sample was 400 bp.

The PCR amplicons were subsequently purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, USA) following the manufacturers' guidelines.

The indexation step, required to pool the samples and to insert them into the flow cell, was performed through a second PCR for the entire set of generated amplicons. In this reaction, the adapter's sequences were inserted in the amplicon extremities. PCR was conducted with the Nextera XT index kit (Illumina®) following the manufacturers' instructions.

The resulting libraries were purified using magnetic beads provided in the Agencourt AMPure XP kit (Beckman Coulter) to remove fragments <100 bp and residual primers. The libraries were quantified by RT-PCR using the KAPA-KK4824 Library Quantification Kit (Illumina®) according to the manufacturer's recommendations. Next, an equimolar pool of samples was prepared by normalization of all samples to the final concentration of 3 nM. High-throughput DNA sequencing (HTS) was conducted on a MiSeq platform (Illumina®).

Metagenomic Sample Preparation and DNA Sequencing

The library preparation for metagenomics analysis was performed using the Nextera DNA Library Prep kit (Illumina®) to randomly fragment the metagenomic DNA using transposase activity. The adapters were inserted using the Nextera XT Index kit (Illumina®) following the manufacturers'

instructions. The libraries were quality-assured by removal of residual adapters, exceeding dNTPs and fragments <100 bp using AMPure XP magnetic beads (Beckman Coulter).

The libraries were quantified by RT-PCR using the KAPA-KK4824 Library Quantification Kit (Illumina®) following fabricant standards. A final concentration of 4 nM was used to normalize the libraries to pool them into the sequencer flow cell. The HTS was conducted on a NextSeq 500 platform (Illumina®) using the running kit NextSeq 500 MID Output (300 cycles) to generate 2 × 150 bp paired-end (PE) reads.

Raw Reads Processing: Contaminant Removal and Quality Control

The first preprocessing step was to overlap the PE reads using the BBmerge tool (Bushnell et al., 2017). The FASTQ files derived from the metataxonomic approach were addressed by the BaseSpace (Illumina®) pipeline with reads without adapters and Phred score values higher than 30, so these files were not subjected to any further quality processing.

On the other hand, the FASTQ files derived from metagenomic DNA sequencing were quality trimmed using the BBDUK tool to remove adapters and fragments <100 bp and Phred score <30 (parameters: hdist = 1, tpe, tbo, qtrim = rl, trimq = 30, maq = 30, minlen = 100).

Due to intrinsic characteristics, the sputum matrix was heavily concentrated with DNA from human cells. Thus, the human DNA reads were removed for the final processing of the Metagenomics Shotgun data. This was performed by downloading the human reference genome (version GRCh38.p12) from the NCBI database and indexing it on the Bowtie2 tool (Langmead and Salzberg, 2012). The FASTQ files were aligned against the indexed reference genome, resulting in two separate files: one with matches and the other with no matches. The unmatched FASTQ files were selected for downstream metagenomics analysis.

Community Taxonomic Composition

The community taxonomical composition was determined by two independent methods: (i) the QIIME1 open-reference method for amplicon analysis and (ii) MetaPhlan2-specific marker gene analysis for the metagenomics-derived data.

In the QIIME1 environment version 1.9.1 (Caporaso et al., 2010), the FASTQ files derived from metataxonomic HTS were converted to FASTA files, and the open-reference method was chosen to perform OTU (operational taxonomic units) picking using the UCLUST algorithm (Edgar, 2010) for sequence clustering. For fungal OTU picking, the UNITE database (version 8.0) was selected as a reference (Nilsson et al., 2019), and for bacterial OTU picking, the last version of the SILVA database (version 132) preformatted for QIIME was chosen (Quast et al., 2013).

The OTU tables were filtered to remove OTUs lower than 0.1% in relative abundance using built-in scripts provided by the pipeline. A bar plot of bacterial composition per patient was drawn in the R environment using the ggplot2 package. The statistical analyses related to alpha and beta-diversity measurements were performed on Microbiome Analyst (Dhariwal et al., 2017) environment selecting the default parameters of the "Marker Data Profiling (MDP)" pipeline.

The MetaPhlan2 pipeline (Segata et al., 2012) was chosen to process the shotgun-derived data using default parameters.

Annotation of Virulence and Antibiotic Resistance Genes

The search for virulence factors (VFs) in the genomes was conducted using the ShortBRED (Short, Better Representative Extract Dataset) tool (Huttenhower et al., 2012). Initially, a FASTA file containing the entire set of VFs curated on the Victors Database (Sayers et al., 2019) was downloaded. Then, the UniRef90 database (Suzek et al., 2007) was downloaded and used as a comprehensive reference database for additional comparisons of protein sequences. To create a VF custom reference database, first, the script ShortBRED-Identify was used. This script performs a global sequence homology identification of protein families related to the target sequences from the Victors Database. These sequences were collapsed to form a unique consensus sequence that possesses short peptide markers that best represented a given protein family. Moreover, applying the script ShortBRED-Quantify, the reads were aligned against the custom reference database represented by the newer protein markers, determined by the ShortBRED-Identify algorithm, quantified in terms of their absolute counts, normalized by marker gene length and finally represented by reads per kilobase per million (RPKM).

Regarding the search of antibiotic resistance genes (ARGs) in the metagenome, the same methodology for VF determination was employed, differing only due to the reference database chosen, which was the CARD (Comprehensive Antibiotic Resistance Database) (Jia et al., 2017).

RESULTS

Three young patients with cystic fibrosis who were part of a long-term follow-up in the "Ambulatório Multidisciplinar de Fibrose Cística (AMFC)" at HC-FMRP-USP were monitored in this study in 2018. The youngest patient (patient B) had a fatal outcome due to severe and difficult-to-treat infection at the beginning of 2019.

The bacterial and fungal microbiotas/microbiomes of the patient's lung were investigated by culture isolation, metataxonomics, and metagenomics shotgun sequencing. The culturable bacteria detected in the specimens of CF patients were for the patient A—methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, and *Burkholderia cepacia* complex; for the patient B—*B. cepacia* complex; and for the patient C—*Staphylococcus aureus*, *P. aeruginosa, Stenotrophomonas maltophilia, Achromobacter* sp., *Achromobacter xylosoxidans*, and *Chryseobacterium indologenes* (**Supplementary Table S1**). The results of fungal culture revealed the frequent isolation of *Aspergillus fumigatus* from the sputum of patients A and C. Additionally, *Penicillium*

sp. and *Torulaspora delbrueckii* were isolated from patient A, and *Candida albicans* was isolated from patient C. From patient B, *Penicillium* sp., *Hanseniaspora* sp., *Torulaspora delbrueckii*, and *Talaromyces amestolkiae* were isolated from sputum samples (**Table 1** and timeline **S1**). Regarding *A. fumigatus* clinical isolates, nucleotide polymorphisms were observed in calmodulin (cmd) and beta-tubulin (tub) genomic DNA sequences, which indicated four different sequence patterns, which we named sequence types (ST) 1 to ST4. Patient A had ST1 isolated from sputum samples PA1 and PA5 and ST2 from sputum samples PA3 and PA5. Patient B had no *A. fumigatus* isolates. Patient C had ST1, ST2, and ST3 isolated from sputum sample PC1, and ST4 from sputum samples PC1, PC2, PC3, and PC4 (**Table 2**). Focusing on sequence types, ST1 and ST2 were isolated from two different sputum samples from patient A, and ST4 was isolated from 4 sputum samples from patient B, thus indicating the colonization of patients A and C by *A. fumigatus* strains.

Concerning the results of HTS, for metataxonomic characterization of sputum samples from CF patients A, B, and C, the BaseSpace pipeline (Illumina®) returned an average of 153,229 and 87,538 quality-processed PE reads of 16S *rRNA* and ITS, respectively. These reads were overlapped to be analyzed on the QIIME1 pipeline, which is useful for determining the microbial community composition and diversity. A low overlap was observed, and only R1 FASTQ files were maintained, following the guidelines of the BBmerge tool, which recommends working only with the R1 reads to assure high quality during downstream processing if there is <15% overlap.

Regarding the HTS of metagenomics shotgun data, the average number of raw PE reads generated was 10,601,533 per sample. All samples were overlapped and preprocessed to remove low-quality base calls and adapter sequences, resulting in an average of 4,540,817 quality-processed PE reads per sample. However, the majority of community DNA was derivate from the hosts (mean of 95.82%) remaining an average of 182,342 quality-filtered PE reads by sample for taxonomic and functional downstream analyses.

Community Composition Shifts

The structures of the microbial communities from CF patients were drawn from metataxonomic and metagenomic results to exploit the best of each technique, aiming to improve the resolution of the study. The metataxonomics focus on specific taxonomic markers, and the targeted amplification of these DNA regions allows for enrichment of OTUs that might otherwise be difficult to detect due to low relative abundances in shotgun datasets (Almeida and De Martinis, 2019). On the other hand, the analyses of single-copy species-specific reads from the shotgun results help in the assignment of OTUs at the species level.

According to the metataxonomic findings, the patients presented variable bacterial compositions (**Figure 1**). For patient A, the high prevalence of *Staphylococcus* was notorious among all sputum samples evaluated. Lower amounts are shown for the *Rickettsiales* and *Burkholderia* groups in samples PA1, PA3, and PA5.

TABLE 1 | Molecular identification of fungal isolates.

Patient	Sputum sample	ID	Species	ITS				Calmodulin				β-tubulin			
				Ident. (%)	e-Value	Max score	Access GenBank	Ident. (%)	e-Value	Max score	Access GenBank	Ident. (%)	e-Value	Max score	Access GenBank
PA	PA1	LMC8001.01	*Aspergillus fumigatus*	100	0.0	933	KC689325.1	100	0.0	952	MK451408.1	100	0.0	917	KJ175502.1
	PA2	NI	NI	–	–	–	–	–	–	–	–	–	–	–	–
	PA3	LMC8001.03	*A. fumigatus*	100	0.0	933	KC689325.1	100	0.0	952	MK451394.1	100	0.0	917	KJ175502.1
	PA4	LMC8001.08	*Penicillium* sp.	99.81	0.0	968	MK226539.1	–	–	–	–	–	–	–	–
	PA5	LMC8001.09	*A. fumigatus*	100	0.0	933	KC689325.1	100	0.0	952	MK451408.1	100	0.0	917	KJ175502.1
	PA5	LMC8001.10	*A. fumigatus*	100	0.0	933	KC689325.1	100	0.0	952	MK451394.1	100	0.0	917	KJ175502.1
	PA5	LMC8001.12	*Torulaspora delbrueckii*	100	0.0	1360	KY105646.1	–	–	–	–	–	–	–	–
PB	PB1	NI	NI	–	–	–	–	–	–	–	–	–	–	–	–
	PB2	LMC8002.01	*Penicillium* sp.	98.48	0.0	926	MH864712.1	–	–	–	–	–	–	–	–
	PB2	LMC8002.02	*Hanseniaspora* sp.	99.37	0.0	1,146	AJ512437.1	–	–	–	–	–	–	–	–
	PB2	LMC8002.03	*Torulaspora delbrueckii*	100	0.0	1,362	KY105646.1	–	–	–	–	–	–	–	–
	PB3	LMC8002.04	*Talaromyces amestolkiae*	100	0.0	961	MH856395.1	–	–	–	–	–	–	–	–
PC	PC1	LMC8003.01	*A. fumigatus*	100	0.0	963	KC689325.1	100	0.0	896	MK451402.1	100	0.0	917	KJ175502.1
	PC1	LMC8003.02	*A. fumigatus*	100	0.0	963	KC689325.1	99.79	0.0	952	MK451394.1	99.8	0.0	924	KJ175502.1
	PC1	LMC8003.06	*A. fumigatus*	100	0.0	963	KC689325.1	100	0.0	952	MK451394.1	100	0.0	917	KJ175502.1
	PC1	LMC8003.09	*Candida albicans*	100	0.0	924	KY101918.1	–	–	–	–	–	–	–	–
	PC1	LMC8003.10	*A. fumigatus*	100	0.0	963	KC689325.1	100	0.0	952	MK451408.1	100	0.0	917	KJ175502.1
	PC2	LMC8003.11	*A. fumigatus*	100	0.0	963	KC689325.1	99.79	0.0	952	MK451394.1	99.8	0.0	924	KJ175502.1
	PC3	LMC8003.13	*A. fumigatus*	100	0.0	963	KC689325.1	99.79	0.0	952	MK451394.1	99.8	0.0	924	KJ175502.1
	PC4	LMC8003.14	*A. fumigatus*	100	0.0	963	KC689325.1	99.79	0.0	952	MK451394.1	99.8	0.0	924	KJ175502.1

ID, fungal identification; NI, no isolation; -, not done; Ident., identity; ITS, internal transcribed spacer.

TABLE 2 | *A. fumigatus* clinical isolate nucleotide polymorphism.

Patient	Sample	ID	Gene (bp)				ST
			cmd	cmd	cmd	tub	
			195	360	568	328	
PA	PA1	LMC8001.01	T	T	A	G	1
	PA3	LMC8001.03	C	G	G	G	2
	PA5	LMC8001.09	T	T	A	G	1
	PA5	LMC8001.10	C	G	G	G	2
PC	PC1	LMC8003.01	T	G	A	G	3
	PC1	LMC8003.02	C	G	A	A	4
	PC1	LMC8003.06	C	G	G	G	2
	PC1	LMC8003.10	T	T	A	G	1
	PC2	LMC8003.11	C	G	A	A	4
	PC3	LMC8003.13	C	G	A	A	4
	PC4	LMC8003.14	C	G	A	A	4

ID, strain identification; cmd, calmodulin; tub, ß-tubulin; ST, sequence type.

For patient B, *Rickettsiales* was the main bacterial order detected, with variable levels of *Streptococcus* and *Burkholderia* genera during the study (**Figure 1**).

Patient C was chronically colonized by *Achromobacter*, but *Staphylococcus* sp. was also detected in PC1 and PC3 samples, with a higher prevalence in the former. *Pseudomonas* genus was present in all but PC3 sputum samples. The PC4 sample was the most diverse, with the additional detection of *Streptococcus, Neisseria, Prevotella, Actinobacillus*, and *Haemophilus* (**Figure 1**).

Regarding intraspecies diversity (α-index), it was higher for patient B samples, as revealed by the larger number of different OTUs detected (**Figure 2A**). The theoretically expected diversity expressed by the Chao1 index (Kim et al., 2017) was very close to the OTU index observed in this study (**Figure 2B**), indicating the good quality of sampling, which means that the number of groups undetected was similar to the number of groups estimated to be undetected at random. However, the lower values of Shannon and Simpson indexes (**Figures 2C, D**) measured for patient B and patient C samples indicate that the majority of OTUs harbored by these patients were represented by rare groups, which is corroborated by the observation of the low relative abundances of the minor OTUs (**Figure 1**). Overall, the higher values observed for Shannon and Simpson metrics for patient C revealed a well-established microbiome, with a higher diversity in comparison with other patients, indicating a uniform relative abundance of OTUs. The comparison among the microbiota of patients (β-diversity) rendered a pattern of clustering per patient, indicating that each individual presented a distinguishable bacterial profile (**Figure 2E**).

Concerning the results of metataxonomics based on ITS gene markers, fungi were detected only in the PC4 sputum sample. This patient exhibited most reads assigned to *Saccharomyces cerevisiae*, *Aspergillus fumigatus*, and *Schizophyllum* sp. (**Figure 3**). Notably, the fungi were present in the sample with the highest bacterial diversity (**Figures 1**, **2C, D**).

Clustering analysis based on metagenomic shotgun data revealed the occurrence of a particular microbiota in each patient, and clinical specimens presented complex microbial compositions (**Figure 4**). Almost all samples of patient A were clustered together, except for the PA3 sample, possibly due to the "not assigned" OTUs. Additionally, the PA5 sample presented high levels of *Burkholderia* complex OTUs, analogous to some samples of patient B, justifying its proximity with the patient B cluster (**Figure 4**). As patient C exhibited a very particular microbial profile, the samples were clustered independently from the other subjects.

Exploring deeply the shotgun data, the patient A microbiota was characterized by the presence of *S. aureus* during the entire study and, in lower proportions, the cooccurrence of *Neisseria* sp., *Neisseria flavescens*, *Haemophilus parainfluenzae*, *Ralstonia* sp., and *Burkholderia* sp. (**Figure 4**).

The patient B microbiota was marked by the dominance of *Burkholderia cepacia* complex and the co-occurrence of *Rothia mucilaginosa*, *Stenotrophomonas maltophila*, *Veillonella* sp., *Streptococcus mitis/oralis/pneumoniae*, *Streptococcus salivarius*, and *Capnocytophaga* sp. (**Figure 4**).

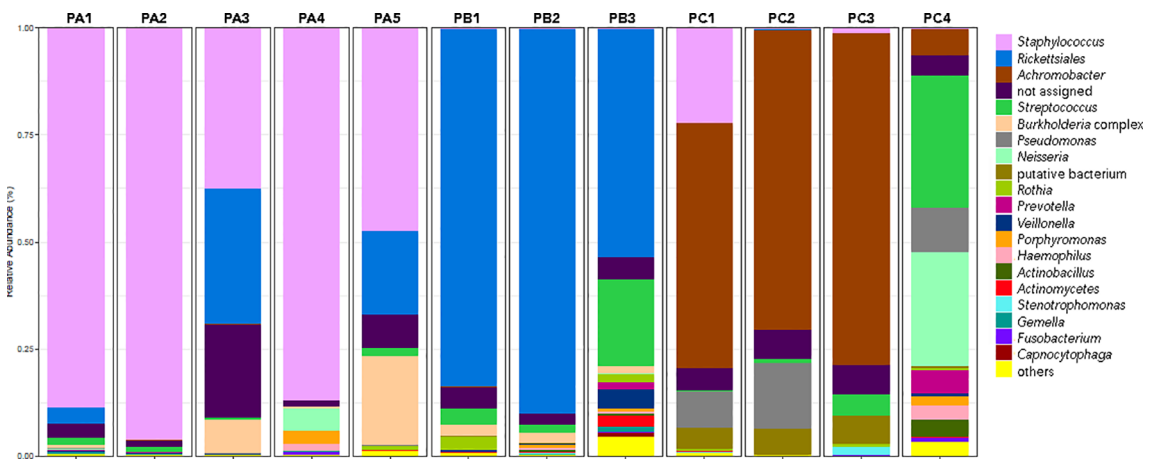

FIGURE 1 | Shifts in CF patient-associated bacteria expressed by the top 21 most prevalent OTUs.

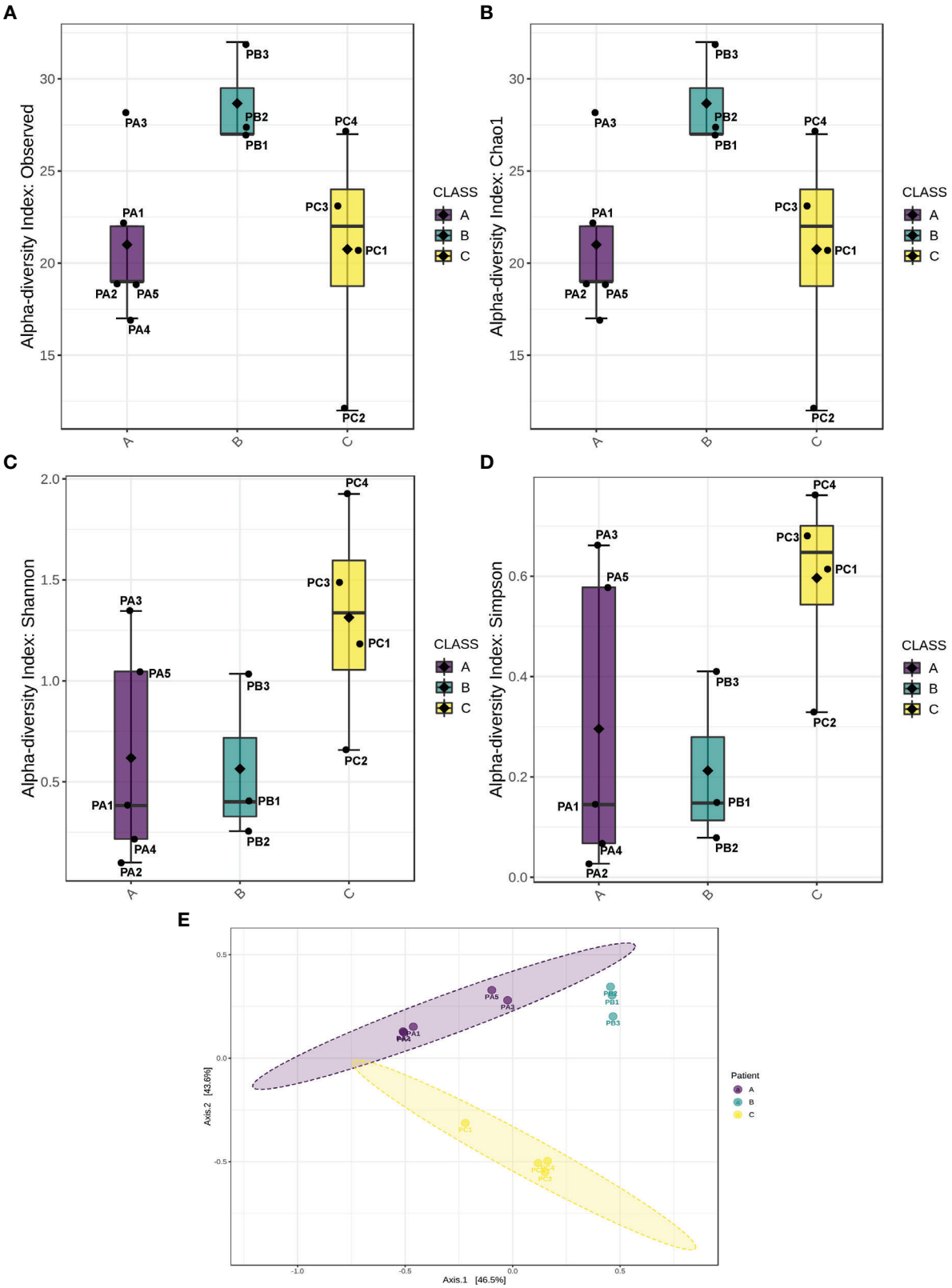

FIGURE 2 | Biological diversity indexes. Indexes of α-diversity (intrasample comparison) represented by observed OTUs **(A)**, Chao1 **(B)**, Shannon **(C)**, and Simpson indexes **(D)**. **(E)** Index of β-diversity (intersample comparison).

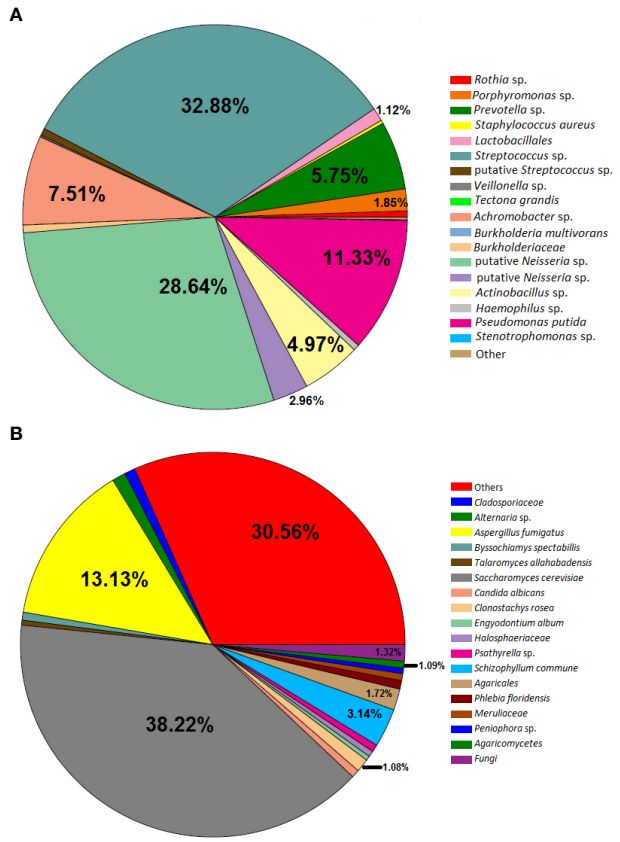

FIGURE 3 | Pie chart depicting bacteria **(A)** and fungi **(B)** detected in Patient C in the sample PC4. The slices without the relative abundance information represent the taxa that have less than 1% relative abundance.

Finally, patient C exhibited the most diverse microbiota, as illustrated by **Figures 2C, D**. *Achromobacter* sp. and *A. xylosoxidans* were present in all sputum samples. *Pseudomonas aeruginosa* and *Bordetella* sp. were observed in 75% (n = 3) of the sputum samples. PC4 was the most diverse sample, with the cooccurrence of low relative ratios of species from the genera *Haemophilus*, *Streptococcus*, *Kingella*, *Prevotella*, *Neisseria*, *Porphyromonas*, *Veillonella*, *Actinobacillus*, and *Rothia* (**Figure 4**).

In summary, the shotgun clustering snapshot corroborated the metataxonomic assignment of OTUs and the β-diversity measurements, which unequivocally translated the real community structure assemblage and justified the high microbial diversity found in patient C.

Virulence and Antibiotic Resistance Genes

The alignment of quality-processed reads against the custom marker genes database created from the comparison of UniRef90 and Victor databases detected sets of reads matching against 33 coding genes related to putative virulence factors. As observed for microbial succession, the prevalence of specific virulence determinants was highly variable according to each patient sampling time. In general, patient A exhibited high amounts of reads related to *Pseudomonas* quinolone signal (PQS), adhesion/invasion, cytolytic toxins, and general pathogenicity-enhancing effectors. In contrast, the most diverse microbiota in terms of virulence repertoire were those from patients B and C, which varied in terms of the presence of type II secretory systems and exopolysaccharide synthesis, presence of type III secretory systems, endotoxin synthesis and cytolytic potential (**Figure 5**).

Considering the ARG profiles, the alignments against the custom CARD and UniRef90 databases returned sets of reads matching 46 putative ARG coding genes. The antimicrobial profiles were variable according to the patient, but each one presented a huge diversity of efflux pump determinants. Patient A presented reads matching to nucleoside, beta-lactam, and aminoglycoside antibiotic classes, while the patient B microbiome was more related to general multidrug resistance effectors, porins with reduced permeability to antibiotics and tetracycline resistance. Finally, patient C presented genes conferring resistance to phosphonic acid derivatives, aminoglycosides, beta-lactams, chloramphenicol, and multidrug-related drugs (**Figure 6**).

DISCUSSION

The lung microbiomes of three young patients with CF with compromised pulmonary function were studied. The investigation is particularly interesting once the microbial community is more dynamic in infants than in adults (Cox et al., 2010; Klepac-Ceraj et al., 2010; Frayman et al., 2017). Virulence factors and antibiotic resistance genes were also explored.

The bacteria isolated from sputum samples were *S. aureus*, *P. aeruginosa*, and *B. cepacia* complex from patient A; *B. cepacia* complex from patient B; and *Achromobacter* sp., *A. xylosoxidans*, *Stenotrophomonas maltophilia*, *P. aeruginosa*, and *Chryseobacterium indologenes* from patient C. Although metataxonomic and metagenomic analyses identified most of these species, reads of *P. aeruginosa* were not observed in patient A sputum samples. Additionally, metataxonomic and metagenomic analyses identified species of the genera *Streptococcus*, *Neisseria*, *Rothia*, *Prevotella*, and *Haemophilus*, among others. The differences between identification by both culture isolation and HTS strategy show that the culture complexity and fastidious nutritional requirements of some pathogens can lead to non-isolation in standard microbial culture (Zemanick et al., 2017), and in the intrinsic composition of metagenomic samples with a high proportion of host-DNA, which sometimes encompasses more than 99% of the dataset, as was observed in this study, limiting the resolution of community composition. A recent study advocates the use of culture-enrichment metagenomics associated with the traditional HTS methods to improve the identification of low-abundant organisms and microbiome characterization, which may overcome this limitation (Lim et al., 2014).

As mentioned above, patient B showed an early poor outcome that culminated in patient death. The same patient had the

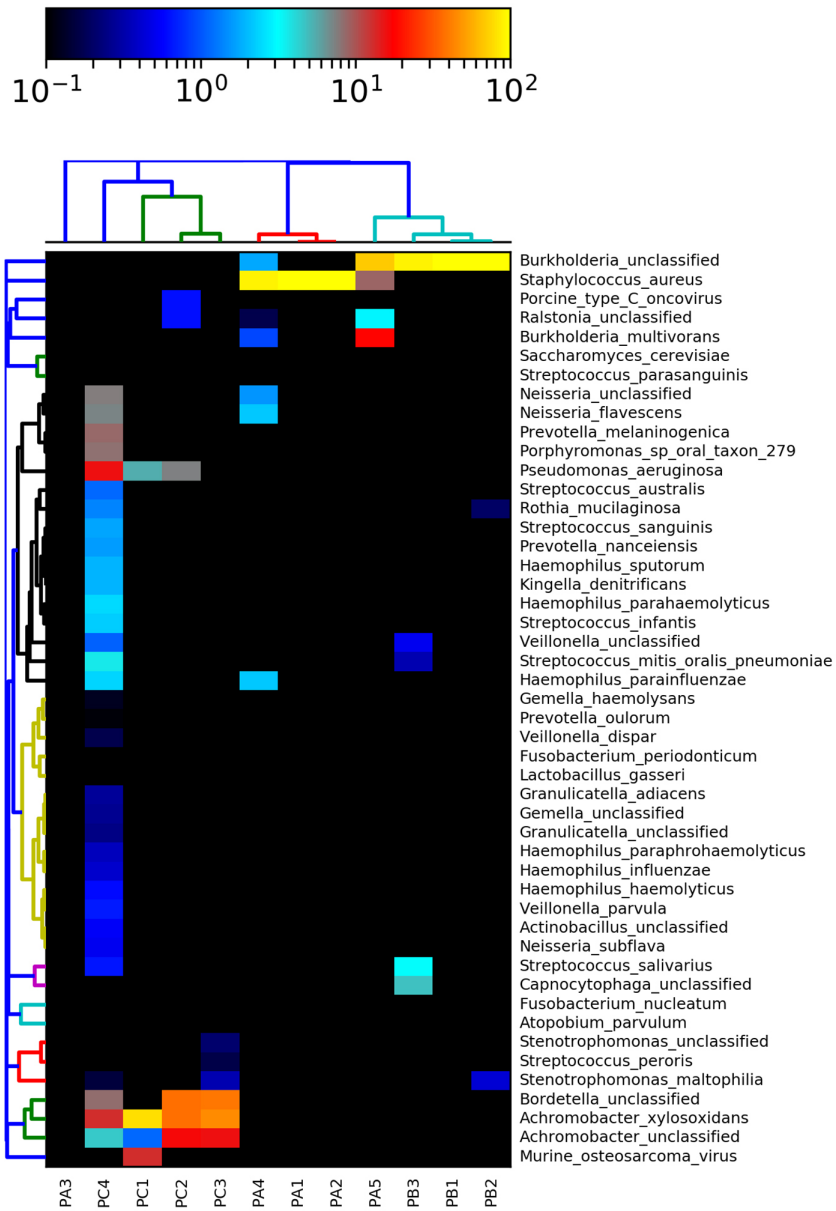

FIGURE 4 | Species-level taxonomic assignment of fungi, bacteria, and viruses in the microbiome based on metagenomics data. Some taxa were not assigned to the species level, so only the genus level was expressed.

lowest lung microbiota diversity, and studies have linked the decreased lung microbiota diversity with a reduction in lung function (Cuthbertson et al., 2020), which can justify the poor outcome. Additionally, the increase in microbiota dominance occurs in the loss of microbiota diversity (Cuthbertson et al., 2020). Members of *Pseudomonas*, *Staphylococcus*, and *Achromobacter* genera found in this study are potential pathogenic microorganisms and are frequently isolated from the airways of CF patients (Coburn et al., 2015; Losada et al., 2016). *B. cepacia* complex members were also found in this study. These members are most frequently isolated from patients with CF and generally linked to pulmonary deterioration (Coburn et al., 2015), high risk to the host due to multidrug resistance, easy transmissibility, the potential to cause septicemia, and an unpredictable clinical outcome, varying from an asymptomatic infection up to fatal pneumonia known as "cepacia syndrome" (Zlosnik et al., 2015; Hector et al., 2016). A previous study in the same hospital showed patients with good clinical outcomes and favorable options for antibiotic therapy (da Costa Capizzani et al., 2017). Even so, possibly the highly impaired lung functions of these young patients are related to the early presence of very pathogenic bacteria, related to adverse outcomes.

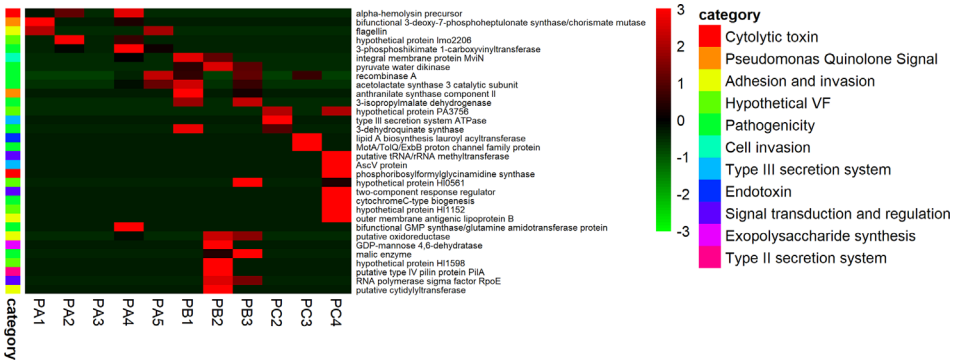

FIGURE 5 | Disease-related potential. Virulence sequences of fragmented genes detected on the CF-associated microbiome.

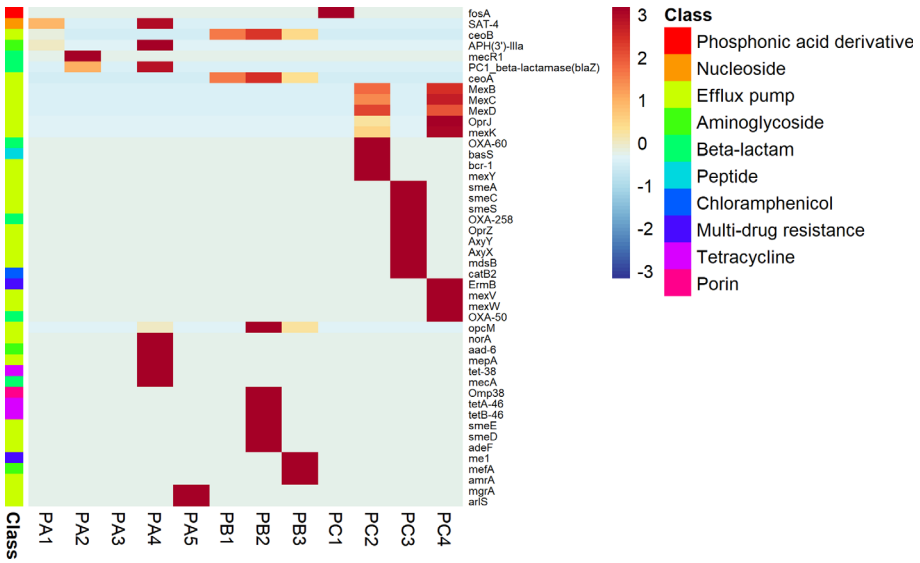

FIGURE 6 | Disease-related potential. ARG sequences of fragmented genes detected on the CF-associated microbiome.

Patient A's lung microbiome had a prevalence of *S. aureus* and *B. cepacia* complex, which was shown by culture isolation, metataxonomics, and metagenomics from the sputum samples. *S. aureus* is a common pathogen recovered from CF samples, and the association of this species with exacerbations in CF is evidenced by high levels of inflammation due to stimulus in interleukin-6 (IL-6) recruitment and eventually provoking lung damage (Hurley, 2018).

The patient B microbiome was primarily dominated by *B. cepacia* complex. This species was identified by both culture isolation and metataxonomics/metagenomics. It is commonly identified in CF patients and represents a high risk to the host due to the carriage of several virulence factors (VFs), such as the expression of secretion systems, several colony variants, and the presence of lipopolysaccharides, which are VFs frequently related to pandrug-resistance phenotypes (Lopes et al., 2015).

Achromobacter xylosoxidans is also a prevalent CF-related microbe and has been considered an emergent bacterium. It was highly detected in patient C lung microbiota, although *A. insuavis* and *A. ruhlandii* species are currently known as key pathogens in CF disease as well. These bacteria present resistance against the innate immune response, high plasticity in front of antimicrobial agents, may acquire resistance in short periods of exposure to antimicrobials, and can alter the expression of a set of genes to promote chronic infection (Edwards et al., 2017).

According to the literature review, the lung microbiota in CF disease may be divided into emergent pathogen microbiota and core microbiota. Commonly, the core microbiota of CF pediatric patients is formed by the genera *Streptococcus*, *Rothia, Prevotella*, *Actinomyces*, *Veillonella*, *Gemella*, *Neisseria*, and *Haemophilus*, which are commensal bacteria and not necessarily related to CF disease (Prevaes et al., 2017). Metataxonomic and metagenomic

analyses identified the canonical core CF microbiome species from the genera *Streptococcus, Neisseria, Rothia, Prevotella*, and *Haemophilus*, among others, in our study. Association studies showed that the taxa *Pseudomonas aeruginosa, Rothia mucilaginosa*, and *Streptococcus pneumoniae* were related to more deteriorated lung function (Paganin et al., 2015). *Rothia* sp. and *Streptococcus* sp. were detected at variable levels by metataxonomics in all sputum samples, but *Pseudomonas aeruginosa* was detected only in the patient C microbiome, concomitant with high levels of *Achromobacter* spp. The presence of all these taxa in the CF microbiome could predict a progressive loss in lung function.

Regarding the core microbiota, attention must be paid to *H. influenza*, which is a bacterium that naturally colonizes the respiratory tract during infancy but has recently been reported as an emergent pathogen frequently related to acute respiratory infections, contributing to the local inflammatory response and premature lung damage. The main concern with *H. influenza* pathogenicity is its strong ability for biofilm formation, which leads to high tolerance to antibiotics (Cardines et al., 2012). On the other hand, there are also reports describing the protective effect of *Haemophilus* spp. colonization to the lungs of adolescent patients with CF (Hector et al., 2016). Additionally, *S. maltophilia* and *Ralstonia* sp. were emergent pathogens detected among the sputum samples (considering both approaches of OTU assignment). Recent publications suggest that *S. maltophilia* plays several roles in lung damage, which can culminate with disease exacerbation, lung transplantation, and even death (Waters et al., 2013). The highly successful adaptation of *S. maltophilia* to CF is related to intrinsic antibiotic resistance, a decrease in biofilm formation, and pathogenicity in chronic disease. Additionally, *S. maltophilia* exhibits marked phenotypic and genotypic heterogeneity and complex regulatory networks among *S. maltophilia* clones in CF disease, which makes it difficult to identify therapeutic strategies to eradicate this bacterium (Esposito et al., 2017).

Ralstonia spp. infections in CF are still poorly reported, probably because it is underestimated, since this pathogen can grow on *Burkholderia cepacia* selective agar, generally with high levels of contamination by other pathogens, such as *P. aeruginosa* and *S. aureus* (Green et al., 2017). The genus *Ralstonia* is a common cause of nosocomial infections in CF patients due to contamination of hospital devices (*i.e.* respiratory therapy) and distilled water used to prepare solutions to be injected in patients. As infection is commonly related to polymicrobial communities in CF, it is difficult to establish the role of *Ralstonia* species in pathogenicity, but a high prevalence of this potential pathogen has been reported in the CF microbiome when the lung microbiome diversity is too low (Prior et al., 2017).

Thus, despite the low number of samples here, the airway of the CF patients presented dominant bacterial genera and inter-individual variability in microbial community composition and diversity, confirming the results of individual signatures of multiple species recorded in other studies (Delhaes et al., 2012; Losada et al., 2016; Güemes et al., 2019). The individual variations of microbial composition are most likely attributed to the patient's domestic environment and how patient's microbiome will respond to therapeutic perturbation (Whelan et al., 2020).

The depleted mucociliary clearance and the thick mucus filling the airways of CF patients easily trap the inhaled fungal conidia. *A. fumigatus* is the most prevalent species involved in CF colonization and disease (Warris et al., 2019) and was culture isolated at a high frequency from the sputum samples of patients A and C. Additionally, *A. fumigatus* with the same sequence type was observed in different sputum samples of each patient. Persistent colonization occurs when the same species is identified twice or more times in the same year (Saunders et al., 2016). Thus, the lung airways of patients A and C have persistent colonization of the lung airways by *A. fumigatus*. On the other hand, transient colonization of *Penicillium* sp., *Hanseniaspora* sp., *Torulaspora delbrueckii*, and *Talaromyces amestolkiae* was observed in the lung airways of patient B once a single culture isolation was registered (Saunders et al., 2016). The isolation of *A. fumigatus* in respiratory secretions of CF patients is a common occurrence. However, the mean age of the patient at the date of the first isolation of *A. fumigatus* ranges from 9 to 16 years old (Whelan et al., 2020). Our results reinforce the literature data once 9-year-old patient B has no *A. fumigatus* isolation from sputum samples, whereas older patients (A and C) present persistent colonization by this fungal species.

The culture method was able to recover fungal species in most sputum samples once it is an enrichment-based strategy and appropriate selective media with longer incubation times, which are not routinely performed in clinical microbiology labs (Hong et al., 2017; Martín-Gómez, 2020). On the other hand, metataxonomic and metagenomic analyses detected fungal reads in only one sputum sample (PC4). It is important to note that fungi are generally present in low amounts in the lung microbiome of CF patients, representing approximately 1% of the total OTUs (Losada et al., 2016), hampering the identification of these microorganisms by the culture-independent method. Thus, considering only metagenomics data, without the enrichment of gene markers by metataxonomics, few fungal reads were assessed, suggesting that metagenomics may underestimate fungi detection since the higher bacterial biomass and accelerated proliferation may overwhelm the fungi reads. The concordance between the molecular methods was in the detection of *S. cerevisiae* reads. *S. cerevisiae* was previously identified in sputum from CF patients (Güngör et al., 2013; Ziesing et al., 2016). A recent study evaluated the fungal composition in low- and high-severity CF patients against healthy subjects through isolation methods and found that *S. cerevisiae* was present in high-severity or healthy subjects, but its meaning in the context of CF physiopathology is unknown.

It is important to highlight the high diversity of bacterial species in the PC4 sample in comparison with the other samples. However, the copresence of fungal members and its influence on bacterial diversity is a matter that needs future investigation. The high microbiome diversity is related to low ratios of pathogenic

bacteria and to an improvement in lung function (Carmody et al., 2015; Losada et al., 2016). However, the presence of pathogenic fungi, such as *A. fumigatus*, one of the major fungi identified in PC4, may not be positive since there is evidence of its role in CF pathophysiology causing a significant decrease in FEV-1 and being frequently correlated with inflammatory episodes (Eickmeier et al., 2020).

Schizophyllum commune was the third most prevalent fungal OTU detected in the patient C metagenome. This basidiomycete is an environmental fungus that commonly grows on organic matter. However, a novel report linked this microorganism to allergic bronchopulmonary mycosis (ABPM) and pulmonary fungal ball (Chowdhary et al., 2013), characterizing it as an emergent pathogen.

Regarding the other fungal taxa detected, to the best of our knowledge, there are no conclusive reports on their influence in CF disease. The well-known yeast *C. albicans* is frequently isolated from the sputum culture of CF patients. It is believed that the use of inhaled steroids and antibiotics predisposes *Candida* sp. colonization (Williams et al., 2016).

In this work, we observed a wide diversity of VFs and ARGs found in all CF microbiomes. As pointed out by a previous assay, the abundance of VFs and ARGs in CF microbiomes are not directly related to a specific bacterial group but are spread among several members of the CF community, indicating that the emergence of these traits should be linked to a particular clinical condition of the CF patient (normal, mild or severe) and enhanced by the antibiotic therapy treatment provided in care units for long-term periods (Bacci et al., 2017).

Antibiotic stewardship indeed may potentiate resistance by triggering downstream responses on the bacterial community, such as the upregulation of efflux pumps in biofilm formation, making bacteria immune to antimicrobials or enhancing the rates of horizontal gene transfer among bacteria (Sherrard et al., 2014). On the other hand, susceptible bacteria can acquire temporary resistance to antibiotics due to microbial interactions in the lung environment. Resistant bacteria can modify antibiotics, diminishing their efficacy, or biofilm formation may limit and dilute the minimum inhibitory concentration values resulting in sublethal doses or fungi that may protect bacteria from forming hyphal barriers (Vandeplassche et al., 2019).

A recent study suggests that a therapy focusing on eliminating anaerobic bacteria re-establishes the canonical CF microbiota, improving lung function and respiratory health (Silveira et al., 2020). At the same time, evidence of a therapy targeted to the weakest cross-feeder, a susceptible bacterium that feeds the entire microbial community, reduces the viability of the resistant ones, improving treatment efficacy (Adamowicz et al., 2018). Currently, the simple detection of virulence factors and antibiotic resistance genes in CF microbiomes is unmeaning if microbial interactions and community response to therapy are not considered.

In summary, the lung airway microbiome of three young CF patients with fungal colonization presents a personal signature with low variation in the microbiome across pulmonary exacerbations and a core set of virulence factors and antibiotic resistance genes. Understanding the microbial community is crucial to improve therapy because it may have the opposite effect, restructuring the pathogenic microbiota. Future studies focusing on the influence of fungi on bacterial diversity and microbial interactions in CF microbiomes will be welcome to fulfill this huge gap in fungi influence in CF physiopathology.

DATA AVAILABILITY STATEMENT

The dataset generated for this study can be found in GenBank accession MT886813 to MT886844, MT891039 to MT891061, and MT911441 to MT911463. The metataxonomic sequence data are available in Bioproject PRJNA644204. The shotgun data are available in Bioproject PRJNA644285.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of "Faculdade de Ciências Farmacêuticas de Ribeirão Preto da Universidade de São Paulo" (FCFRP-USP), under protocol number 2.492.043, in accordance with the HC-FMRP-USP as a co-participating institution. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

OA performed the data curation, formal analysis, investigation, methodology, visualization, and wrote, reviewed, and edited the original draft. CC wrote, reviewed, and edited the manuscript. LuT was in charge of the methodology. PB was in charge of the methodology and wrote, reviewed, and edited the manuscript. AC conceptualized the study and wrote, reviewed, and edited the manuscript. EM supervised the study and wrote, reviewed, and edited the manuscript. LiT was responsible for the formal analysis, visualization, and writing and reviewing the manuscript. MK conceptualized the study, and was in charge of the methodology, project administration, supervision, formal analysis, funding acquisition, project administration, resources, visualization, writing, reviewing, and editing original draft. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.598938/full#supplementary-material

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ELISA Test for the Serological Detection of *Scedosporium/ Lomentospora* in Cystic Fibrosis Patients

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The detection and diagnosis of the opportunistic fungi *Scedosporium* spp. and *Lomentospora prolificans* still relies mainly on low-sensitive culture-based methods. This fact is especially worrying in Cystic Fibrosis (CF) patients in whom these fungal species are frequently isolated and may increase the risk of suffering from an infection or other health problems. Therefore, with the purpose of developing a serologic detection method for *Scedosporium/Lomentospora*, four different *Scedosporium boydii* protein extracts (whole cell protein extract, secretome, total cell surface and conidial surface associated proteins) were studied by ELISA to select the most useful for IgG detection in sera from CF patients. The four extracts were able to discriminate the *Scedosporium/ Lomentospora*-infected from *Aspergillus*-infected and non-infected patients. However, the whole cell protein extract was the one selected, as it was the one with the highest output in terms of protein concentration per ml of fungal culture used, and its discriminatory capacity was the best. The ELISA test developed was then assayed with 212 sera from CF patients and it showed to be able to detect *Scedosporium* spp. and *Lomentospora prolificans* with very high sensitivity and specificity, 86%–100% and 93%–99%, respectively, depending on the cut-off value chosen (four values were proposed A_{450nm}= 0.5837, A_{450nm}= 0.6042, A_{450nm}= 0.6404, and A_{450nm}= 0.7099). Thus, although more research is needed to reach a standardized method, this ELISA platform offers a rapid, low-cost and easy solution to detect these elusive fungi through minimally invasive sampling, allowing the monitoring of the humoral response to fungal presence.

Keywords: enzyme linked immunosorbent assay, *Scedosporium*, cystic fibrosis, serodiagnosis, *Lomentospora*

HIGHLIGHTS

In recent years huge efforts have been made to develop new serological techniques to improve diagnosis of fungal infections. However, most of the advances are focused on high prevalence fungal pathogens such as *Aspergillus* or *Candida*. Regarding less common fungi like *Scedosporium/Lomentospora*, which are considered emerging pathogens and are gaining clinical relevance due to the severity of the infections they cause, there are no commercial detection methods available. This manuscript describes an ELISA test developed using a whole cell protein extract from *Scedosporium boydii* that offers a great sensitivity and specificity to detect *Scedosporium/Lomentospora* in Cystic Fibrosis (CF) patients using serum samples. Compared with the commercial kits for other fungi, which usually show a 75%–96% of sensitivity, the clinical usefulness of the test developed is remarkable considering that values obtained are of 86%–100% for sensitivity and 93%–99% for specificity. These results place it in a good position as a candidate to be used as a diagnostic tool. Thus, this ELISA platform is a rapid, low-cost, and easy solution to detect *Scedosporium/Lomentospora*, allowing the monitoring of the antifungal humoral response.

INTRODUCTION

Cystic fibrosis (CF) is the major genetic disorder among the Caucasian population (Mirtajani et al., 2017). This multisystem disease is caused by mutations in the CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) gene encoding a chloride-conducting transmembrane channel, which participates in electrolytic transport and mucociliary clearance of the airways. CFTR dysfunction results in an increased viscosity of secretions and underlies an altered immune response of these patients (Rowe et al., 2005). Although several organs are adversely affected, morbimortality is essentially associated with lesions in the lungs (Castellani & Assael, 2017). The airways of CF patients are affected by the accumulation of a thick layer of sticky bronchial mucus, which acts as a culture media for microorganisms, entrapping airborne bacteria and fungal spores, immobilizing them and facilitating their growth. In this sense, these pathogens cause chronic respiratory infections, turning the lung into an inflammatory microenvironment and eventually leading to pulmonary damage (Elborn, 2016).

Even though bacteria, such as *Pseudomonas aeruginosa* or *Staphylococcus aureus*, are known to be the major causative agents of these infections, several fungal species colonize the respiratory tract of CF patients. However, while the relevance of bacteria is well known, the clinical significance of fungal recovery from respiratory secretions remains unclear (Schwarz et al., 2018). *Candida albicans*, among yeasts, and *Aspergillus fumigatus*, among filamentous fungi, are the fungal species most frequently isolated from CF respiratory samples. However, fungi from *Scedosporium* genus are increasingly reported in the CF context and currently rank second, just behind *A. fumigatus*, among the filamentous fungi colonizing CF airways (LiPuma, 2010).

Despite airway colonization by *Scedosporium* spp., or the strongly related *Lomentospora prolificans* (Ramirez-Garcia et al., 2018), it is usually well tolerated, it may lead to a true respiratory infection with variable degree of tissue invasion, fungal sensitization or allergic bronchopulmonary mycoses (Martín-Gómez, 2020). In fact, these fungal pathogens seems to be more representative during scenarios of moderate-to-severe alteration of lung function, and their presence on CF airways has been associated with a decline in Forced Expiratory Volume in 1 s (FEV1) (Soret et al., 2020). In addition, the chronic detrimental presence of these pathogens may cause fatal disseminated infections when the patient undergoes an immunosuppression period, for example after a lung transplantation (Symoens et al., 2006).

Unfortunately, lung infection or colonization by *Scedosporium/Lomentospora* is nowadays a diagnostic and therapeutic challenge in CF patients. Despite the many methods of *Aspergillus* detection being currently available, the detection of *Scedosporium/Lomentospora* relies upon low sensitivity culture-based traditional methods. Recently, some advances have been made on molecular diagnosis, but these methodologies are not standardized and not accessible to everyone, and serodiagnosis strategies are performed only in specialized laboratories, but these are not commercially available (Mina et al., 2017). Therefore, the precise diagnosis of these fungi is actually a significant challenge. Furthermore, the clinical features and histopathology of infected tissue samples are similar to those of aspergillosis, so confirmation data of these species may often be underestimated (Mello et al., 2019). Hence, detection and correct discrimination of *Scedosporium* infections from others is of crucial importance because the treatments may be quite different. Indeed, *Scedosporium/Lomentospora* species are considered intrinsically resistant to most of the currently available antifungal drugs (Pellon et al., 2018).

In this sense, to contribute to the finding of new diagnostic weapons that allow prevention, early diagnosis, and ultimately a more effective treatment, our research group analysed different protein extracts of *Scedosporium boydii* as serodiagnostic tools to detect *Scedosporium/Lomentospora* and discriminate them from other fungal pathogens relevant in the CF context. Therefore, in this study we designed and tested a customized serological assay for the detection of *Scedosporium*-specific IgG antibodies in sera from CF patients.

MATERIALS AND METHODS

Microorganisms and Culture Conditions

The fungal strains used in this study were *Scedosporium boydii* CBS 116995, *Lomentospora prolificans* CECT 20842, *Aspergillus fumigatus* Af293 and *Candida albicans* NCPF 3153. All strains were maintained cryopreserved at -80ºC and cultured as required on Potato Dextrose Agar (PDA) (Pronadisa, Madrid, Spain).

To harvest conidiospores of *S. boydii* and *L. prolificans*, PDA plates grown at 37ºC for 7 days were washed twice with sterile saline solution (0.9% [w/v] NaCl) (SS). The suspension of conidia was

filtered through sterile gauze to avoid cell debris and centrifuged. Conidia of *A. fumigatus* were collected from PDA tubes grown at 37ºC for 4 days using sterile SS-Tween20 (0.9% [w/v] NaCl, 0.02% [v/v] Tween20), and washed twice by centrifugation. Finally, *C. albicans* was grown in PDA tubes at 37ºC for 24 h, and yeast cells were gathered next day by resuspending the culture with phosphate buffered saline (PBS). The concentration of each fungal cell was adjusted as needed using a hemocytometer.

Human Serum Sample Collection and Categorization

A collection of 212 sera from CF patients (corresponding to 102 different patients) were used in this study with the approval of the Ethics Committee from the University of the Basque Country (UPV/EHU; reference M30/2018/081).

The categorization of the sera was based on the results of mycological examination of a sputum sample collected in parallel to the sera and inoculated on Sabouraud gentamicin chloramphenicol agar, and simultaneously on Modified Thayer Martin agar or Sabouraud chloramphenicol agar supplemented with 0.5 g/L cycloheximide for the specific recovery of *Scedosporium* species from these polymicrobial samples. All plates were incubated at 37ºC for up to 15 days before to consider the sputum samples as free of fungi. According to these, three groups of sera were defined: Group Scedo+ (n = 23) consisted of sera from CF patients with positive cultures for *Scedosporium/Lomentospora*, Group Asp+ (n = 86), CF patients with *Aspergillus* spp. being the only filamentous fungi recovered from sputum; and group Scedo-/Asp- (n = 103) as control, consisted of sera from CF patients without any filamentous fungus recovered from samples. Sera from patients with co-infection of *Scedosporium/Lomentospora* and *Aspergillus* spp. were included in the group Scedo+.

In addition, five sera from each group described above were selected to evaluate the usefulness of different protein extracts for *Scedosporium/Lomentospora* serodiagnosis, and to study cross-reactivity with other fungal pathogens. To do that, two criteria were followed: each serum corresponded to a different patient without any coinfections.

Fungal Protein Extracts

Four different protein extracts were obtained for this study: whole cell protein extract (Total WCP), extract of secreted proteins (Secretome), cell surface associated proteins (Total CSP) and conidial surface proteins (Conidial CSP). All extracts were obtained for *S. boydii*, but the Total WCP was also obtained for *L. prolificans, A. fumigatus*, and *C. albicans*. The resulting protein extracts were stored at -80ºC until required.

The extraction processes (**Figure 1**) were carried out in triplicate and the quality of the extract was verified by SDS-PAGE in 12% polyacrylamide gels, stained afterwards as previously described (Dyballa and Metzger, 2009) with Coomassie Brilliant Blue G250 (CBB), and digitalized using ImageScanner III (GE Healthcare, Chicago, IL, USA). Protein concentration was quantified using Pierce 660 nm Protein Assay Reagent (Thermo Fisher Scientific,

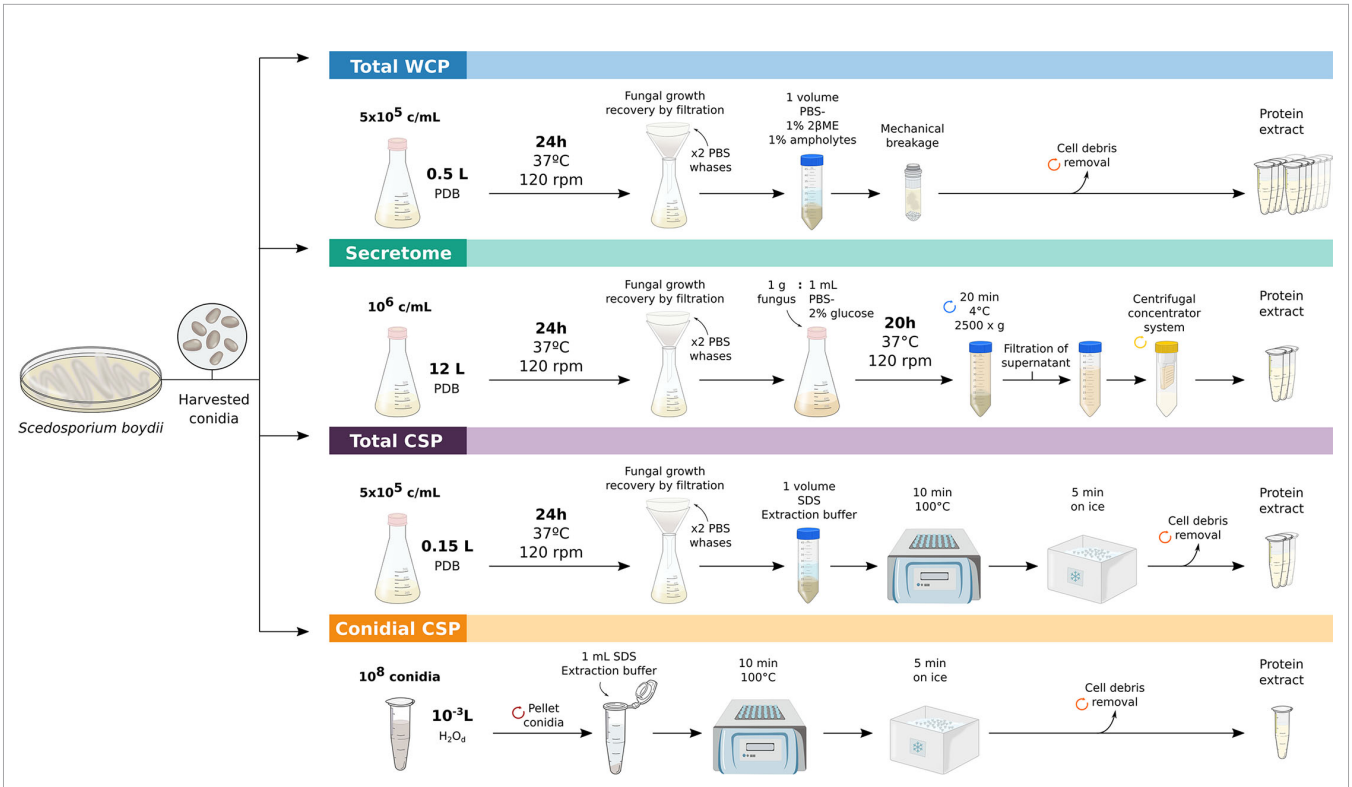


FIGURE 1 | Workflows employed for obtaining *S. boydii* protein extracts. Key-steps in the obtaining of crude total extract (Total WCP), secretome, total extract of cell-surface associated proteins (Total CSP) and conidial cell-surface associated proteins (Conidial CSP) of *S. boydii*.

Rockford, IL, USA). Likewise, to avoid interference from some reagents present in the extraction buffers, protein extracts were precipitated with a solution of acetone and 10% (w/v) trichloroacetic acid, and resuspended in distilled water, prior to protein concentration measurement.

Whole Cell Protein Extract of Conidia and Hyphae (Total WCP)

To obtain total protein extracts, 5 x 10^5 cells/ml (conidia or yeasts) were inoculated into Potato Dextrose Broth (PDB) (Pronadisa, Madrid, Spain) cultured at 37ºC and 120 rpm for 24 h. Fungal growth was recovered by filtration and washed twice with PBS to remove traces from the medium. Fungal material was resuspended in PBS supplemented with 1% (v/v) β-mercaptoethanol and 1% (v/v) ampholytes pH 3-10 (GE Healthcare, Freiburg, Germany). Finally, cell disruption was achieved by bead-beating with glass beads for 20 min at 30 Hz using the MillMix20 (Tethnica, Slovenia), following the standardized protocol described previously (Pellon et al., 2016). Cell debris was discarded by centrifugation, and the resulting protein suspension was sonicated on ice for 2 min at 40% amplitude and 2 s pulses.

Extract of Secreted Proteins (Secretome)

Extraction of *S. boydii* secretome was carried out following the methodology described in Buldain et al., 2019, with slight modifications. Briefly, 10^6 conidia/ml were inoculated into PDB and grown for 24 h at 37ºC and 120 rpm. Fungal material was collected, washed twice with sterile PBS and cultured for 20 h at 37ºC and 120 rpm in PBS supplemented with 2% glucose in a proportion 1 g fungus: 1 mL medium. The culture was centrifuged, the supernatant filtered through a sterile gauze and then through a 0.22 µm membrane. Finally, the cell-free supernatant was concentrated using a 100,000 MWCO VIVASPIN centrifugal concentrator system (Sartorius, Göttingen, Germany). The resulting suspension was sonicated under the same conditions as the Total WCP extract described above.

Cell Surface Associated Proteins (Total CSP and Conidial CSP)

Proteins associated with the cell surface were collected by employing the protocol described by Pitarch et al. (2002), but with some modifications. To be precise, the culture of 5 x 10^5 conidia/ml PDB for 24 h at 37ºC and 120 rpm was recovered by filtration and washed twice with PBS. Fungal material was resuspended in a volume of SDS extraction buffer and boiled for 10 min at 100ºC, cooled 5 min on ice, centrifuged and the supernatant containing Total CSP was recovered.

To obtain the Conidial CSP extract, the same procedure was carried out but using 10^8 conidiospores as fungal material, which were directly resuspended in 1 ml of extraction buffer.

Enzyme Linked Immunosorbent Assay (ELISA) for the Detection of *Scedosporium/Lomentospora*

Specific anti-*S. boydii* IgG was measured using a customized enzyme-linked immunosorbent assay (ELISA) method. ELISA was performed by coating wells of high binding micro test plates (Sarstedt, Nümbrecht, Germany) overnight at 4ºC with 10 µg/ml of protein extract diluted in sterilized PBS (100 µl per well). The following day the wells were washed three times with 200 µl PBS and blocked for 1 h at 37ºC by adding 200 µl of 5% (w/v) skimmed milk powder solution in PBS containing 0.05% (v/v) Tween 20 (PBST). Thereafter, three washes with PBST preceded the incubation at 37ºC for 1 h with 100 µl of human sera diluted 1:200 in PBST. In parallel, PBST without serum was added to some wells as negative control. Then these plates were washed three times with PBST, and 100 µl of HRP labeled anti-human-IgG (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:10,000 in PBST was added to each well. After 1 h incubation at 37ºC, three washes with PBS preceded the incubation with 50 µl TMB substrate solution (Thermo Fisher, Waltham, MA, USA) for 30 min in the dark at 24ºC. Finally, the reaction was stopped by adding 50 µl of 0.5 M H_2SO_4 and absorbance (Abs) was measured at 450 nm using a Synergy TM HT plate reader (BioTek, Winooski, VT, USA).

A pool made up of the five Scedo+ sera selected was included in each experiment as a positive control to monitor batch-to-batch variations and to normalize the data after the study.

Data Processing, Statistical Treatment, and Analysis

For data analysis, absorbance (Abs) values obtained in negative control wells were subtracted from remaining wells. Serum samples were measured in duplicate, and three replicates were performed for each ELISA experiment. With the aim of avoiding plate to plate bias variation, each Abs value was divided by the Abs value of the positive control included in all the plates and the result was expressed as Relative Abs.

Successive statistical analyses were run in SPSS Statistics software version 24 (IBM, Armonk, NY, USA) and the data plotted using Prism7 software (GraphPad, San Diego, CA, USA). Data distribution and its normality was studied by box plot analysis (see **Supplementary Material 1**). Two outlying values, classified into the Scedo- group, were identified and excluded from the analysis hereinafter. Normal distribution of data was detected by the Shapiro-Wilk test (< 50 samples) or the Kolmogorov-Smirnov test (> 50 samples), and homogeneity of the variance was proven by Levene test. Mean IgG response was compared between the three sera groups (Scedo+, Asp+, and Scedo-/Asp-) by performing a one-way analysis of variance (ANOVA) for normal distributed data or by Kruskal-Wallis for data with non-normal distribution, followed by Bonferroni's multiple comparison test. Likewise, Scedo+ group's mean specific IgG response was compared to the mean response of *Scedosporium* negative samples (Asp+ and Scedo-/Asp-) by performing Student's *t* test or the Mann-Whitney U test for data with a normal and non-normal distribution, respectively. All the analyses were performed taking into account a confidence interval (CI) of 95%, for this a p-value < 0.05 was considered statistically significant.

The optimal diagnostic cut-off value to discriminate positive and negative results was selected by taking into consideration the

following seven criteria defined in the bibliography (Habibzadeh et al., 2016; Mina et al., 2017; Unal, 2017): Youden Index (*J*), Concordance Probability Method (CZ), Index of Union (IU), Closest to [0.1] criteria (ER), Control mean Abs plus 2 standard deviation (SD) ($\bar{X}$ + 2*SD*), and Sensitivity = Specificity (SE = SP), and the maximum positive likelihood ratio (MLR+).

To calculate the cut-off value of Abs by some of these criteria an analysis of the Receiver Operating Characteristic (ROC) curve was performed, and the performance of the test was checked based on the area under the ROC curve (AUC). To do this, Abs obtained with each serum sample of Scedo+ group were considered as Patient Values, meanwhile Abs of sera from Asp+ and Scedo-/Asp- groups were included in Control Values. In this way, the ROC area under the curve (AUC) was drawn with 95% CI.

Finally, the accuracy and test performance of the ELISA was evaluated by comparison with the "Gold Standard" method (mycological culture of the sputa), calculating validation parameters of Sensitivity (SE), Specificity (SP), Positive Predictive Value (PPV), Negative Predictive Value (NPV), and Efficiency (EFF). In addition, agreement between the two techniques was analyzed by Cohen's Kappa index (K), which excludes the possibility of agreement occurring by chance.

RESULTS

Fungal Prevalence in CF Patients' Sputum Samples

The epidemiological study of the group of sera used in this work, carried out according to the microbiological examination of patients' sputum (**Figure 2**), showed that *Candida* yeasts were detected in 51.43% of the patients, *C. albicans* being the most frequently isolated (74.07%). Regarding filamentous fungi, 31.43% of the patients resulted in positive cultures for species in the genus *Aspergillus, A. fumigatus* being the most prevalent (66.67%), followed by *Aspergillus terreus* (39.39%) and species of *Aspergillus flavus* complex (3.03%). Meanwhile, 11.43% of CF patients were positive for *Scedosporium/Lomentospora*, with the highest impact from the *S. apiospermum* species complex (83.33%) and a significant prevalence of *L. prolificans* (50%). Likewise, 36.19% of the population studied showed negative cultures for these three fungal pathogens.

Reactivity of Human Sera Against *S. boydii* Protein Extracts

With the aim of analysing the immunoreactive capacity and the discriminatory power of *S. boydii* protein extracts, specific IgG reactivity of five sera from different patients selected from each of the three groups of CF sera were measured by ELISA using four different types of protein extracts as antigen: Total WCP, Secretome, Total CSP, and Conidial CSP (**Figure 3**). In this sense, all extracts allowed the differentiation of the three groups, with the differences between Scedo+ and the other two groups, separately or together, statistically significant. From the four extracts, the best results were obtained with the Total WCP (**Figure 3A**) as discrimination of sera was the most accurate, and there was no overlap between groups.

Although the secretome extract offered almost as accurate results, when evaluating the efficiency of the extraction methods (**Figure 4**) in terms of processing time, initial culture volume

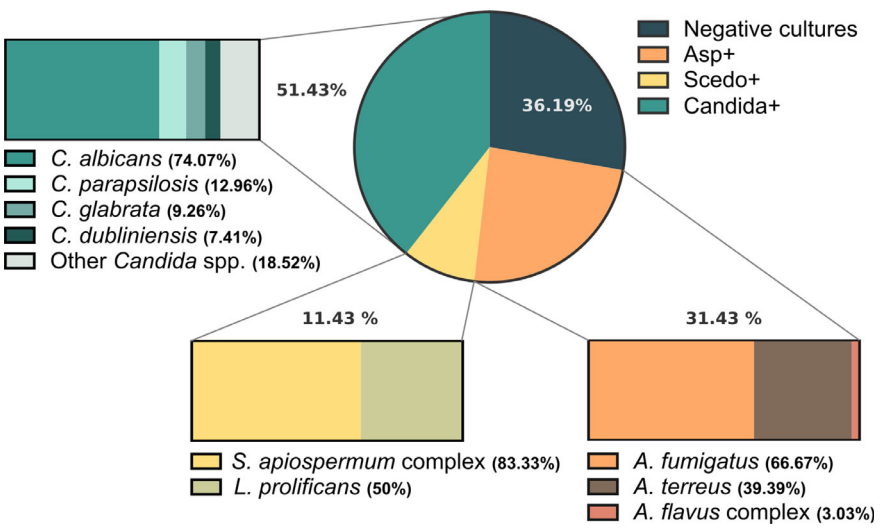


FIGURE 2 | Frequency of isolation of fungal species from respiratory samples of Cystic Fibrosis (CF) patients when culturing sputum samples on Sabouraud gentamicin chloramphenicol agar and Modified Thayer Martin agar or Sabouraud chloramphenicol cycloheximide agar for the selective recovery of *Scedosporium* species from these polymicrobial samples. The circle depicts the general prevalence of *Aspergillus* spp., *Scedosporium/Lomentospora* and *Candida* spp., as well as the frequency of patients with negative cultures for these fungi: 31.43%, 11.43%, 51.43%, and 36.19%, respectively. Moreover, the species-specific prevalence is represented within the boxes.

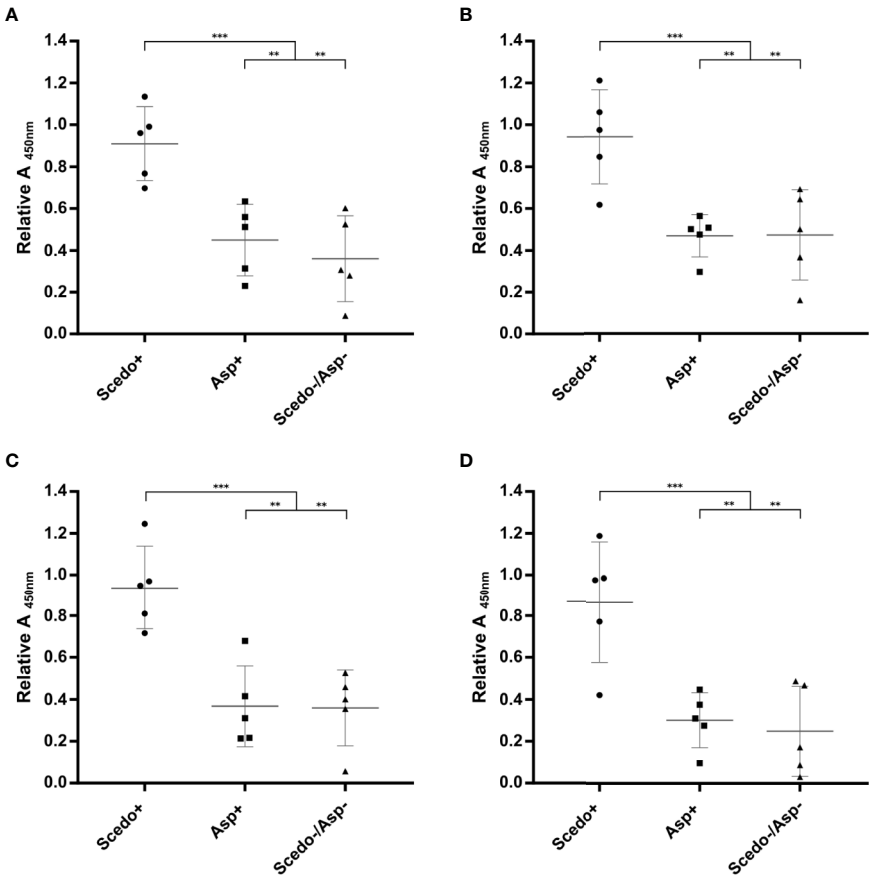


FIGURE 3 | Screening of the immunoreactivity of Cystic Fibrosis (CF) patients' sera against *Scedosporium boydii* protein extracts. Total WCP **(A)**, Secretome **(B)**, Total CSP **(C),** and Conidial CSP **(D)**. Specific IgG antibody response is represented for the five sera selected from each group (Scedo+, Asp+ and Scedo-/Asp-) and against each protein extract, by relative Abs value at 450 nm obtained for each serum. Wide horizontal lines depict the media, and short horizontal lines represent the standard deviation (SD). Median value obtained for group Scedo+ is significantly different from that obtained for each of the other two groups, and for the sum of both (as negative samples) (***$p < 0.001$; **$p < 0.01$).

required, protein concentration, and volume of useful extract, the secretome extraction method was very time-consuming and yielded a lower concentration of protein.

IgGs Cross-Reactivity Study With Total WCP Extract of *L. prolificans*, *A. fumigatus*, and *C. albicans*

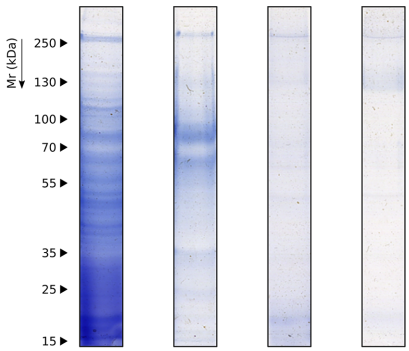
Selected sera were also tested against total WCP extract of the related species *L. prolificans*, and the most representative species of the two genera most frequently isolated in CF, *A. fumigatus*, and *C. albicans* (**Figure 5**). ELISA values showed that there is a high cross-reactivity between groups of patients when the antigens of *A. fumigatus* and *C. albicans* are used. Meanwhile, *L. prolificans* extract was able to discriminate between groups, with the differences being statistically significant. This demonstrates that there is high cross-reactivity between *Scedosporium* and *Lomentospora* because protein extracts of both fungi succeeded in discriminating Scedo+ patients (**Figure 5A**). Moreover, sera from Scedo+ groups seemed to cross react with *Aspergillus* extract (**Figure 5B**), although the contrary was not observed, as sera from Asp + did not detect Scedo+ extract at the same level (Fig 5A). In the case of the *C. albicans* extract, sera from the three groups showed high reactivity against it (**Figure 5C**).

Total WCP Extract of *S. Boydii* as a Valuable Tool for Serological Detection of *Scedosporium/Lomentospora*

In order to evaluate *S. boydii* Total WCP extract as a serodiagnostic tool for *Scedosporium*/*Lomentospora* detection, sera from the collection were tested individually using ELISA (**Figure 6**).

When plotting all the values obtained in the ELISA assay, the three categories of sera were well distinguished. Indeed, differences between the median value of the specific IgG response for group Scedo+ compared to that obtained for Asp+ and Scedo-/Asp- were statistically significant, and also differed significantly from that obtained considering all *Scedosporium* negative samples together (Asp+ and Scedo-/Asp-) (***$p < 0.001$).

Protein extract	Total CWP	Secretome	Total CSP	Conidial CSP
Time of procedure (h)	26	50	25.15	0.45
Protein concentration (µg/mL)	1745 ± 294	670 ± 73	180 ± 18	184 ± 53
Final volume of extract per 100 mL of broth culture (mL)	5	0.025	2	—*
Broth culture volume required for 1 mg extract (mL)	11.5	6.25 x 10^3	278	—*



(*) Broth culture not required, suspension of conidia directly harvested from culture plate

FIGURE 4 | Evaluation of the efficiency of the methods for obtaining the different protein extracts of *S. boydii*. Approximate performance values of extraction methods: time of procedure, final volume of useful extract, protein concentration, and quality of the extract.

The diagnostic performance of the test was assessed by conducting an ROC analysis that showed an AUC value of 0.9942, meaning a high discrimination capacity (**Figure 7**). Nevertheless, to assert whether a result was positive (detection of *Scedosporium*/*Lomentospora*) or negative, a decision threshold had to be established, so seven different criteria were used: *J*, CZ, IU, ER, $\bar{X}$ + 2*SD*, SE = SP, MLR+ (**Table 1**).

According to the above-mentioned criteria, four cut-off values were identified: Abs_{450nm} > 0.5837 using *J*, CZ, IU, and ER criteria, and Abs_{450nm} > 0.6042, > 0.6404 and > 0.7099 using SE = SP, $\bar{X}$ + 2*SD*, and MLR+, respectively. When evaluating the test performance, the SE was ≥ 86.9%, SP ≥ 93.6% and the PVN ≥ 98.4% regardless of the cut-off point selected. However, the PPV suffers a large variation, from 65%–95%, depending on the cut-off value. The K index was calculated as 0.76, 0.75, and 0.78 for the 0.5837, 0.6042, and 0.6404 cut-offs respectively, indicating a substantial agreement excluding chance. For the 0.7099 cut-off the K index calculated was 0.89 which indicates an almost perfect agreement. The cut-offs and the corresponding validation parameters are detailed in **Table 1**.

Monitoring of Specific IgG Levels Against *Scedosporium*

In order to determine the utility of the designed serological test, not only for serodiagnosis using a single sample point but also in the monitoring of CF patients, the Abs value of several serum samples corresponding to the same patient were plotted in a time-dependent manner according to the date of sample. The monitoring of patients (P) with at least three serum samples obtained over a period of more than 15 days is shown in **Figure 8** and distributed in three different graphs according to the CF group in which they were previously classified. In this way, the evolution of the humoral IgG specific response against *S. boydii* can be observed and therefore, the evolution of the disease. In fact, some interesting trends where found when looking at the patients represented.

Scedo+ monitored patients (**Figure 8A**) showed a high specific IgG response, which in all cases exceeded the cut-off values determined in this study. Specifically, P1 and P2 showed values that remained stable as clearly positive over the sampling interval. Conversely, P3 experienced an initial period of stability, but the final sample value decreased and crossed the threshold towards a negative outcome, and therefore, suggests a possible clinical improvement that should be confirmed with subsequent additional samples.

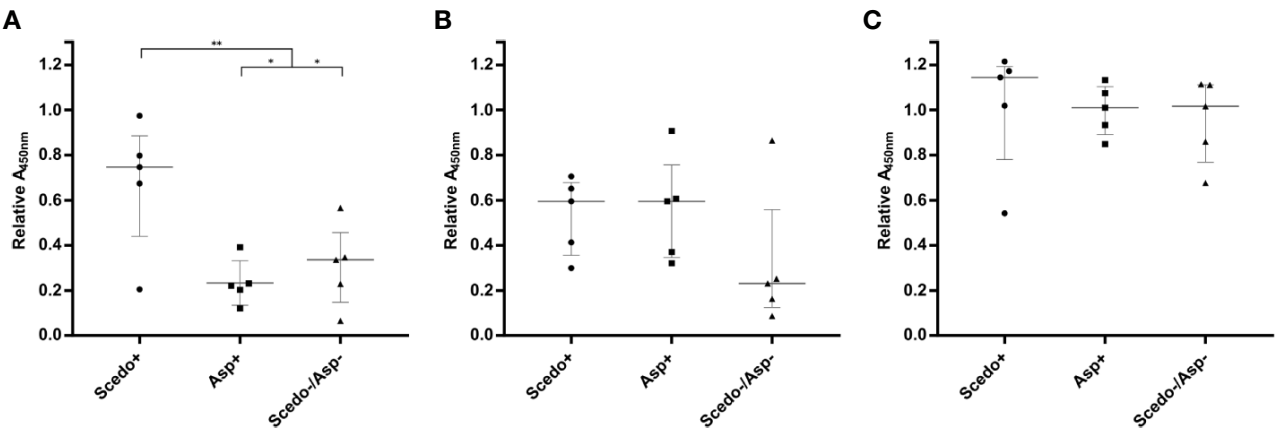


FIGURE 5 | Immunoreactivity of CF patients' serum IgGs to other fungal pathogens. Immunoreactivity of specific serum IgG against Total CWP of *L. prolificans* **(A)**, *A. fumigatus* **(B),** and *C. albicans* **(C)** is represented as the relative Abs value at 450 nm obtained for each serum. Wide horizontal lines depict the median, and short horizontal lines represent the interquartile range (IQR), except for graph A where media (SD) is represented. Comparison of the median value among Scedo+ group and each of the other two groups, and for the sum of both as negative samples (**p < 0.01; *p <0.05).

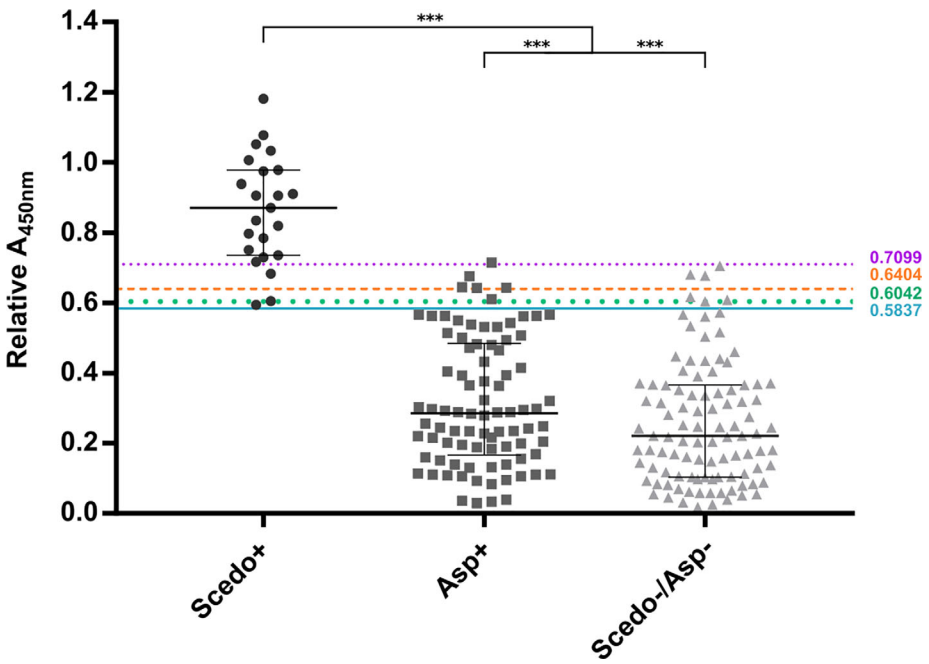


FIGURE 6 | IgG response against *Scedosporium boydii* Total WCP extract of cystic fibrosis patients. Patients with positive sputum cultures for *Scedosporium/Lomentospora* are classified into group Scedo+, with positive cultures for *Aspergillus* spp. into group Asp+, and cultures without any recovery of these fungi into group Scedo-/Asp-. Antibody responses are represented by the relative Abs value at 450 nm obtained for each serum. The four cut-off values obtained are depicted as solid, dotted and dashed lines. Wide horizontal lines depict the median, and short horizontal lines represent the interquartile range (IQR). Median value obtained for group Scedo+ is significantly different from that obtained for each of the other two groups, and for the sum of both (as negative samples) (***$p < 0.001$).

Regarding the Asp+ group (**Figure 8B**) most of the serological profiles plotted corresponded to representative patients from the group, whose values were clearly under the diagnostic threshold. Nevertheless, two of the monitored patients showed a fairly high humoral response. P6 was close to the cut-off, even in certain sample times that crossed the threshold, while P5 exhibited a serological response that matches the profile of culture-confirmed Scedo+ patients. In this sense, these patients should remain under surveillance since they are at risk of presenting an undiagnosed *Scedosporium* colonization.

Finally, the monitoring of the patients from the Scedo-/Asp- group is illustrated in **Figure 8C**. Although most of the patients maintained negative results during the sampling period, P1, P2, and P3 showed intriguing serological profiles due to the high values detected. While P6 started with values close to the threshold but eventually developed a downward trend, P2 and P3 started the sampling with clearly negative results, however, high positive values were obtained in later samples. Which in turn, indicates that these patients, and especially P3, need to be carefully monitored since they may have an undetected presence of *Scedosporium*.

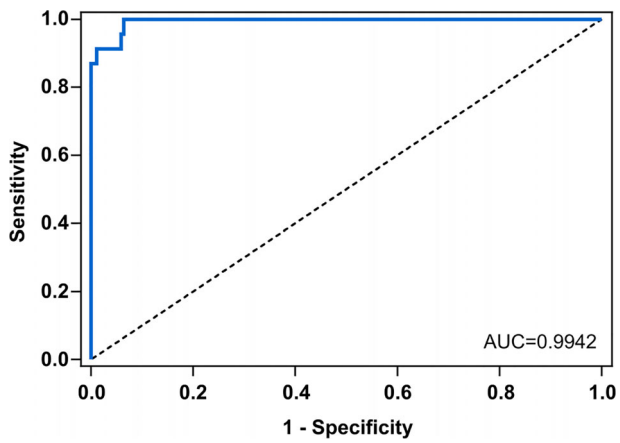


FIGURE 7 | Receiving operating characteristics curve (ROC) for anti – *Scedosporium boydii* Total WCP IgG ELISA. Scedo+ patients vs. negative patients (Asp+ and Scedo-/Asp-). The area under the curve (AUC) for *S. boydii*-specific IgG was 0.9924. Line of identity is indicated with the dashed line.

DISCUSSION

The range of fungal species detected in CF patients continues to increase in line with new discoveries in diagnostic methodologies (Tracy & Moss, 2018). The development of selective culture media has become a cornerstone for improving the classic gold standard of culture-based diagnosis, enhancing the recovery of low prevalence and/or slow growing fungal pathogens (Pham et al., 2015; Hong et al., 2017; Coron et al., 2018). However, there are still huge limitations that hinder the correct detection of these

TABLE 1 | Performance of *Scedosporium boydii* specific IgG at different cut-off values with 25 serum samples of Cystic Fibrosis patients with *Scedosporium/Lomentospora* positive sputum cultures and 190 negative sera.

Cut-off value (Relative A_{450nm})	Criteria	Test validation parameters (%)					
		SE	SP	EFF	PPV*	NPV*	K
0.5837	*J*, CZ, IU, ER	100	93.58	94.29	65.71	100	0.76
0.6042	SE=SP	95.65	94.12	94.29	66.67	99.44	0.75
0.6404	$(\bar{X} + 2SD)$	91.30	95.72	95.24	72.41	98.90	0.78
0.7099	MLR+	86.96	99.47	98.10	95.24	98.41	0.89

J, Youden Index; CZ, Concordance Probability Method; IU, Index of Union; ER, Closest to [0,1]; $\bar{X}$, mean value of control sera absorbance; SD, standard deviation; MLR+, maximum positive likelihood ratio; SE, sensitivity; SP, specificity; EFF, efficiency or correct classification rate; PPV, predictive value of positive test; NPV, predictive value of negative test; K, Cohen's Kappa index.

(*) Theoretical values considering a prevalence of Scedosporium/Lomentospora of 11.43% observed in this study.

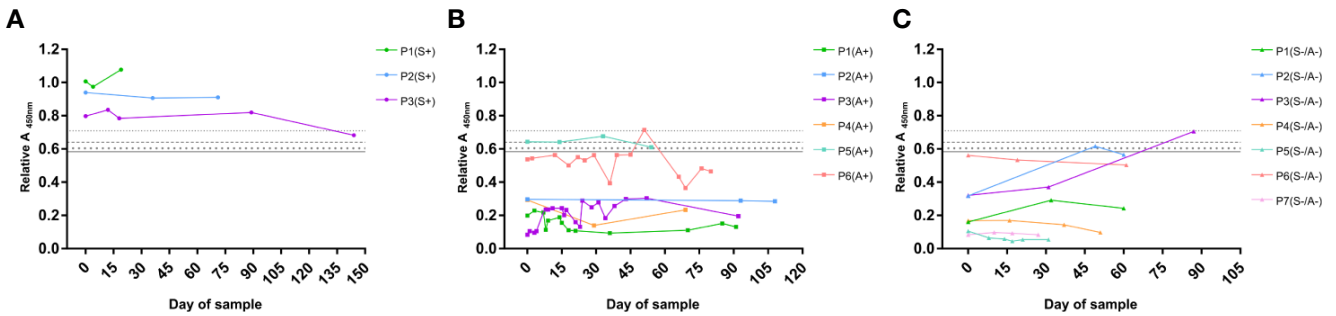


FIGURE 8 | Monitoring of specific IgG serological response of CF patients against *S. boydii*. Example of representative patients of the Scedo+ group **(A)**, Asp+ group **(B)**, and Scedo-/Asp- group **(C)**. Relative Abs values of the ELISA test using a total WCP of *S. boydii* are depicted on a time scale.

fungi, such as the lack of standardized guidelines for processing respiratory samples and the absence of commercial available culture media designed for these challenging isolations (Chen et al., 2017). On the other hand, serological tests have led to non-culture-based diagnosis of fungal infections since the 1950s because of their advantages, such as the ease of minimally invasive sample collection (Richardson and Page, 2018). Unfortunately, at present, there is a lack of specific serological kits for low-prevalence fungal pathogens that enable rapid and easy detection.

For many years, efforts have been focused on *Aspergillus fumigatus* as it is the most prevalent filamentous fungi in CF airways (Richardson & Page, 2017). In this sense, crude antigenic extracts from conidia and hyphae have been widely used for *Aspergillus* serological detection (Page et al., 2016). On the other hand, species from *Scedosporium* genus are gaining more and more attention because of their high chronicity in CF airways as well as their associated pathogenicity. However, despite their disturbing clinical relevance, accurate detection methods that allow an adequate diagnosis of these threatening pathogens is currently lacking (Chen et al., 2017). Mycological culturing continues to be the most widespread method. This requires the use of selective media that are not available everywhere, and incubation times for these slow-growers are excessively long (Coron et al., 2018). This problem is reflected in the epidemiological studies that suffer worrying variations in the prevalence rates, caused to some extent, by the use of different and non-standardized procedures. In fact, there is a growing awareness of this issue and this has led to the publication of research into the frequency variation depending on the detection method employed (Borman et al., 2010; Sedlacek et al., 2015; Hong et al., 2017; Boyle et al., 2018; Hedayati et al., 2019). These diagnostic hurdles in turn result in delayed diagnosis, and consequently, a late introduction of an effective treatment. Therefore, considering the mounting concern about the critical need for standardized and reliable detection methods, efforts must be made to develop rapid and robust tests to ensure an early detection of *Scedosporium*, and consequently the establishment of an effective treatment and patient monitoring. With this in mind, the aim of this study was to analyse the utility of different protein extracts of *S. boydii* for a serological detection of *Scedosporium/Lomentospora* in CF patients by ELISA.

To achieve this, 212 serum samples corresponding to 105 CF patients were used. Patients were classified into three groups, Scedo+, Asp+, and Scedo-/Asp-, based on the fungus isolated from the sputum, *Scedosporium, Aspergillus*, or neither, respectively. Mycological cultures showed that *Candida* was the most frequently isolated fungus among yeasts, and *Aspergillus* among filamentous fungi. Nevertheless, *Scedosporium* species were isolated from 11.43% of CF patients' sputa, these results were in agreement with the prevalence rates of 8%–16% published in different epidemiological studies (Cimon et al., 2000; Blyth et al.,

2010; Schwarz et al., 2017). Moreover, species of *S. apiospermum* complex were isolated in 85.33% of Scedo+ patients, which is consistent with the *Scedosporium* species distribution in CF, since *S. apiospermum* and *S.boydii* are the most frequently isolated species (Bouchara et al., 2019). Finally, *L. prolificans* was detected in 50% of Scedo+ patients, which is a notoriously high prevalence rate when compared with the worldwide incidence data (0%–40%), but it is in concert with the geographical restriction of the fungus, Spain and Australia being the countries with the highest incidence (Seidel et al., 2019).

The first step in the design of the ELISA for the serological detection of *Scesdosporium/Lomentospora* was the selection of an easy-to-obtain protein extract with a good level of discrimination. Some research has deciphered potential *Scedosporium* virulence markers (reviewed in Santos et al., 2009) with different cellular locations that are related to both morphological phases (hypha and conidium). Bearing in mind the wide variety of antigens, the immunoreactivity and discriminatory capacity of four *S. boydii* protein extracts, which included total protein extract (Total WCP), secretome extract, cell surface associated proteins (Total CSP), and cell surface associated proteins only from conidia (Conidia CSP), were compared by indirect ELISA against fifteen patients (five from each CF group). The results obtained with the four extracts showed that they were able to discriminate Scedo+ sera from Asp+ and Scedo-/Asp- groups. However, of the four extracts, Total WCP was selected to continue with the study as it was the one with the highest output in terms of protein concentration per mL of fungal culture used, and the discriminatory capacity was the best because none of the five patients tested in the Scedo+ group overlapped with any of the other two groups.

Among the discarded extracts, secretome was expected to be interesting because of the chronicity of *Scedosporium* colonizations of CF airways and the specificity of the secreted proteins according to bibliography (Santos et al., 2009; Bertrand et al., 2010). Moreover, metalloproteases such as superoxide dismutase, some proteolytic enzymes, as well as ectophosphatases are secreted to the external environment and play a protective role for the fungus and orchestrate the cleavage of key host components (Larcher et al., 1996; Da Silva et al., 2006; Da Silva et al., 2012). In agreement with this idea, the results obtained using this extract were very good, but similar to the ones obtained with the Total WCP and, additionally, there were some problems with gathering high protein concentrations, a long and complex process was necessary to obtain an extract with low yields.

Regarding Total CSP and Conidial CSP, it is well known that some cell wall associated proteins of *Scedosporium*, such as glucans, peptidorhamnomanns and glucosylceramides, show a high immunoreactive capacity (Pinto et al., 2004; Da Silva Xisto et al., 2019). Moreover, since the typical entry of the fungus into the host body is through inhalation of conidia, it is reasonable for proteins of the cell wall of the conidium to play an important role in the host-pathogen interactions and colonization/infection of airways (Buldain et al., 2016). Consequently, as with the secretome, the results obtained were also good but yielded low protein concentration and in addition, some overlapping between groups with the Conidial CSP was observed. This cross-reactivity could be explained by the fact that some surface proteins of the conidia are common to different moulds, are important for the survival of the fungus, and possess immune-modulatory functions (Voltersen et al., 2018).

Hence, further experiments were carried out using Total WCP. Crude antigenic extracts have been widely used in immunodiagnostic systems, some of them being currently commercially available for the detection of *Aspergillus* (Page et al., 2016; Richardson and Page, 2017). The advantage of these kinds of extract is that they involve every interesting antigen. On the contrary, one of their main handicaps is their cross-reactivity against panfungal antigens of closely related fungi (Buldain et al., 2019). In this sense, biochemical studies try to characterize specific proteins for diagnostic purposes, but as little is known about the physiology and biochemistry of *Scedosporium*, and the number of studies performed in the field is small, only a few proteins of interest have been characterized. Among these are a serine protease from the subtilisin family, two enzymes involved in ROX detoxification, cytosolic Cu,Zn-superoxide dismutase, a catalase and some heat shock proteins (Santos et al., 2009).

Moreover, the potential of crude antigenic extracts of *Scedosporium/Lomentospora*, which is the first step in the serodiagnosis race, has not yet been evaluated. In this way, Total WCP was tested against the totality of sera by the same optimized ELISA assay, and the results show that it was able to discriminate the Scedo+ patients successfully. In this sense, ROC AUC analysis possesses a good discriminatory capacity for the test (AUC=0.9942) but assessing the criteria for cut-off determination (Habibzadeh et al., 2016; Unal, 2017), four threshold values were represented. The most affected parameter was the theoretical PPV*, because a PPV* of 65% was obtained with the lowest threshold values but it increased to 95% in the highest one. Theoretical NPV*, efficiency and specificity values remained more or less stable in all the cases, while sensitivity varied from 86%–100% with the highest and the lowest cut-offs, respectively. Despite the variations observed, the ELISA assay showed a remarkably high specificity, which means an absolute capacity to discriminate CF patients with no presence of *Scedosporium*. Moreover, it was observed that being stricter with the selection of the cut-off value resulted in improved ability to detect *Scedosporium* positive patients. Nevertheless, choosing one cut-off value may be risky considering the limited number of Scedo+ samples. In this sense, increasing this sample population might be helpful to establish a definitive cut-off point.

Turning to the commercial kits for *Aspergillus* detection that show a 75%–96% sensitivity (Richardson and Page, 2017), the developed test exhibits a remarkable clinical usefulness considering that values of 86 - 100% of sensitivity and 93%–99% of specificity were obtained. Moreover, it is worth mentioning that *Lomentospora*- positive culture patients also showed a specific response against *S. boydii* extract, so the developed ELISA platform is a valuable tool to detect the intrinsic multi-resistant fungus *L. prolificans* as well. In the last few years, other authors have made efforts to shed light on

serodiagnosis of *Scedosporium*. In fact, Bouchara and co-workers in 2017 developed an ELISA with two recombinant proteins, *Scedosporium* catalase A1 and cytosolic Cu,Zn-superoxyde dismutase, described as antigens with diagnostic utility in *Aspergillus*. In this study, they managed to detect *Scedosporium* infections, and differentiate it from an *Aspergillus* infection. Moreover, precipitin assays can be performed but they take up to one week for the results to be obtained, lack sensitivity (Fujiuchi et al., 2016), and can only be performed in a few specialized laboratories (Cimon et al., 2000).

Finally, the study of the ELISA test as a patients' monitoring tool showed the value of the technique for the observation of the evolution of the fungal presence since the humoral response of the patient can be tracked over time, although this point should be studied in more depth in the future. Nevertheless, the best diagnosis is the variety of tests with different purposes that complement each other and offer a real vision of the patient's condition and evolution. In this sense, our research group aimed to explore new diagnostic resources by developing an indirect ELISA test using *S. boydii* whole cell protein extract. Detecting humoral response against *Scedosporium* in CF patients regardless the result of the culture may be helpful for clinicians to maintain these patients under surveillance, and to anticipate the establishment of their antifungal treatment.

CONCLUSIONS

In this study a crude antigenic extract of *S. boydii* was selected to detect *Scedosporium/Lomentospora* serologically. The ELISA test developed is able to detect the *Scedosporium* spp. and *L. prolificans* in CF patients' sera, with a very high sensitivity and specificity, up to 100% and 99%, respectively. Thus, this ELISA platform offers a rapid, low-cost and easy solution to detect these elusive fungi through minimally invasive sampling, with high output and specificity, and allows the monitoring of the evolution of the infection, the recovery and the effectiveness of the antifungal therapy. In spite of the potential of these results, more research is needed in this field to detect specific antigens from the fungal extract to improve sensitivity and specificity, to minimize cross-reactivity with other closely related fungal pathogens, and ultimately to reach a standardized method. Nevertheless, it is worth bearing in mind that the best diagnosis is achieved with a combination of methods that allow a complete vision of the infection.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee from the UPV/EHU. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LM-S, IB, LA-F, and MA carried out the experiments. J-PB and MM-G obtained and classified the sera samples. AR, FH and AR-G conceived the experiments and supervised the work. LM-S, AR, FH, and AR-G analyzed the data. LM-S and AR-G wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Frequent Pet Contact as Risk Factor for Allergic Bronchopulmonary Aspergillosis in Cystic Fibrosis

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Aspergillus fumigatus (*Af*) frequently colonizes the respiratory tract of patients with cystic fibrosis (CF). *Af* is associated with loss of pulmonary function and allergic bronchopulmonary aspergillosis (ABPA), a hypersensitivity fungal lung disease. Environmental factors have impact on CF patients' lung function variation. The aim of this nationwide questionnaire survey was to investigate the amount of CF patients with frequent pet contact including pet species and to examine the potential impact of frequent pet contact on the occurrence of *Af* colonization and ABPA diagnosis in these patients. The survey was carried out in 31 German CF centers in 2018. A total of 1232 who completed the surveys were included, and statistical analysis was performed by chi-squared test. Within the study cohort 49.8% of subjects (n = 614; CF patients < 18years: 49.4%, n = 234; ≥ 18years: 50.1%, n = 380) reported frequent contact to pets, of which 60.7% reported frequent contact to dogs, 42.3% to cats and other animals. Of those with frequent pet contact, 71.8% (n = 441) had contact to one pet or more pets from the same family. *Af* colonization was not significantly associated with frequent pet contact. ABPA diagnosis was documented in 16.7% (n = 206) of all included CF patients and was significantly associated with frequent pet contact (18.9%, n = 116, p = 0.042), confirming previous single center examinations. Particularly, patients with frequent contact to dogs showed an increased ABPA prevalence of 21.3%. Frequent pet contact might be a risk factor for ABPA. CF patients who are sensitized to *Af* should be informed about the increased risk to develop an ABPA by frequent pet contact. Patients with recurrent onset of ABPA should be evaluated in terms of frequent pet contact.

Keywords: aspergillosis, *Aspergillus fumigatus*, allergic bronchopulmonary aspergillosis, respiratory infection, pet, cystic fibrosis, cat, dog

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INTRODUCTION

Cystic fibrosis (CF) is a life-limiting recessive genetic disease. Mucus retention, chronic infections and inflammation in the airways lead to progressive respiratory impairment (Elborn, 2016). Beside bacterial species, fungal colonization is commonly observed in the respiratory tract of patients with CF (Ziesing et al., 2016). *Aspergillus fumigatus* (*Af*) is the most common filamentous fungus in CF

(Ziesing et al., 2016; Schwarz et al., 2018). *Af* colonization is more common in adolescence and adulthood (Pihet et al., 2009; Warris et al., 2019). The respiratory tract of 10.3 to 60 % of CF patients is colonized by *Af* (Pihet et al., 2009; Ziesing et al., 2016; Warris et al., 2019; Hong et al., 2020). The presence of this environmental filamentous fungus in CF sputum is associated with worse respiratory quality of life (Hong et al., 2020). Inhaling *Af* spores into the lungs may cause multiples diseases including invasive pulmonary aspergillosis, aspergilloma (Mousavi et al., 2016) and growth of *Af* hyphae within the bronchial lumen triggers an immunoglobulin E (IgE)-mediated hypersensitivity response that results in airway inflammation, bronchospasm, and bronchiectasis (Armstead et al., 2014; Janahi et al., 2017). ABPA has a distinct Th-2 mediated pathophysiology and is associated with accelerated lung function decline (Tracy et al., 2016; Hong et al., 2020). ABPA is a frequent event in patients with CF (Tracy et al., 2016), with an age dependent occurrence (Maleki et al., 2020) and a prevalence of 3 to 25 % (Mastella et al., 2000; Patel et al., 2019; Maleki et al., 2020). Differences on reported rates of *Af* colonization and ABPA diagnosis might be influenced by regional variation in environmental load of *Af*, therapeutic regimes, seasonal or annual variation, the origin of samples (Ziesing et al., 2016). ABPA is challenging to diagnose and remains underdiagnosed in many patients (Janahi et al., 2017). ABPA is associated with increased lung function decline, more frequent hospitalizations and significant CF morbidity (Maturu and Agarwal, 2015; Keown et al., 2019). 45% of households in Germany have pets (Heimtierhaltung, 2018). Human contact with cats, dogs, and other pets results in several million pet-related infections each year. These parasitic, fungal, bacterial, viral or arthropod dependent infections range from self-limiting skin conditions to life-threatening systemic illnesses (Rabinowitz et al., 2007). Environmental factors have been shown to impact respiratory health. Exposure to environmental allergens like pet dander has been associated to worse respiratory outcome in other lung diseases such as asthma and pets may be potential sources for methicillin-resistant *Staphylococcus aureus* (MRSA) infection (Morrow et al., 2014). With regard to CF, case reports describe interspecies transmission of *Pseudomonas aeruginosa* (Mohan et al., 2008) and *Bordetella bronchiseptica* (Ner et al., 2003; Register et al., 2012) between pet cat/pet dog and CF patients. Furthermore, questionnaire data highlighted the association between cat ownership and higher frequency of nasal polyps as well as combined cat–dog ownership and higher rate of wheezing in CF patients (Morrow et al., 2014). The ubiquitous fungus *Af* possesses versatile features enabling them to survive in various environmental conditions, with a wide range of hosts including humans and animals (Mousavi et al., 2016). Retrospective single center data analysis reveals that ABPA is associated with pet ownership in CF (Thronicke et al., 2016). Therefore, pet ownership might pose a potential risk to patients with CF. From the perspective of preventive medicine, reservoirs of *Af* and the potential origin of infection in CF patients should be evaluated. Because of the difficulties of recognizing ABPA in the context of CF, due to overlapping clinical, radiographic, microbiologic and immunologic features (Stevens et al., 2003), advances in the understanding of possible risk factors may have a positive effect on patient prognosis. Limited work has been done examining fungal infections in CF regarding frequent pet contact. This questionnaire survey was conducted to determine frequent pet contact and pet species in CF, to examine the relationship between frequent pet contact and *Af* colonization as well as ABPA diagnosis in children and adult patients with CF.

MATERIALS AND METHODS

Design and Development

This questionnaire survey was conducted at 31 German CF centers in 2018. The target population was patients with CF living in Germany. CF patients were recruited by their CF center team during their hospital stay independent of age and clinical status. Participation was voluntary. Ethical aspects were considered, and approval for the study was gained by the Ethics Committee of the Charité–Universitätsmedizin Berlin (EA2/057/18).

The questionnaire "Risk factors for *Af* infection in CF patients" included several items, which are not documented in the medical data base German Cystic Fibrosis Registry. Contact to pets (during last 12 months; several times per week, no/rare contact) and pet species were queried per questionnaire beside patient's age (years) and sex (male, female, intersex), *Af* colonization within the last 12 months (negative, positive, multiple positive tests, unknown) and history of ABPA (negative, positive, unknown). For a positive of *Af* colonization within the 12 month of observation period, at least one positive microbiological indication was required. The ABPA diagnosis was determined in every single CF center and was based on the minimal 2003 Cystic Fibrosis Foundation (CFF) consensus criteria (Stevens et al., 2003). No other *Af* associated phenotypes were considered. Besides *Af*, no other *Aspergillus* spp. were included. Behavioral patterns, hygiene routines, onset, or history of pet contact and clinical data were not investigated. Only the part with respect to pet contact has been separately analysed for this manuscript. The questionnaire was sent to 94 CF treating centers in Germany in 2018 and data of 1,477 patients have been received from 31 CF centers.

Statistical Analysis

Only completed questionnaires with distinct information on *Af* colonization, ABPA diagnosis and contact to pets were included in the final analysis. Frequent pet contact was defined as contact to animals/pets several times per week. Patients with no or rare animal/pet contact, *i.e.* less than once a week, were categorized as "no pet". In the analysis, it was not distinguished whether the patients had contact to one animal or several animals of one species (*e.g.* fish). No scientific classifications were included for pets. In some cases, no further pet specifications were available, *e.g.* fish/aquarium, bird, reptile, or wild animal. Prevalence determination for single animal species was only performed for dogs and cats, due to case numbers. Means and standard deviations were calculated metrical variables. Frequency and percentage were used for categorical variables. Associations of categorical variables were analyzed with chi-square test. Since the

study is exploratory no level of significance is specified, and the p-values are not Bonferroni adjusted. A p-value ≤0.05 is considered to be significant even if the first kind familywise error rate is not bounded by 5%. All figures were created using Graphpad Software, Inc. GraphPad Prism 8.4.0. All statistical analyses were performed using IBM SPSS Statistics Version 24 and Graphpad Software, Inc. GraphPad Prism 8.4.0.

RESULTS

A total of 1232 completed questionnaires were analyzed. Patients' characteristics were shown in **Table 1**. 600 patients with CF (48.7%) were female. The mean age was 23 ± 14 years. Prevalence of 29.9% was found for *Af* and was significantly higher in adult patients (36.9%) compared to patients <18 years (18.6%; p < 0.001). Prevalence of 16.7% was found for ABPA. Likewise *Af* colonization, significantly higher values were observed in adults (22.7%) compared to children (7.2%; p < 0.001). N = 614 (49.8%) of the CF patients had frequent contact to pets. Among those, 234 patients were <18 years and 380 patients were ≥18 years of age. No difference was obtained in the frequency of frequent contact to pets in children (49.4%) and adults (50.1%; p = 0.794; **Table 1**).

Of those with frequent contact to pets, 71.8% (n = 441) had contact to one pet (or more pets from the same family, *e.g.* fish; **Table 2**), 18.7% (n = 115) had contact to two pets and 7.0% (n = 43) had frequent contact to three up to five different pets. The animals mentioned in the questionnaire were listed in **Table 2**. The majority of CF patients had frequent contact to dogs (60.7%) and cats (42.3%), followed by horses and rabbits (**Table 2**).

The association of Af colonization with frequent pet contact missed significance (no pet n = 175; 28.3 %; Figure 1A). Af prevalence was similar for CF patients with frequent contact to pets (n = 193; 31.4 %; p = 0.232), with contact only to a single pet (n = 135; 30.6 %; p = 0.418), only dog pets (n = 71; 29.6 %; p = 0.713) or cat pets (n = 49; 32.2 %; p = 0.629; **Figure 1A**). In children a non-significant increase from 17.1 % (n = 41) to 20.1 % (n = 47) of Af colonization was observed in the context of frequent pet contact (p = 0.401; **Figure 1A**). In adults, Af prevalence increased from 35.4 % (no pet n = 134) to 38.2 % (pet contact n = 146), without reaching any significance (p = 0.397; **Figure 1A**).

ABPA was significantly more pronounced in patients with frequent pet contact (no pet n = 90; 14.6 %; pet contact n = 116; 18.9 %; p = 0.042; single pet n = 87; 19.7 %; p = 0.026; **Figure 1B**), in patients with frequent contact only to dogs (n = 51; 21.3 %; p = 0.018) and in adult patients with frequent pet contact (no pet n = 73; 19.3 %; pet contact n = 99; 26.1 %; p = 0.027; **Figure 1B**). No significant difference for ABPA diagnosis was observed in patients <18 years with frequent pet contact (no pet n = 17; 7.1 %; pet contact n = 17; 7.3 %; p = 0.939) and for patients with frequent contact only to cats (n = 25; 16.4 %; p = 0.559; **Figure 1B**).

DISCUSSION

The present study explores frequent contact to pets, *Af* colonization and ABPA diagnosis in CF patients. 1232 patients with CF were included in the analysis. *Af* was documented in 368 and ABPA in 206 CF patients, both with significantly higher occurrence in adult individuals. Frequent pet contact was not found to be associated with *Af* colonization but was significantly associated with ABPA. Notably, the rate of frequent pet contact was similar in children and adult patients with CF. Our findings contribute to the limited literature on frequent pet contact and fungal infections in CF. A retrospective single centre analysis, including 55 pet owners, examined the association of ABPA with pet ownership (Thronicke et al., 2016). Due to the limited sample size, no correlation for ABPA (11 cases) in various pet groups were found (Thronicke et al., 2016). A wide range of animal species is kept as pets by CF patients, with dogs (60.7%) and cats (42.3%) as the most frequent ones (**Table 2**). This trend corresponds to the general tendency published elsewhere (Damborg et al., 2016). Environmental factors have been shown to impact respiratory health in CF (Morrow et al., 2014). After parasitic infections, fungal skin infections from cats and dogs are probably the most common pet-associated diseases (Rabinowitz et al., 2007). Case reports describe interspecies transmission of bacteria between cats/dogs and CF patients (Ner et al., 2003; Mohan et al., 2008; Register et al., 2012). Morrow et al. investigated cat and dog exposure in 703 CF patients (Morrow et al., 2014): 47.2% reported dog, and 28.1% reported cat ownership (Morrow et al., 2014). Combined cat–dog ownership was associated with wheezing, but no differences in lung function, self-reported environmental allergies, or ABPA were reported (Morrow et al., 2014). Here, a significant association between frequent contact to dogs and ABPA was observed (**Figure 1**). Due to missing information in the questionnaire, we cannot distinguish between different dog breeds or short/long haired dogs. Pet dogs have been considered to

TABLE 1 | Patients characteristics.

	All CF patients	CF patients <18 years	CF patients ≥ 18 years	P value
	1232 (100.0%)	474 (38.5%)	758 (61.5%)	
Female sex, n (%)	600 (48.7%)	236 (49.8%)	364 (48.0%)	0.546
Age (years), mean ± SD (range)	23 ± 14 (0–88)	9 ± 5 (0–17)	31 ± 10 (18–88)	
Af colonization, n (%)	368 (29.9%)	88 (18.6%)	280 (36.9%)	<0.001
ABPA, n (%)	206 (16.7%)	34 (7.2%)	172 (22.7%)	<0.001
Frequent contact to pets, n (%)	614 (49.8%)	234 (49.4%)	380 (50.1%)	0.794

TABLE 2 | Pet contact in CF.

	Including all CF patients with frequent contact to pets, independent of the number of different pets (multiple choices possible) n = 614			Only CF patients included with frequent contact to a single pet or pets from one category (no multiple choices) n = 441		
	Number of CF patients, n (%)	*Af* colonization, n (%)	ABPA, n (%)	Number of CF patients, n (%)	*Af* colonization, n (%)	ABPA, n (%)
Dog, cat						
Dog	373 (60.7%)	118 (31.5%)	70 (18.7%)	240 (54.4%)	71 (29.6%)	51 (21.3%)
Cat	260 (42.3%)	92 (35.4%)	40 (15.4%)	152 (34.7%)	49 (32.2%)	25 (16.3%)
Rabbit, rodent[a]	68 (11.1%)	21	6	24 (5.4%)	9	2
Horse, cattle, sheep, goat	46 (7.5%)	18	8	9 (2.0%)	1	3
Reptile, amphibian, spider[b]	26 (4.2%)	11	9	6 (1.4%)	5	4
Bird[c]	18 (2.9%)	7	4	7 (1.6%)	0	1
Fish[d]	10 (1.6%)	2	1	2 (0.5%)	0	0
Other[e]	2 (0.3%)	1	1	0	0	0

[a]*Rabbit, Hamster, Guinea pig, Gerbil, Mouse, Chinchilla.*
[b]*Tortoise, Frog, Reptile (no further specification), Chameleon, Iguana, Lizard, Newt, Snake, Spider.*
[c]*Budgerigar, Bird (no further specification), Chicken, Duck, Parrot.*
[d]*Fish, Aquarium (no further specification), Shrimp/Prawn.*
[e]*Hedgehog, Wild animal (no further specification).*

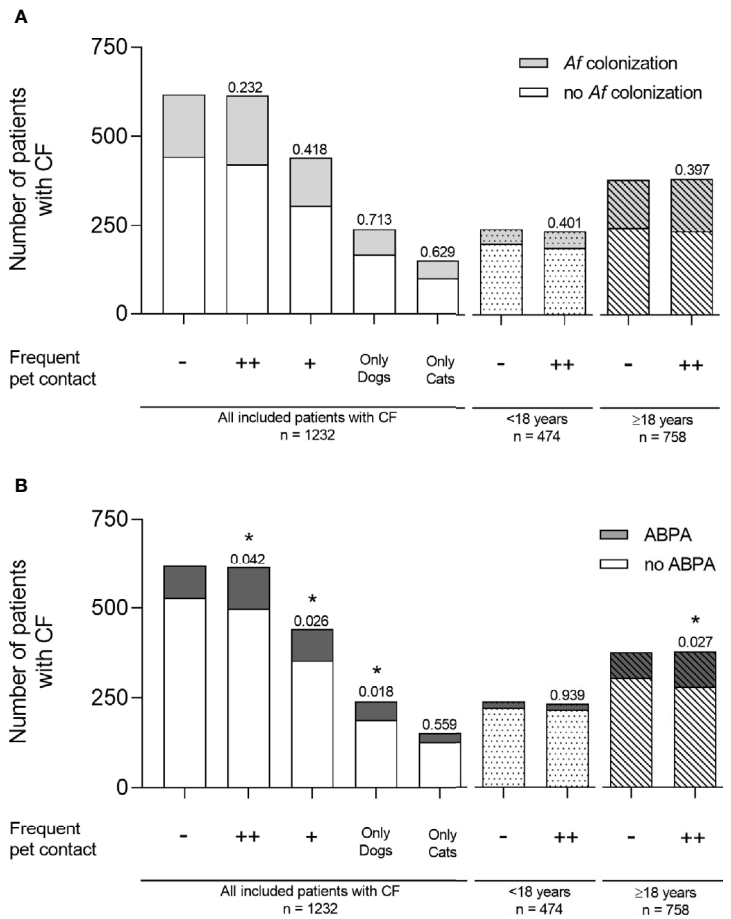

FIGURE 1 | *Af* colonization and ABPA diagnosis in CF patients with or without frequent contact to pets. **(A)** *Af* colonization (light gray) and **(B)** ABPA diagnosis (dark gray) were shown for all included CF patients with no frequent pet contact (–), with frequent pet contact to one or more (++) different pets, with contact to only one pet (+), and with frequent contact to dogs only or cats only. Bars without fill pattern represent all included patients with CF. Dotted bars represent children aged under 18 years and striped bars represent adult patients ≥18 years. Significance levels of *$p < 0.05$ were compared to controls with no frequent pet contact. Statistical analysis was performed with chi-square test. Data are shown as absolute numbers.

be involved in the contamination of indoor air by serving as a source of providing molds at houses (Jang et al., 2007). Both from skin and hairs, *Aspergillus* spp. was the most commonly found genus in dogs with isolation rates of 25% (Jang et al., 2007). Due to sample size limitations, other single pet groups were not included in statistical analysis (**Table 2**). In contrast to frequent pet contact, *Af* colonization and ABPA diagnosis were significantly pronounced in adult CF patients in this study (**Table 1**). In this nationwide, multicenter sample of CF patients, 49.8% reported to have frequent pet contact, mostly with one pet or pets from the same family (**Table 1**). This is slightly higher than documented in the general German population (45%) (Heimtierhaltung, 2018). Maybe, this circumstance is due to the fact, that frequent contact to pets has been associated with both emotional and physical health benefits (Carmack, 1991; Hemsworth and Pizer, 2006) especially in chronic illness and long-term conditions (Brooks et al., 2013). Furthermore, potential bias could result from particular interest of patients with frequent pet contact to participate in this study. Domesticated animals can affect the indoor microbiome by introducing exogenous microbial members into buildings (Leung and Lee, 2016). Pets may also act as vectors for various infectious agents (Rabinowitz et al., 2007). Close contact between pets and people offers favorable conditions for transmission by direct contact (*e.g.* petting, licking) or indirectly through contamination of domestic environments (Damborg et al., 2016). Even asymptomatic animals may transmit infections (Rabinowitz et al., 2007). To identify the reservoirs of *Af*, and thus a possible origin of infection in patients (Pihet et al., 2009) health care professionals should actively enquire about household pets and provide accurate information and practical advice on how to minimize the risk of infection (Hemsworth and Pizer, 2006; Rabinowitz et al., 2007). Although few studies have assessed the effectiveness of such measures, specific prevention guidelines involve common-sense measures, such as adequate handwashing and proper disposal of animal waste (Rabinowitz et al., 2007). Regarding high risk patients (*e.g.* immunocompromised patients including organ transplant recipients), contact with reptiles, including turtles, lizards, snakes as well as exotic and sick pets should be avoided (Rabinowitz et al., 2007). However, the benefits of the human-animal bond must be considered. Health care providers should be sensitive to the emotional attachment between patients and their pet and the psychological benefits of frequent pet contact (Carmack, 1991). With proper handling immunocompromised patients should be able to continue enjoying the significant benefits of frequent pet contact (Hemsworth and Pizer, 2006). In contrast to other studies (Morrow et al., 2014; Thronicke et al., 2016) the intensity of contact to pets was assessed and was defined as frequent contact, several times per week. This was implemented since the development of *Af* disease conditions is dependent on prolonged pathogen–host-interactions (Mousavi et al., 2016). Furthermore, a large sample size of 614 patients with frequent pet contact, including different age groups, was analyzed, and the number and species of pets present in the household was documented. The most significant limitation of our study is the absence of clinical data like lung function, serological markers, or allergy measures. Survey associated bias has to be considered. Moreover, the common behavioral patterns and hygiene routines associated with contact to pets were not enquired. Finally, the onset of pet exposure or history of pet contact was not collected. The potential influence of other environmental factors like different residential area types, will be discussed in detail in a separate article of this thematic special issue (see "*Urban life as risk factor for aspergillosis*"). Frequent contact to pets should be queried actively during clinical visits, and CF patients should be informed about the risk to develop an ABPA. Especially CF patients with recurrent onset of ABPA should be examined in terms of frequent contact to pets.

CONCLUSION

Frequent pet contact might be a risk factor for ABPA in patients with CF. These results should be included in patient guidance and preventive measures, especially for *Af* sensitized patients or patients with recurrent ABPA.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Charité–Universitätsmedizin Berlin (EA2/057/18). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

CG, PE, ST, UD, KN, and CS contributed to the conception and design of the study. UD and CG organized the database. KN and CG performed the statistical analysis. CG, PE, ST, UD, KN, and CS wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Comparative Analysis of Clinical Parameters and Sputum Biomarkers in Establishing the Relevance of Filamentous Fungi in Cystic Fibrosis

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Background: The relationship between fungal culture (FC) positivity and airway inflammation in CF is largely unknown. Identifying the clinical significance of filamentous fungi in CF using both clinical parameters and biomarkers may change our antimicrobial therapeutic strategies.

Objectives: To investigate the clinical characteristics and airway biomarker profile in relation to the detection of filamentous fungi in respiratory samples obtained from CF patients.

Methods: A prospective cohort study over 24 months, including children and adults with CF. Participants provided sputum and/or bronchoalveolar lavage samples, which underwent processing for bacterial and fungal culture, leukocyte differential cell count and biomarker analysis for neutrophil elastase (NE), interleukin-8 (IL-8), galactomannan and tumor necrosis factor receptor type 2 (TNF-R2). We performed FC using neat sputum plugs, an approach shown to be more sensitive compared to routine laboratory testing.

Results: Sixty-one patients provided 76 respiratory samples (72 sputum and 4 BAL). Median age was 17 years (range 6 months–59 years). FC positivity was noted in 49% of the cohort. FC positivity was greater during pulmonary exacerbation compared to the stable state (67 versus 50%). Participants aged 5–30 years had a lower FEV1 within the FC positive group. A significant association between FC positivity and non-tuberculosis mycobacterial (NTM) culture was observed on non-parametric testing ($p = 0.022$) and regression analysis ($p = 0.007$). Exposure to indoor mold was a predictor for FC positivity ($p = 0.047$). There was a trend towards increased lung clearance index (LCI), bronchiectasis and intravenous antibiotic use in the FC positive group. There was no significant difference in biomarkers between FC positive and negative patients.

Conclusion: *Aspergillus. fumigatus* is the commonest filamentous fungi cultured from CF airways. We found no difference in the airway biomarker profile between FC positive and

negative patients. The role of galactomannan and TNFR2 as fungal specific biomarkers in CF remains uncertain. FC positivity is associated with a lower FEV_1 in younger patients, a lower LCI, NTM positivity, bronchiectasis, and intravenous antibiotic exposure. Larger trials are needed to determine the role of galactomannan and TNF-R2 as potential fungal biomarkers in CF.

Keywords: biomarkers, inflammation, galactomannan, TNF-R2, IL-8, neutrophil, fungi, children

INTRODUCTION

Cystic fibrosis (CF) lung disease involves impaired mucociliary clearance leading to pulmonary inflammation, infection, and a more rapidly declining lung function. The CF airway milieu contains higher levels of pro-inflammatory cytokines in comparison to healthy controls (Sagel, 2009). Whilst the association between pathogenic bacteria and airway inflammation, infection and disease progression is well established (Hauser et al., 2011), less is known about the role of fungi in the CF airways and their impact on airway inflammation and lung disease progression. Allergic bronchopulmonary aspergillosis (ABPA) is a well-established complication in CF associated with sensitization to *Aspergillus fumigatus* (Thia and Balfour Lynn, 2009). In the absence of ABPA, it is not clear whether the detection of fungi in the CF airway in asymptomatic patients reflects simple colonization (innocent bystander effect) or infection contributing to increased airway inflammation (Liu et al., 2013; Chotirmall and McElvaney, 2014).

A. fumigatus is the commonest filamentous fungus isolated from CF airways (Pihet et al., 2009; Sudfeld et al., 2009). Improved mycological methods enable the detection of other fungi, including *Aspergillus terreus, Scedosporium apiospermum* complex, *Exophiala dermatitidis*, and *Penicillium* species (Borman et al., 2010).

The clinical significance of *A. fumigatus* colonization in CF is uncertain. Whilst Saunders et al. (2016) report a steeper decline in Forced Expiratory Volume in 1 second (FEV1) in their fungal culture positive pediatric CF cohort, studies by Baxter et al. (2013b) and De Vrankrijker et al. (2011) found no association between *A. fumigatus* airway colonization and lung function. Other studies have reported greater risk of pulmonary exacerbations requiring hospitalization (Amin, 2010) and higher rates of bronchiectasis (McMahon et al., 2012).

The lack of standardization in processing respiratory samples for fungal culture, differing fungal recovery rates between standard culture methods and the insensitivity of certain fungal culture methods (Chen et al., 2017) is likely to contribute to these contradictory findings. The use of modified culture methods using higher amounts of sputum inoculum have shown increased sensitivity in the detection of fungi (Pashley et al., 2012; Fraczek et al., 2014).

Exploring the relationship between FC positivity and inflammatory airway biomarkers may help in clarifying the clinical significance of fungi. Attempts at profiling the inflammatory cytokine profile in conjunction with clinical and microbiological profiles in CF is limited to a few well-established biomarkers (Frayman et al., 2017) such as neutrophil elastase (NE) and interleukin-8 (IL-8) found in higher concentrations in the CF airway (Frayman et al., 2017). There is a need to identify biomarkers that are specific for pulmonary infections including fungal infection. There is emerging evidence for the novel biomarkers galactomannan and tumor necrosis factor receptor 2 (TNF-R2) for monitoring non-invasive fungal lung disease in CF and asthma (Baxter et al., 2013a; Ghebre et al., 2017)

Neutrophil-driven airway inflammatory responses play a central role in CF. Activation of the innate immune system following pathogenic exposure results in phagocytosis, release of NE and cytokines (Hartl et al., 2012; Lammertyn et al., 2017). IL-1β and TNF-α facilitate recruitment of immune cells to the site of inflammation. Recruitment cytokines such as IL-8 have high neutrophil attracting capacity (Strieter, 2002), further exacerbating the inflammatory process and resulting in large amounts of neutrophilic intracellular protease and oxidant being released. Activation cytokines (interferon-γ, IL-2, IL-12, and TNF-α) augments the T-helper cell response.

Inhalation of fungal spores may result in germination and hyphal growth within the bronchioles (Chotirmall and McElvaney, 2014). Fungi may subsequently colonize, infect, or induce a spectrum of hypersensitivity reactions within the airways. Galactomannan, a fungal cell wall component, is released during hyphal tissue invasion by *Aspergillus* species and can be detected in body fluids (Mennink-Kersten et al., 2004). Current literature focusses on the role of galactomannan in invasive pulmonary aspergillosis, but only a few studies to date have aimed to evaluate its role in non-invasive fungal lung disease (Nguyen et al., 2007; Baxter et al., 2013a).

Differentiating between filamentous fungi as innocent bystanders and fungal infection, using a combination of clinical parameters and airway biomarkers, may inform antimicrobial strategies. This is particularly important given emerging evidence that CF transmembrane conductance regulator (CFTR) modulators may not alter susceptibility to *A. fumigatus* (Fritsch et al., 2019). Consequently the role of fungi in CF is likely to present an ongoing clinical challenge, whilst at the same time limiting our therapeutic options due to complex drug interactions (Tracy and Moss, 2018).

We report a prospective observational cohort study. Our principal aim was to describe the clinical characteristics and airway inflammatory biomarker profile (NE, IL-8, TNF-R2 and galactomannan) in filamentous fungi culture-positive CF patients. We hypothesized that fungal culture positivity in CF is associated with worse lung function and increased airway inflammation.

MATERIALS AND METHODS

Setting and Study Population

Children and adults attending tertiary CF centers at the University Hospitals of Leicester NHS trust, UK, were eligible for enrolment if they had a well-documented diagnosis of CF based on the presence of two disease-causing CFTR mutations and/or a sweat chloride ≥60 mmol/L. Exclusion criteria included CF transplant patients. Participants were approached during routine clinical encounters inclusive of inpatient admissions and outpatient visits. Inclusion criteria for this study included having sputum and/or bronchoalveolar (BAL) samples collected. All respiratory samples were obtained when clinically indicated. Samples during the stable state are obtained at all routine clinic visits on a two-monthly basis or as part of their annual comprehensive review process. Further respiratory samples were obtained during periods of exacerbation as defined by Fuch's criteria (Fuchs et al., 1994). BAL sampling was undertaken when there was no clinical response to a 4–6-week course of broad spectrum oral antibiotics without a causative pathogen on respiratory samples.

Written informed consent was obtained from study participants ≥17 years of age. For participants <17 years of age written informed consent was obtained from parents or caregivers. Pediatric patients older than 7 years old also provided written assent. This study was approved for review and anonymized use of patient clinical data as part of the Leicester longitudinal study of respiratory infections and microbiomics in CF by the East Midlands Research Ethics Committee, reference number 12/WM/0285.

Study Design

Prospective cohort study, including pediatric and adult CF patients, from September 2016 to August 2018. The study protocol included a baseline visit which occurred at a routine clinical encounter during a non-exacerbation period. We obtained paired exacerbation and stable respiratory samples for all study participants who experienced a pulmonary exacerbation during the study period.

Sputum and Data Collection

Pediatric study participants aged five years and over underwent sputum induction (Paggiaro et al., 2002; Ahmed et al., 2019) lung function and serological testing as part of their annual review process during a period of stable disease. Sputum was induced with hypertonic saline in our purpose built negative pressure room dedicated to children. If an insufficient sputum sample was obtained during sputum induction, spontaneously expectorated sputum samples and/or BAL samples were collected according to standardized procedures (Miller et al., 2005). BAL samples were obtained in theatre under anesthesia as recommended by the ERS task force (De Blic et al., 2000). Adult patients do not routinely undergo sputum induction and therefore spontaneously expectorated sputum was used during a stable clinical state.

Electronic medical records were used to obtain study data including: patient demographics, CFTR genotype, medications, lung function, serological, clinical microbiology, and radiology results (bronchiectasis on CT chest). Pediatric patients also underwent a multiple nitrogen washout test (Lum et al., 2007; Davies et al., 2008; Nuttall et al., 2019) as part of their annual review process. We assessed the respiratory status (stable versus exacerbation) of all CF patients at the time of sputum collection.

Respiratory Sample Processing and Storage

For the detection of filamentous fungi, a sensitive approach was used whereby neat sputum plugs were inoculated onto potato dextrose agar plates containing 16 μl/ml chloramphenicol, 4 μl/ml gentamicin, and 5 μl/ml fluconazole (PGCF) (Pashley et al., 2012). Samples were incubated at 37°C for up to seven days. We identified fungi based on macroscopic and microscopic features (Greer et al., 1997). For bacterial cultures the British National Standard method for investigating sputum was used in accordance with the UK CF Trust Microbiology Standards Working Group recommendations (Public Health England, 2019). Sputum was homogenised with 0.1% dithiothreitol (DTT) and 10 μl of this mixture was diluted in 5 ml distilled water. A sterile loop was used to simultaneously inoculate 1 μl onto the following culture media: (1) mannitol salt/chromogenic agar, (2) cystine-lactose-electrolyte-deficient (CLED) agar, (3) *Burkholderia cepacia* selective agar, and (4) *Mycobacterium species* selective agar at 35–37°C. All samples were incubated for 48 h to five days, except for *Mycobacterium* selective culture, which requires incubation for up to eight weeks.

Supernatant from sputum and/or BAL samples were obtained following homogenisation with 0.1% DTT, followed by vortexing, filtering and a centrifugation step as per our local SOP (Bhatt, 2016).

Cohort Characterization

Participants were categorized into the following groups; A) fungal culture positive and B) fungal culture negative based on absence of filamentous fungi on culture. Further sub-group analysis included paired samples obtained during pulmonary exacerbation and a clinically stable state. Pulmonary exacerbation was defined by Fuch's criteria and stable disease included the absence of Fuch's criteria (Fuchs et al., 1994). Colonization with a known CF pathogen was defined as ≥50% respiratory samples positive for the same bacteria on routine culture within a 12-month period (Liu et al., 2013).

Participants were deemed to have allergic bronchopulmonary aspergillosis (ABPA) (Barton et al., 2008) if they had suggestive clinical features including; a total serum IgE >500 kU/L and a raised specific IgE to *A. fumigatus* of >0.35 kUA/L (Thia and Balfour Lynn, 2009).

Sputum Cell Differentials

Cytoslides were prepared by fix air-drying in 100% methanol for five minutes followed by staining using RapiDiff II (Bios Europe) staining kit according to protocol (Parker, 2018). The cytoslides were used to determine the leukocyte differential cell count (Brightling et al., 2000).

ELISA Biomarker Assays

Inflammatory cytokine profiles were measured in sputum supernatants; We estimated NE using the PMN Human Elastase ELISA kit (Invitrogen™), IL-8 using the Human IL-8 ELISA Kit (ThermoFisher Scientific), galactomannan using the Platelia™ Enzyme Immunoassay *Aspergillus* antigen© (Bio-Rad) kit and TNF-R2 using the Human Tumor Necrosis Factor Receptor II (soluble TNF-R2) ELISA kit (Invitrogen ™). All ELISA assays were performed in accordance with individual manufacturer's instructions. Results of NE, IL-8, and TNF-R2 assays were interpreted using standard curves appropriate for individual kit protocols. Galactomannan was measured using ratios of optical densities (OD). A positive result was based on a GM optical density index (ODI) of ≥0.5.

An optimization step was undertaken due to homogenization of our respiratory samples with DTT. All inflammatory mediator kits underwent preparation of standards with and without DTT for comparison. This validation step did not demonstrate any significant variations in the concentration of standards for biomarker assay kits.

Statistical Analysis

We present summary statistics as median (range) for continuous data and as frequency (%) for categorical data. The comparison of baseline characteristics across both groups of fungal culture was performed using Mann-Whitney U test for continuous data and Chi-squared tests for categorical data. Analysis of normally distributed data including the association of lung function with age was performed using the independent samples t-test. Statistical significance was at the 0.05 level. The association between clinical characteristics across both categories of fungal culture was investigated with the use of a binomial regression model and reported as p-values (Wald test) and odds ratios (OR). The following independent variables; age, antibiotic use, bronchiectasis, lung function and immunological markers on serology were used for this logistic regression model to determine the association with fungal culture positivity (dependent variable).

RESULTS

Cohort Characteristics

Sixty-one of the 73 patients enrolled provided respiratory specimens and were subsequently eligible for inclusion in our study. Twelve patients were excluded as no respiratory sample for fungal culture was obtained (**Figure 1**). A total of 76 respiratory specimens were processed for fungal culture, which comprised of 72 sputum and four BAL samples. Median age at recruitment was 17 years (range 6 months–59 years) with 29 male participants and a median percent predicted FEV_1 of 77% (range 21–120%). All 61 participants provided a sputum sample during the stable state, of whom 43 patients also produced respiratory samples suitable for biomarker analysis. Of the respiratory samples that underwent biomarker analysis (n = 43), only three samples were derived from BAL. Fifteen patients provided a further respiratory sample during clinical exacerbation to enable comparison with their paired stable state sample.

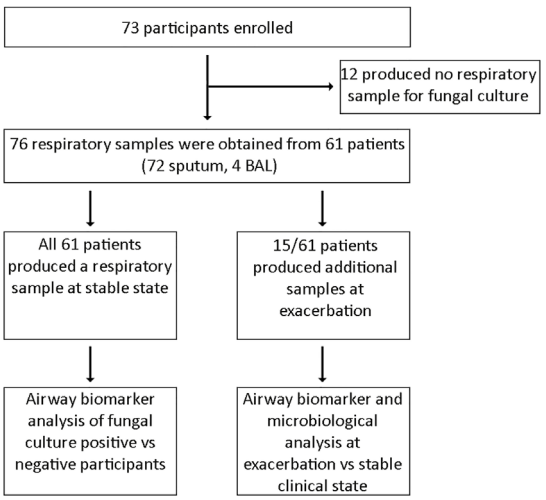

FIGURE 1 | Consort diagram.

Clinical Characteristics in Relation to Fungal Culture

Thirty-one patients (49%) cultured filamentous fungi from their sputum and/or BAL during a clinically stable state. Concomitant growth of *A. fumigatus* and yeast was the commonest (60%) within the fungal culture positive group, followed by *A. fumigatus* alone (40%). No other filamentous fungi were detected.

The median age within the fungal culture positive cohort was 15 years (range 7–53 years). No child under age seven years was observed to be fungal culture positive. Patient demographics, clinical data and cultured pathogens are presented in **Table 1**. No significant differences were observed between the distribution of age, BMI, genetics, indoor mold exposure (as defined by the presence of self-reported household mold) and pancreatic status between the two groups.

We observed significantly higher rates of non-tuberculous mycobacteria (NTM) isolates within the fungal culture positive sub-group (p = 0.022). This finding was not noted for co-colonization with other bacteria such as *Staphylococcus aureus* or *Pseudomonas aeruginosa*.

We report greater use of inhaled corticosteroids within the FC positive cohort compared to the FC negative cohort (p = 0.008).

A binomial regression analysis was carried out to predict whether fungal culture positivity is associated with clinical characteristics including age, sex, mold exposure, routine microbiology, medication use, and lung function. Fungal culture positivity was 14 times more likely in the presence of NTM (p= 0.007) and five times more likely with indoor mold exposure (p = 0.047). Although not statistically significant, the use of three-monthly intravenous antibiotic was four times more

likely to be associated with fungal culture positivity. Significant associations for the predictor of fungal culture positivity were not demonstrated for other independent variables highlighted in **Table 2**. Missing values within the binomial regression model were accounted for by use of median values (N <4 for independent variables including prophylactic, nebulized and intravenous antibiotic use, lung function, microbiology and serological markers). Due to multiple missing values for the independent variables LCI and bronchiectasis, a regression model could not be utilized without introducing significant bias.

The trend in lung function measures in relation to age and fungal culture positivity is shown in **Figure 2**. The mean FEV1 is lower within the fungal culture positive cohort at all ages with the exception of the over 30-year group. A significantly lower FEV1 was noted within the 21–30-year old fungal-culture positive group (p = 0.015). Although not statistically significant, we noted a higher FEV1 within the over 30-year old fungal-culture positive subgroup.

Although not significant, we also note an increased LCI within the fungal culture positive cohort at all ages in comparison to the fungal culture negative cohort.

Similar rates of ABPA were noted between the two cohorts with 13% in the fungal culture positive group (n = 4) and 19% in the fungal culture negative group (n = 6).

Biomarker Analysis

Neutrophil Elastase

All samples were processed for NE however only 33/43 (77%) yielded a result as ten readings were unavailable due to insufficient sample. Twenty four of the 33 samples (73%) had a detectable NE value of ≥0.1 ng/ml (reference range 0.16–10 ng/ml). The remaining samples had a value of <0.1 ng/ml, which fell outside of the detectable range on the standard curve. There was no significant difference in NE between the fungal culture positive group (median value 0.1 ng/ml, range 0.1–10) and the fungal culture negative group (median 0.1 ng/ml, range 0.1–2.3, p = 0.107) (**Figure 3A**).

Two patients had high NE levels (> 10 ng/ml), both of whom were fungal culture positive. The first patient had serological evidence of ABPA with a serum IgE of 4433 kU/L, *Aspergillus* specific IgE of 25.1 kUA/L, and an *Aspergillus* specific IgG of 130 mg/L. This patient was receiving three-monthly intravenous antibiotics and nebulized antibiotics as part of M*ycobacterium abscessus* eradication regime. The second patient had mixed growth of *A. fumigatus* and *Haemophilus influenzae* on sputum culture, no pre-existing history of fungal lung disease, normal serological markers, and was not receiving regular intravenous antibiotics.

TABLE 1 | Clinical characteristics in CF with and without positive sputum for filamentous fungi at stable state.

Variable	Fungal culture positive		Fungal culture negative		P value
	Total N		Total N		
Male n (%)	30	13 (43%)	31	16 (52%)	0.517
Age (years)	30	15 (7–53)	31	16 (0.5–59)	0.919
DF508 homozygous n (%)	30	13 (43%)	31	14 (45%)	0.886
Pancreatic insufficient n (%)	30	26 (87%)	31	28 (90%)	0.654
BMI (kg/m^2)	30	20.8 (13.5–30.1)	31	19.5 (15.3–27.6)	0.573
Indoor mold exposure n (%)	29	12 (40%)	30	9 (29%)	0.659
Ivacaftor n (%)	30	1 (3%)	31	3 (10%)	–
Prophylactic antibiotics n (%)	29	16 (55%)	30	17 (56%)	0.993
Nebulized antibiotics n (%)	29	14 (48%)	30	14 (47%)	0.992
Inhaled steroids n (%)	28	19 (68%)	31	10 (32%)	**0.008**
Oral steroids n (%)	29	3 (10%)	31	1 (3%)	–
3 monthly ivab n (%)	29	11 (38%)	30	5 (17%)	0.185
SA colonization n (%)	29	6 (21%)	30	4 (13%)	0.753
PA colonization n (%)	29	8 (28%)	30	9 (30%)	0.979
Previous NTM n (%)	29	12 (41%)	30	3 (10%)	**0.022**
Bronchiectasis on CT n (%)	17	13 (76%)	11	7 (64%)	0.225
ABPA n (%)	29	4 (14%)	30	6 (20%)	–
Total IgE (KU/L)	29	156 (5–4,433)	29	76 (3–4,273)	0.260
Asp. IgE (KUA/L)	27	0.33 (0–41)	30	0.05 (0–37.8)	0.378
Asp. IgG (mg/L)	29	58 (15–185)	28	52 (6.8–181)	0.139
FEV_1 predicted (%)	30	72 (21–122)	28	80 (24–131)	0.920
FEV_1/FVC (%)	30	70 (32–97)	28	72 (41–92)	0.767
LCI	9	11.6 (7.75–20.72)	11	7.84 (6.41–13.85)	0.972
Sputum lymphocyte count (%)	13	0 (0–0.5)	11	0 (0–5.75)	0.289
Sputum macrophage count (%)	13	2.75 (0–32)	11	2 (0.25–28.25)	0.511
Sputum eosinophil count (%)	13	0.1 (0–2.75)	11	0.25 (0–14.5)	0.631
Sputum neutrophil count (%)	13	94.88 (67.75–99.5)	11	92.75 (10.25–98.5)	0.887

IVAB, Intravenous antibiotics; ICS, inhaled corticosteroid; SA, Staphylococcal aureus; PA, Pseudomonas aeruginosa, NTM, non-tuberculous mycobacteria; IgE, Immunoglobulin E; Asp IgE, Aspergillus specific IgE; Asp IgG, Aspergillus specific immunoglobulin G; FEV1, Forced expiratory volume in one second; FVC, Forced vital capacity; LCI, Lung clearance index. Colonization is defined as >50% samples positive over 12-month period.

Categorical data is presented as frequency (n) and analyzed using Chi-squared test. Continuous data is presented as median and range. Non-parametric data was analyzed as comparison of medians across groups using the Mann-Whitney U test. Statistical significance was defined as P value < 0.05.

Bold values signify statistical significance at P < 0.05.

TABLE 2 | Binomial regression analysis of clinical characteristics in relation to fungal culture positivity.

Variable	P value	Exp(B)/OR 95% CI
Age (years)	0.542	1.024 (0.948–1.107)
Sex	0.220	0.354 (0.067–1.860)
Pancreatic insufficiency	0.469	0.167 (0.396–7.486)
Indoor mold exposure	**0.047**	5.217 (1.025–26.545)
Prophylactic antibiotic use	0.546	0.635 (0.146–2.773)
Nebulized antibiotic use	0.801	0.822 (0.180–3.750)
3 monthly ivab exposure	0.108	4.761 (0.712–31.836)
FEV_1 predicted (%)	0.351	0.985 (0.953–1.017)
S. aureus	0.514	1.845 (0.293–11.601)
P. aeruginosa	0.869	1.175 (0.170–7.984)
NTM	**0.007**	14.263 (2.064–98.540)
Total IgE (KU/L)	0.264	1.001 (0.999–1.002)
Asp. IgE (KUA/L)	0.307	0.930 (0.810–1.069)
Asp. IgG (mg/L)	0.401	1.007 (0.990–1.025)

IVAB, Intravenous antibiotics; Forced expiratory volume in one second; IgE, Immunoglobulin E; Asp IgE, Aspergillus specific IgE; Asp IgG, Aspergillus specific immunoglobulin G. P values are derived from the Wald test with statistical significance defined as <0.05.

Interleukin-8

Sufficient sputum was available for 23 samples to be tested for IL-8 (reference range 15.6–1,000 pg/ml). All assays yielded a detectable result of >62 pg/ml (reference range 15.6–1,000 pg/ml) (**Figure 3B**). Sixteen of the 23 respiratory samples (70%) tested were from fungal culture positive patients. The comparative median and ranges of IL-8 between the fungal culture positive and negative groups are reported in **Figure 3**. There was no significant difference in IL-8 between the fungal culture positive and negative sub-groups (p = 0.871).

Tumor Necrosis Factor Receptor 2

All respiratory samples were tested for TNF-R2 (reference range 2–142 ng/ml). Eight patients (19%) had a detectable level of ≥2 ng/ml (**Figure 3C**). Five of the eight were fungal culture positive. Both groups had a median TNF-R2 below the detectable range of the assay.

Participants with detectable TNF-R2 levels were not noted to have any discernible patterns to lung function impairment, lung clearance index, serological markers, intravenous antibiotic use, or radiological evidence of bronchiectasis on CT.

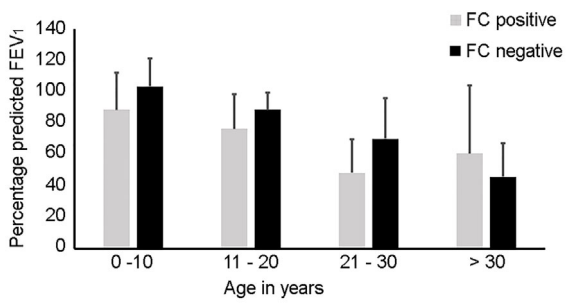

FIGURE 2 | Histogram representing differing age groups in relation to fungal culture (FC) positivity and percentage predicted Forced Expiratory Volume in 1 second (FEV_1). The lung function data is presented as the standard error of mean per age category. The mean FEV_1 is lower within all age groups within the FC positive group with the exception of the over 30-year sub-group; 0–10 years (FC positive mean 89%, 95% CI 64–113; FC negative mean 103%, 95% CI 84–122, p = 0.25), 11–20 years (FC positive mean 76%, 95% CI 63–90; FC negative mean 89%, 95% CI 77–100, p = 0.225), 21–30 years (FC positive mean 48%, 95% CI 29–68; FC negative mean 70%, 95% CI 48–92, p = 0.015) and >30 years (FC positive mean 61%, 95% CI 8–129; FC negative mean 46%, CI 28–64, p = 0.434).

Galactomannan

There was enough sputum for galactomannan testing available for 32 patients. Only four had a positive result with ≥0.5 ODI, three of which fell into the fungal culture positive group (**Figure 3B**).

Paired Sputum Analysis

Fifteen patients provided paired sputum samples obtained at routine exacerbation and stable state visits. Ten (67%) were positive for filamentous fungi during an exacerbation state and seven (50%) during the stable state. All but two participants' samples were culture positive for *A. fumigatus*. The other two samples were positive for either *Penicillium species* or *Aspergillus niger*, both during the acute exacerbation state. **Figure 4** demonstrates a higher proportion of concomitant bacterial growth during the exacerbation state. Ninety-three percent had mixed bacterial and fungal growth during acute exacerbation in comparison to 60% during the stable state. Seven patients had the same bacterial species detected during exacerbation and stable states and represented chronic colonization. No participants during exacerbation were fungal culture negative and exhibiting only normal respiratory flora, compared to 20% (n = 3) at stable state.

Due to a lack of sufficient sputum volume, only three paired samples were available for biomarker comparison between pulmonary exacerbation and stable disease. NE (1.2 ng/ml exacerbation vs 0.05 ng/ml during stable state) and GM (0.5 OD exacerbation vs 0.14 OD stable) were higher during pulmonary exacerbation (p < 0.001), with a trend towards higher IL-8 levels during the exacerbation state (1,842.4 pg/ml exacerbation vs 1,335.3 pg/ml during stable).

DISCUSSION

Our study is the first to describe CF airway biomarkers in relation to fungal culture positivity. We report the relationship between airway fungal culture positivity, airway biomarkers and clinical, radiological and microbiological characteristics in children and adults with CF.

The clinical relevance of fungal airway colonization and its impact on lung disease progression in CF is unclear. In keeping with previous reports, we found that *A. fumigatus* was the most frequently isolated fungal species (Pihet et al., 2009). Colonization with *A. fumigatus* has been associated with more advanced CF lung disease (Ramsey et al., 2014), lower lung function (Amin, 2010; Saunders et al., 2016) and greater risk of pulmonary exacerbations requiring hospitalization

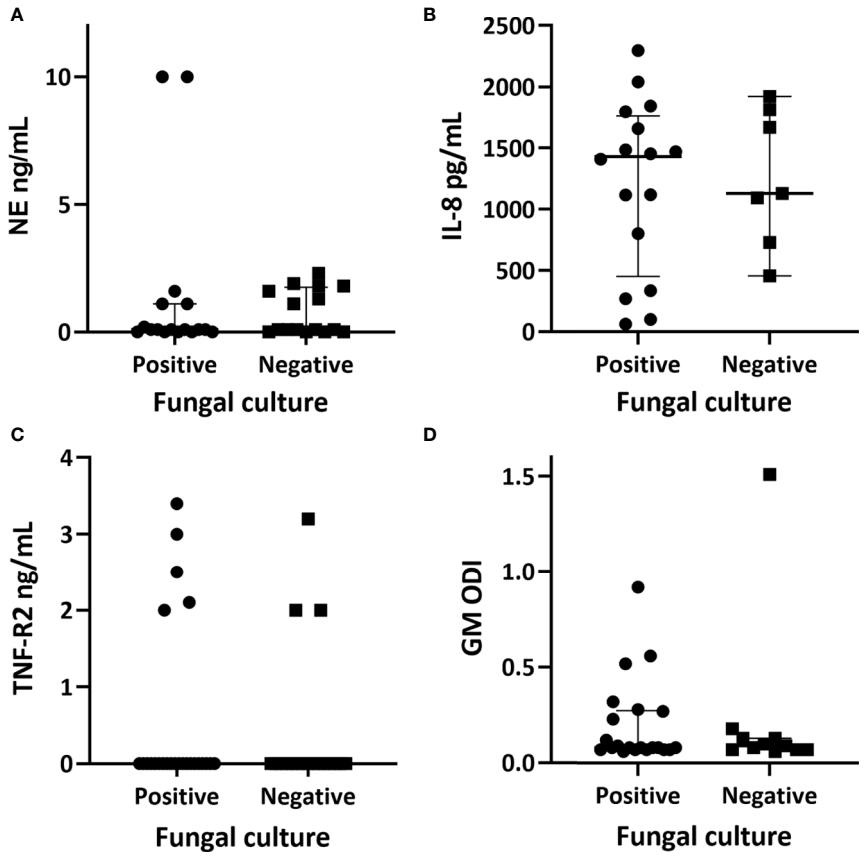

FIGURE 3 | Quantitative airway biomarker measurements in relation to fungal culture positivity. **(A)** neutrophil elastase with the fungal culture positive group having a median value of 0.1 ng/ml (range 0–10) and the culture negative group had a median of 0.1 (range 0–2.3); p = 0.107. **(B)** interleukin-8 with a median of 1,430.45 pg/ml (range 62.9–2,296.2) within the fungal culture positive group and a median of 1,128.3 (range 455.2–1,921.7) within the fungal culture negative group; p = 0.871. **(C)** tumour necrosis factor receptor 2 (TNF-R2) with a median of <2 ng/ml within both subgroups. **(D)** galactomannan values represented as optical density index (ODI) with the fungal culture group having a median of 0.09 (range 0.06–0.92) and the culture negative group with a median of 0.08 (range 0.066–1.44); p = 0.143. Individual data, median and interquartile range are shown. Comparison of medians between groups were undertaken using the Mann-Whitney U test for non-parametric data.

(Amin, 2010). In our study, the fungal culture positive group, with the exception of the over 30-year old sub-group, had a lower mean FEV1 across all younger age groups. Our findings of a higher FEV1 within the FC positive subgroup for the over 30-year age group, may be a reflection of participants surviving with milder CF lung disease.

LCI derived from multiple breath washout is a more sensitive marker of lung disease progression compared to spirometry, allowing non-invasive monitoring of small airways disease. A study by Walicka-Serzysko et al. demonstrated a higher LCI in CF patients with *A. fumigatus* (Walicka-Serzysko et al., 2019). We also found higher LCI's in fungal culture positive patient but this did not reach statistical significance.

The primary focus of previous studies have included exploring the association between *A. fumigatus* and clinical outcomes, there is a paucity of information exploring fungal airway colonization and its impact on markers of airway inflammation. It is well established that the CF airway milieu contains higher levels of pro-inflammatory cytokines in comparison to healthy controls (Sagel, 2009). Evidence for pulmonary inflammation in surveillance BAL such as raised NE and IL-8 in the CF airways is present, even in the absence of clinically apparent lung disease (Armstrong et al., 2005; Frayman et al., 2017; Khan et al., 2019). Sly et al. have reported a link between high NE levels within the CF airways and the subsequent development of bronchiectasis (Sly et al., 2009). Whether fungal colonization exacerbates underlying airway inflammation, resulting in higher levels of airway biomarkers and therefore further driving lung disease progression requires further study. Exploring this relationship is complicated by the difficulty in separating the effect of bacteria and fungi present in respiratory cultures.

In our study, only one-third of patients were fungal culture positive without the presence of concomitant pathogenic

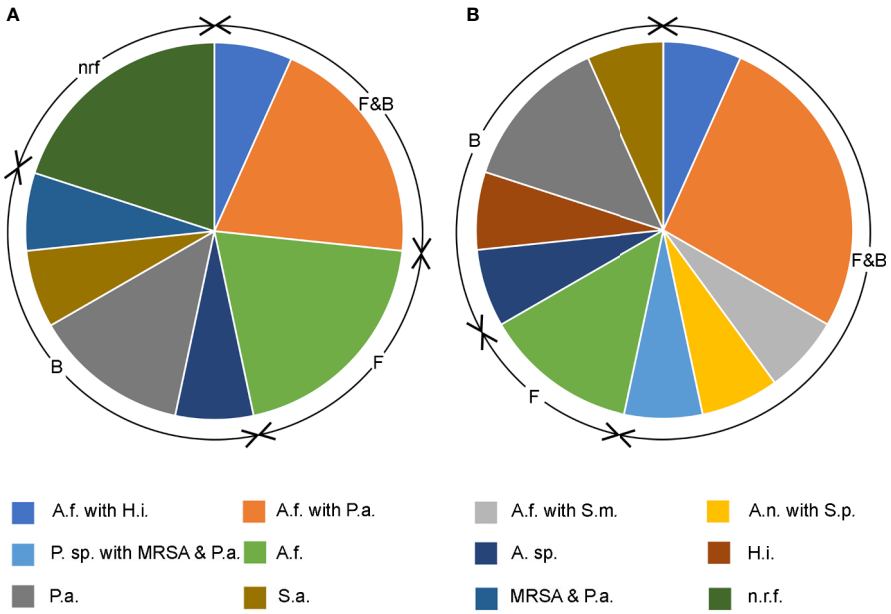

FIGURE 4 | Pie charts showing bacterial and filamentous fungal isolates cultured from respiratory samples during **(A)** stable state and **(B)** acute exacerbation. F&B; concomitant fungal and bacterial growth, F; fungal only, B; bacterial only, nrf; normal respiratory flora. Fungi cultured: A.f. *Aspergillus fumigatus*, A.n. *Aspergillus niger*, P. sp. *Penicillium* sp. Bacteria cultured: A. sp. *Achromobacter* sp., H.i. *Haemophilus influenzae*, P.a. *Pseudomonas aeruginosa*, S.a. *Staphylococcus aureus*, MRSA methicillin resistant *S. aureus*, S.m. *Strenotrophomonas maltophilia*, S.p. *Streptococcus pneumoniae*.

bacteria, making the direct attribution of inflammation to a positive fungal culture problematic. We found no significant difference between NE and IL-8 between the fungal culture positive and negative groups. Two patients with very high levels of NE were fungal culture positive but these low numbers preclude any firm conclusion. Studies have shown improvements in NE and IL-8 levels following treatment of an exacerbation (Ordonez et al., 2003; Colombo et al., 2005). Evidence suggests NE activity is higher during exacerbation and is responsive to antibiotics (Chalmers et al., 2017). Whilst we acknowledge our small number of paired patient samples due to difficulty in obtaining sufficient sputum volumes, we report higher levels of NE and IL-8 during pulmonary exacerbation compared to the stable state.

Galactomannan may be a useful screening test for invasive pulmonary aspergillosis (Kimura et al., 2009; Talento et al., 2017). The diagnostic usefulness of galactomannan assays in patients with non-invasive fungal lung disease in CF, such as ABPA and chronic pulmonary aspergillosis remains uncertain (Nguyen et al., 2007; Kitasato et al., 2009; Fayemiwo et al., 2017). We found that only four patients (13%) within our cohort had a positive result for galactomannan. There were no significant difference in the absolute values of galactomannan between the fungal culture positive and negative groups. Contrary to our study findings, galactomannan has been reported as a sensitive method of detecting *Aspergillus* in sputum in comparison to fungal cultures (Baxter et al., 2013a), even in the absence of invasive disease. Baxter et al. used sputum samples homogenized with Sputasol (commercial product containing DTT) and sonication. In comparison we used homogenized sputum supernatants without sonication, which may have decreased the overall yield.

There is emerging evidence implicating TNF-R2 as a possible airway biomarker in fungal associated asthma. Ghebre et al. noted significantly higher TNF-R2 levels in adults with asthma who were fungal culture positive (Ghebre et al., 2017). Our study is the first to analyze TNF-R2 as a potential biomarker of inflammation in adults and children with cystic fibrosis. Overall, we detected TNF-R2 in only 19% of our patients. Within the limitations of our modest number of patients with detectable TNF-R2 levels, we found no association with adverse clinical outcomes. Whilst Ghebre et al. concluded that TNF-R2 may diminish the inflammatory response to *A. fumigatus*, the exact mechanism for this and its clinical implications in CF is unknown. Interestingly, Ghebre et al. concluded that TNF-R2 appears to be a strong discriminator of exacerbation in children and adults with asthma (Ghebre et al., 2019). Further studies in CF are required to determine if a similar relationship exists between fungal culture positivity, exacerbation states and higher TNF-R2 levels.

We found that the fungal culture positive subgroup had greater previous and/or current positive sputum isolates for non-tuberculous mycobacteria. This is in keeping with previous literature where it has been proposed that *Mycobacterium* superinfection can occur alongside aspergillosis (Cui et al., 2013). The proposed theories include a synergistic relationship which enhances their resistance to environmental

change such as use of broad-spectrum antibiotics (Kerr, 1994; Cui et al., 2013).

Within our CF cohort, we report higher rates of fungal culture positivity with indoor mold exposure. Evidence suggests a link between indoor fungal contamination and ABPA. Reports linking airway fungal colonization and mold exposure in CF are scarce (Rocchi et al., 2015). Fairs et al. reported environmental mold exposure may predispose asthmatics to airway colonization (Fairs et al., 2013) and whether this also occurs in CF patients is unclear and requires further study.

Although not statistically significant we observed a higher rate of bronchiectasis in the fungal culture positive cohort with 76% having radiological evidence of this on CT chest, in comparison to 64% of the culture negative cohort. Similar associations have been reported between *A. fumigatus* and higher radiological scores with more significant bronchiectasis (McMahon et al., 2012).

Whilst over 35% of our fungal culture positive cohort received three-monthly intravenous antibiotics, only 16% of fungal culture negative group were exposed to regular antibiotics. Several studies have shown an association between the use of broad-spectrum antimicrobials and *A. fumigatus* airway colonization (Burns et al., 1998; Hodson et al., 2002). The use of regular intravenous antibiotics are typically reserved for patients with more severe lung disease and/or bacterial colonization. The concept of regular exposure to broad spectrum antimicrobials reducing competition for surfaces and limited nutrients, therefore promoting fungal adhesion, growth and colonization has therefore been proposed (Oever and Netea, 2014). However, a study by Baxter et al. reported a significant decline in the bioburden of *Aspergillus* when they compared pre- and post-antibiotic sputum samples from 26 adult CF patients (Baxter et al., 2013b). Therefore, the use of antimicrobials for well-established CF pathogens and its impact on *A. fumigatus* remains poorly understood.

We report a significantly higher rate of inhaled corticosteroid use within the FC positive cohort. This is a well-established finding in existing literature whereby the use of inhaled and/or systemic corticosteroids are known to promote *A. fumigatus* growth (Noni et al., 2015).

Our study limitations include a modest sample size, in particular for the paired sample analysis. The presence of significant numbers of non-sputum producing children in our study cohort means that we were unable to obtain sufficient volumes to complete ELISA biomarker and sputum cell differential analysis for all patients. We included a combination of sputum and BAL samples to enhance our sample size and to allow inclusion of non-sputum producing children within our study cohort. In order to minimize bias resulting from salivary contamination of respiratory samples, we included BAL samples when clinically indicated and where we were unable to obtain spontaneous or induced sputum samples.

Secondly, published studies report a wide range of prevalence in the detection of filamentous fungi (Nagano et al., 2010; Pashley et al., 2012) due to sampling techniques and culture conditions, it is likely that the true prevalence of fungi in CF is underestimated. To overcome this we used a modified fungal culture approach, which has been shown to be a more sensitive for the detection of filamentous fungi (Pashley et al., 2012). Whilst it is possible that our approach is detecting low levels of fungi that are not truly colonizing the lower airways, the association between higher culture rates and worse lung function in both this and the adult asthma studies (Fairs et al., 2010; Agbetile et al., 2012) that have utilized this more sensitive culture method would suggest the detection may be clinically relevant. An alternative approach to culture may be to utilize a molecular technique such as quantitative polymerase chain reaction (qPCR), which is commercially available for *A. fumigatus* and a limited number of other *Aspergillus* species (Guegan et al., 2018). Both modified fungal culture and *Aspergillus* quantitative PCR have been shown to be more sensitive than the NHS routine approach (Public Health England, 2019) for identifying *A. fumigatus* from respiratory secretions (Fraczek et al., 2014). It should be noted, however, that the current commercial qPCR assays, whilst based on quantitative technology, are used for determining the presence or absence of a fungus only, and will amplify DNA from dead as well as live fungal material.

Our study reports on the short-term clinical outcomes associated with FC positivity in CF given the cross-sectional methodology used. Whilst the long-term outcomes of ABPA and serological sensitization are well-described with evidence of lung function decline (Kraemer et al., 2006), increased need for intravenous antibiotics (Peetermans et al., 2014) and bronchiectasis (Agarwal et al., 2010), literature on the outcomes of *Aspergillus* colonization and non-invasive infection is scarce. It is clear that further prospective longitudinal studies are required to describe the clinical outcomes of *A. fumigatus* colonization and non-invasive infection.

Finally, statistical analysis for LCI and bronchiectasis were excluded from the binomial regression model due to the risk of bias from missing data. A limited number of patients had LCI results available as this is not routine practice in our adult CF cohort and is carried out as part of the annual comprehensive review process in children. However, not all children are capable of completing this procedure. We also note a limited number of patients underwent a CT chest as this was only carried out as part of routine care when indicated. In addition, our results obtained for NTM and mold exposure have high confidence intervals despite clinical significance, reflecting the highly variant estimates derived for these parameters.

In conclusion, we found no difference between the airway biomarker profile between sputum fungal culture positive and negative patients with CF. The role of galactomannan and TNFR2 as more fungal specific airway biomarkers in CF remains uncertain. Fungal culture positivity is associated with a lower FEV1 in patients less than 30 years old, a lower LCI, higher rates of NTM positivity, bronchiectasis and intravenous antibiotic exposure. Larger trials using a broader range of inflammatory markers in conjunction with fungal culture and quantitative PCR measures are needed.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Leicester longitudinal study of respiratory infections and microbiomics in CF by the East Midlands Research Ethics Committee, reference number 12/WM/0285. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

DP, CP, and EG co-designed the study. DP was responsible for analyzing the data and writing the manuscript. CP supervised the laboratory analyses (modified culture and ELISA) and EG the clinical aspects of the study. DP was responsible for patient recruitment, the design and custodianship of the clinical database, and processing clinical samples. DP and KD undertook the ELISA analysis. All authors contributed to the article and approved the submitted version.

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Prospective Evaluation of *Aspergillus fumigatus*-Specific IgG in Patients With Cystic Fibrosis

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Background: In Cystic Fibrosis (CF), the airways are often colonized by opportunistic fungi. The most frequently detected mold is *Aspergillus fumigatus* (*Af*). *Af* diseases are associated with significant morbidity and mortality. The most common clinical picture caused by *Af* is allergic bronchopulmonary aspergillosis (ABPA), triggered by an immunological reaction against *Af*. *Af* bronchitis and invasive aspergillosis rarely occur in CF as a result of spore colonization and germination. Since pulmonary mycoses and exacerbations by other pathogens overlap in clinical, radiological, and immunological characteristics, diagnosis still remains a challenge. The search for reliable, widely available biomarkers for *Af* diseases is therefore still an important task today.

Objectives: *Af*-specific IgG m3 is broadly available. Sensitivity and specificity data are contradictory and differ depending on the study population. In our prospective study on pulmonary *Af* diseases in CF, we determined specific IgG m3 in order to test its suitability as a biomarker for acute *Af* diseases and as a follow-up parameter.

Methods: In this prospective single center study, 109 patients with CF were screened from 2016 to 2019 for *Af*-associated diseases. According to diagnostic criteria, they were divided into four groups (control, bronchitis, ABPA, pneumonia). The groups were compared with respect to the level of *Af*-specific IgG (ImmunoCAP Gm3). We performed a receiver operating characteristic (ROC) curve analysis to determine cut-off, sensitivity and specificity. Twenty-one patients could be enrolled for a follow-up examination.

Results: Of the 109 patients, 36 were classified as acute *Af*-disease (*Af* bronchitis, ABPA, *Af* pneumonia). Of these, 21 patients completed follow up-screening. The median *Af*-specific Gm3 was higher in the acute *Af*-disease groups. There was a significant difference in *Af*-specific IgG m3 compared to the control group without acute *Af*-disease. Overall, there was a large interindividual distribution of Gm3. A cut-off value of 78.05 mg/L for Gm3 was calculated to discriminate controls and patients with ABPA/pneumonia with a specificity of 75% and a sensitivity of 74.6%. The follow up examination of 21 patients showed a decrease of Gm3 in most patients without statistical significance due to the small number of follow up patients.

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Conclusion: *Af* specific IgG may be a useful biomarker for acute ABPA and *Af* pneumonia, but not for *Af* bronchitis in CF. However, due to the large interindividual variability of Gm3, it should only be interpreted alongside other biomarkers. Therefore, due to its broad availability, it could be suitable as a biomarker for ABPA and *Af* pneumonia in CF, if the results can be supported by a larger multicenter cohort.

Keywords: cystic fibrosis, aspergillosis, *Aspergillus fumigatus*, allergic bronchopulmonary aspergillosis, ABPA, Gm3, *Aspergillus fumigatus*-specific IgG

INTRODUCTION

Cystic fibrosis (CF) is the most common lethal genetic disease in Caucasians and affects approximately between 70,000 and 100,000 people worldwide. The leading cause of death is the progressive lung disease, driven by a complex and diverse inflammatory immune syndrome induced by recurrent and chronic bacterial and fungal infections of the lung (Stephenson et al., 2015; Bell et al., 2020).

A. fumigatus (Af), the most frequently detected mold fungus in CF airways, is the fungal pathogen with the most impact on morbidity and mortality in CF (Pihet et al., 2009; Amin et al., 2010; Middleton et al., 2013; Schwarz et al., 2018a). The defective chloride channel in CF together with impaired mucociliary clearance and structural changes resulting from infectious complications (e.g. cavitary lung disease, bronchiectasis) result in a local mucosal immunodeficiency (Cohen and Prince, 2012), which allows airway pathogens like *Af* conidia to colonize and germinate in the airways. Allergic bronchopulmonary aspergillosis (ABPA), bronchitis, aspergilloma and invasive infection (pneumonia) are the known disease entities caused by *Af* in CF (Moss, 2010; Baxter et al., 2013a; Middleton et al., 2013; Hong et al., 2018).

ABPA is an immune mediated lung disease with a prevalence of up to 15% in CF. It manifests with a poorly controlled obstructive disease and recurrent pulmonary infiltrates with or without bronchiectasis (Stevens, 2003). According to the 2003 CFF (Cystic Fibrosis Foundation) consensus criteria, classic diagnosis of ABPA comprises clinical deterioration not attributed to other causes, serum IgE >1000 IU/ml, a positive skin prick test or positive specific IgE, antibodies against *Af*, precipitating or IgG antibodies against *Af*, and radiological changes that are not treatable by antibiotics. For the minimal ABPA criteria, serum IgE >500 IU/ml and fewer of the criteria mentioned above are sufficient to suspect ABPA (Stevens, 2003). Invasive pulmonary *Af* infections primarily occur in severely immunocompromised patients, but they also can occur in CF if additional risk factors are present (e.g. severe lung disease, diabetes, steroid therapy) (Kousha et al., 2011; Hong et al., 2018). The prevalence is extremely low, thus precise data are not available. In CF, invasive pulmonary *Af* infections often show a subacute, slowly progressing clinical course. and is induced by local invasion of *Af (*Schwarz et al., 2018b). Radiographic imaging and clinical courses are often non-specific (Felton and Simmonds, 2014; Kosmidis and Denning, 2015). This bears the risk of delayed diagnosis in CF. *Af* bronchitis is another *Af* entity with an estimated prevalence of 9% in CF. Increasing evidence exists that *Af* in CF can cause airway symptoms without hypersensitivity and without tissue invasion of fungal hyphae (Felton and Simmonds, 2014; Brandt et al., 2018). Shoseyov et al. were the first to report on *Af* bronchitis in six patients (Shoseyov et al., 2006). The group suggested that bronchitis should be considered, and antifungal therapy initiated when deteriorating respiratory function is not responding to antibacterial therapy, *Af* is growing in sputum cultures and ABPA was excluded. Chrdle et al. proposed a definition using clinical criteria: symptomatic chronic lower airway disease, detection of *Af* in sputum or bronchial alveolar lavage (BAL) by culture or PCR, and detection of Aspergillus-specific IgG antibodies (Chrdle et al., 2012). In summary, repeatedly detected *Af* in sputum samples and persistent respiratory symptoms without signs of *Af* hypersensitivity and without radiographic evidence of infection are characteristics of *Af* bronchitis in patients with CF (Felton and Simmonds, 2014; Kosmidis and Denning, 2015). Due to the polymicrobial colonization, overlapping clinical, radiographic, and laboratory characteristics, it is still difficult to clearly distinguish *Af* conditions from other causes of bronchopulmonary exacerbation in CF (Stevens, 2003). This bears the risk of *Af* diseases being missed and can lead to situations in which a potentially harmful differential therapeutic strategy must be attempted in order to achieve successful treatment. Therefore, the search for reliable, widely available biomarkers for *Af* diseases is still an important task today (el-Dahr et al., 1994; Kurup, 2005; Latzin et al., 2008; Hafen GM et al., 2009; Delhaes et al., 2010; Scheffold et al., 2018; Keown et al., 2019). In this light, it seems useful to evaluate single biomarkers in terms of their significance for *Af* diseases. *Af* IgG antibodies are used for diagnosis of chronic pulmonary aspergillosis (CPA) and aspergillus diseases in patients with chronic lung disorders and are referred to as diagnostic criteria in many guidelines (Stevens, 2003; Delhaes et al., 2010; Agarwal et al., 2017). They are found in many patients with ABPA, bronchitis and invasive aspergillosis, and several assays are available. In the clinical routine, in recent years, technologies like ImmunoCAP have continuously replaced older methods like immunoprecipitation (Van Hoeyveld et al., 2006; Baxter et al., 2013b). The ImmunoCAP technique provides quantitative measures and reproducible results and therefore a higher sensitivity and specificity in detecting aspergillus

conditions (Baxter et al., 2013b). However, there is still a lack of standardization in terms of cut-off values, as several working groups examining *Af* IgG in *Af* related conditions obtained discrepant results, which is in part due to different study designs and different laboratory methods used, but this may also reflect the different host-pathogen-interaction of heterogeneous study populations with different underlying diseases (Agarwal et al., 2017; Sehgal et al., 2018; Alghamdi et al., 2019; Sehgal et al., 2019). We therefore evaluated specific *Af* IgG in ABPA, *Af* bronchitis and invasive aspergillosis in CF.

MATERIALS AND METHODS

This prospective study was conducted between January 2016 and December 2019 at Christiane Herzog Cystic Fibrosis Center, Charité—Universitätsmedizin Berlin, Germany. Ethical aspects were considered and approval for the study was gained by the Ethics Committee of Charité—Universitätsmedizin Berlin, Germany (Number EA2/121/16).

We collected sera from 109 patients with CF, ages 6–69 years, independent of their history of *Af* related conditions. To obtain both a control group and a disease group, *Af*-specific IgG was determined either as part of a diagnostic workup when *Af* conditions were suspected or without such suspicion in pulmonary exacerbated and in clinically stable patients during routine checkup. *Af*-specific IgG (ImmunoCAP Gm3) was determined in our routine laboratory. Depending on the diagnosis criteria for *Af* related conditions in CF, the patients were categorized into four groups: control, *Af* bronchitis, ABPA, and *Af* pneumonia. As a few patients were assessed several times during the course of the study, some follow-up measurements for Gm3 are available and listed below.

Diagnosis Criteria for *Af* Related Conditions

Diagnosis of ABPA was based on the minimal 2003 Cystic Fibrosis Foundation (CFF) consensus criteria: i) serum IgE >500 IU/ml; ii) *Af*-specific IgE >0.35 kU/L; iii) clinical deterioration not attributed to other causes; and one of the following criteria: i) presence of *Af* IgG (Gm3 ImmunoCAP) antibodies in serum; ii) new or recent abnormalities on chest radiography or chest CT that have not cleared with antibiotics and standard physiotherapy. In one patient, diagnosis of ABPA was assumed despite a total IgE <500 kU/L due to a threefold increase in total IgE and positive diagnosis criteria.

Af bronchitis was defined as: i) clinical deterioration with repeatedly positive sputum and *Af* PCR or repeatedly positive sputum cultures (≥2 during the past 6 months) for *Af*, ii) no antibiotic treatment response, iii) total serum IgE level <200 kU/L, iv) no observation of new pulmonary infiltrates, and v) appropriate antifungal treatment response.

Criteria for diagnosis of *Af* pneumonia were i) positive sputum *Af* PCR or repeatedly positive sputum cultures for *Af*, ii) new or recent abnormalities on chest radiography or chest CT that have not cleared with antibiotics and standard physiotherapy, iii) no antibiotic treatment response, and iv) total serum IgE level <200 kU/L. In two patients, diagnosis of pneumonia was postulated due to repeatedly positive sputum cultures for *Af* combined with typical radiographic features.

Subjects were excluded if they met any of the following criteria: i) intake of systemic glucocorticoids >5 days for ABPA diagnosis and/or antifungal therapy >5 days within the last 4 weeks; ii) failure to provide informed consent. Patients on standard therapy for *Af* diseases in CF (corticosteroid therapy and/or antifungal treatment) were excluded to enable a clear diagnosis and follow up examination after therapy.

For the available follow up measurements of Gm3, data >30 and <180 days after diagnosis and initiation of therapy were included if the patients were stable at that time and did not meet the diagnostic criteria for *Af* disease.

Af-Specific IgG (Gm3)

Af-specific IgG (Gm3) concentration was measured in our routine laboratory by the commercial ImmunoCAP system (Phadia, Thermo Fisher Scientific, USA), which uses automated fluorescent enzyme immunoassay (FEIA) technology. The m3 antigen is a crude extract from whole *Af* conidia and mycelia. According to manufacturer data, the reference value is <39 mg/L.

Statistical Analysis

Median and ranges were calculated for metrical variables. Distribution of data was assessed with Shapiro–Wilk test for normal distribution. For comparison of two groups *t* test or Mann–Whitney test was applied as appropriate. Comparison of more than two groups with normally distributed data sets was performed with one-way ANOVA including Tukey´s multiple comparison test. Not normally distributed data were analyzed by Kruskal Wallis test. Frequency and percentage were used for categorical variables. Associations of categorical variables were analyzed using chi-square test. Cut-off value determination for IgGm3 was based on receiver operating characteristic (ROC) analysis. A P value < 0.05 was accepted to indicate statistical significance. Data analysis were performed with GraphPad Prism version 8 (GraphPad Software).

RESULTS

Baseline Characteristics

One hundred nine sera of patients with CF were analyzed for *Af* IgG (Gm3 ImmunoCAP), with 63 patients assigned to the control group, 22 patients to the bronchitis group, 18 patients to the ABPA group and six patients to the pneumonia group. The median age of all participants was 26 years (range: 6–69 years). The median BMI was 19.8 kg/m^2, with the lowest BMI in the pneumonia group (17.5 kg/m^2). The median percent predicted FEV_1 was 54 (range: 16.0–133.1), with the highest median percent predicted FEV_1 in the ABPA group (60) and the lowest median percent predicted FEV_1 in the pneumonia group (22, p < 0,01). 24.8% of the patients had diabetes, with the highest proportion in the pneumonia group (66.7%, no statistical significance). The mean total IgG was 13.2 kU/L and was markedly higher in the pneumonia group (16.3 kU/L, no

statistical significance). Co-colonization with *Candida* spp. was documented in 85.3% of all patients (control group 84.1%, bronchitis 90.9%, ABPA and pneumonia each 83.3%, no statistical significance). Detection of other fungal pathogens was documented in 11% of all patients (22.7% bronchitis, 5.6% ABPA, 0% pneumonia, no statistical significance) (see **Table 1**).

Af-Specific IgG (Gm3) Results

Median Gm3 for the control group was 48.9 mg/L ± 40.15 (2.7 - >200 mg/L), for the bronchitis group 51.2 mg/L ± 38.6 (18.2 - 145 mg/L), for the ABPA group 97.5 mg/L ± 56,34 (24.2 - >200 mg/L), and for the pneumonia group 95.3 mg/L ± 22.5 (78.2 - 140 mg/L, $p < 0.01$, (see **Figure 1**).

A statistically significant difference was found between median Gm3 of control vs. ABPA group ($p < 0.05$), and between median Gm3 of control vs *Af* pneumonia group ($p < 0.05$). No significant difference was found between median Gm3 of control vs. bronchitis group (see **Figure 1**).

In the *Af* bronchitis and ABPA group, several patients did not meet the manufacturer's cut-off value of >39 mg/L: in the bronchitis group, 31.8% (n=7) patients had Gm3 levels below the cutoff, and in the ABPA group, 22.2% (n=4) patients had Gm3 levels below the cutoff. In contrast, in the control group, a majority of 58.7% (n=37) patients had Gm3 levels above the cutoff. In the *Af* pneumonia group, all patients had Gm3 levels above the manufacturer`s cut-off of >39 mg/L. (see **Figure 1**).

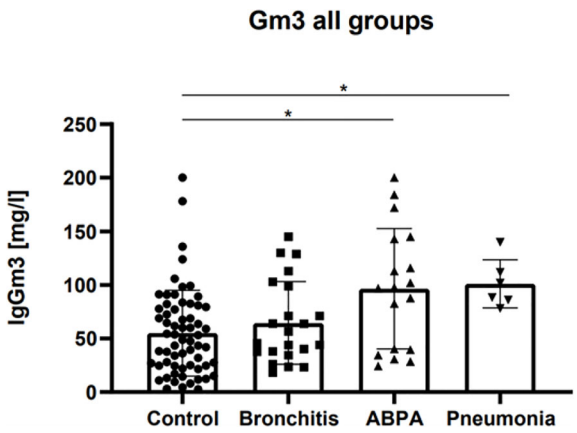

FIGURE 1 | Gm3 (mg/L) for control group, *Af* bronchitis group, ABPA group and *Af* pneumonia group before treatment. Adjusted p-values: control vs. bronchitis: ns, control vs. ABPA: $p < 0.05$, control vs. pneumonia: $p < 0.05$. * represents the level of significance ($p \leq 0.05$).

Follow-Up Gm3

Twenty-one patients could be enrolled for a follow-up examination. In the *Af* disease groups, it was assessed between 30 and 180 days after diagnosis. For the control group, mean

TABLE 1 | Clinical characteristics of included patients.

*Variable	Total	Control	Bronchitis	ABPA	Pneumonia	*P*-value
Number of patients, n	109	63	22	18	6	
Age, years, median (range)	26.0 (6–69)	27.0 (6–69)	26.0 (8.0–57.0)	21.5 (9.0–49.0)	30.5 (15.0–43.0)	ns
Female sex, n (%)	62 (56.9%)	37 (58.7%)	14 (63.6%)	9 (50.0%)	2 (33.3%)	ns
CFTR dF508 homozygous, n (%)	49 (45.0%)	26 (41.3%)	12 (54.5%)	8 (44.4%)	3 (50.0)	ns
BMI, kg/m^2, median (range)	19.8 (12.4–34.2)	20.2 (12.4–34.2)	20.2 (14.5–29.5)	18.8 (14.6–26.9)	17.5 (15.3–23.5)	ns
Percent-predicted FEV1, median (range)	54.0 (16.0–133.1)	60.0 (17.0–133.1)	41.0 (16.3–96.6)	60.0 (27.0–90.0)	22.0 (16.0–57.0)	<0.01
Diabetes, n (%)	27 (24.8%)	12 (19.1%)	6 (27.3%)	5 (27.8%)	4 (66.7%)00	ns
Pancreatic insufficiency, n (%)	100 (91.7%)	57 (90.5%)	20 (90.9%)	17 (94.4%)	6 (100%)	ns
Total IgG, kU/L, median (range)	13.2 (2.0–73.0)	13.2 (6.0–73.0)	12.3 (2.0–21.9)	13.6 (6.2–25.2)	16.3 (13.0–22.0)	ns
Gm3, mg/L, median (range)	60.0 (3.0–200)	48.9 (2.7–200)	51.2 (18.2–145)	97.5 (24.2–200)	95.3 (78.2–140)	<0.01
Gm3 > 39 mg/L, n (%)	72 (66,1)	37 (58,7)	15 (68,2)	14 (77,8)	6 (100)	
Total IgE, kU/L, median (range)	34.5 (2.0–4359)	26.5 (2.5–199)	30.2 (2.1–262)	1048 (344–4359)	44.4 (2.0–84.5)	<0.01
Aspergillus specific IgE, kU/L, median (range)	0.1 (0.1–51.7)	0.1 (0.1–0.1)	0.1 (0.1–3.2)	25.7 (10.8–51.7)	0.1 (0.1–1.0)	<0.01
Steroids, n (%)	17 (15.6%)	0 (0%)	8 (36.4%)	7 (38.9%)	2 (33.3%)	<0.01
Antifungal treatment (Caspofungin, Itraconazole, Posaconazole), n (%)	8 (7.3%)	0 (0%)	2 (9.1%)	5 (27.8%)	1 (16.7%)	<0.01
Chronic *Pseudomonas aeruginosa*, n (%)	71 (65.1%)	37 (58.7%)	17 (77.3%)	13 (72.2%)	4 (66.7%)	ns
Aspergillus fumigatus positive sputum, n (%)	45 (41.3%)	21 (33.3%)	17 (77.3%)	5 (27.8%)	2 (33.3%)	<0.01
Aspergillus PCR positive, n (%), n (%)	60 (55.0%)	30 (47.6%)	16 (72.7%)	12 (66.7%)	2 (33.3%)	ns
Candida spp. positive sputum, n (%)	93 (85.3%)	53 (84.1%)	20 (90.9%)	15 (83.3%)	5 (83.3%)	ns
Detection of other fungal pathogens*, n (%)	12 (11.0%)	6 (9.5%)	5 (22.7%)	1 (5.6%)	0 (0%)	ns

**Except Candida and Aspergillus species.*

Gm3 was assessed at two arbitrary points in time. The mean difference of the control group was 1.6 mg/L. For the bronchitis group, the mean Gm3 difference after initiation of therapy was 13.4 mg/L, and for the ABPA group, it was 12.6 mg/L (no statistical significance). In both the bronchitis and ABPA group, single patients showed a rise in Gm3 in the follow up (see **Figure 2**).

Cut-Off Determination

To determinate a cut-off value, only CF control subjects (n = 63) and CF patients with ABPA or pneumonia (n = 24) were included. ROC curve analysis showed an area under the curve of 0.7675 (95% Confidence Interval = 0.6524–0.88826, see **Figure 3**) A cut-off value of 78.05 mg/L for Gm3 was calculated to discriminate controls and patients with ABPA/pneumonia with a specificity (white square line) of 75% and a sensitivity (black square line) of 74.6% (see **Figure 4**).

DISCUSSION

In this study, we evaluated the utility of *Af* specific Gm3 for the diagnosis of *Af* associated diseases in CF. Mean Gm3 levels revealed a significant difference between control group vs. ABPA and vs. pneumonia group, but not vs. bronchitis group. *Af* specific IgG may therefore be a useful biomarker for acute ABPA and *Af* pneumonia in cystic fibrosis, but not for *Af* bronchitis in cystic fibrosis.

However, we also found a wide distribution of Gm3 in the control, bronchitis and ABPA group. Thus, for the diagnosis of ABPA and *Af* pneumonia in CF, Gm3 should only be interpreted alongside other biomarkers.

Interestingly, a significant proportion of the Gm3 of our control group exceeded the manufacturer`s cut-off-level of 39 mg/L. Due to the high exposure of the CF lung to respiratory pathogens, the immune response in CF—here in the form of Gm3—may fundamentally differ from that of healthy individuals. Specific cut-offs may therefore be required in CF to describe pathogen-mediated conditions. Positive and even high titers of

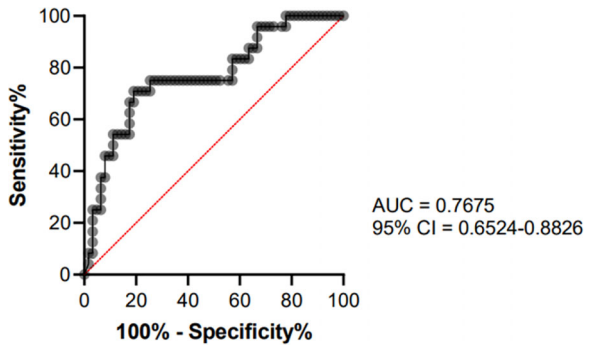

FIGURE 3 | ROC curve analysis showing the performance of Gm3 for distinguishing control group from ABPA/*Af* pneumonia group.

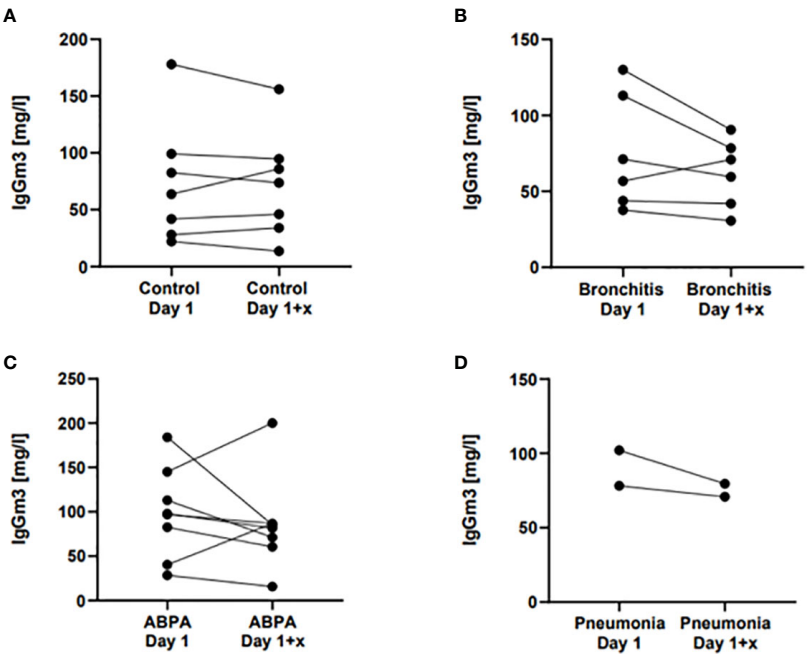

FIGURE 2 | Gm3 follow-up measurements for **(A)** control group, **(B)** *Af* bronchitis group, **(C)** ABPA group and **(D)** *Af* pneumonia group, for each group at time of diagnosis (Day 1) and after treatment (Day 1 +x), for control group at any two consecutive times. Adjusted p-values: no statistical significance.

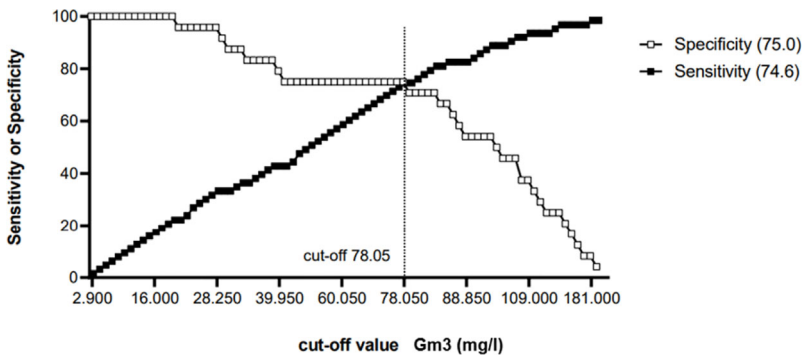

FIGURE 4 | Cut-off-determination: Gm3 = 78.05 mg/L with a specificity (white square line) of 75% and a sensitivity (black square line) of 74.6%.

Gm3 do not necessarily represent *Af*-associated disease. On the other hand, the pathogenic impact and the need for treatment of chronic colonization with *Af* in CF is still under discussion. In our cohort, 33% of the control group patients had *Af* positive sputum (see **Table 1**). There could be cases of bronchitis in the control group that did not meet the definition of bronchitis. Another reason for high Gm3 levels in the control group may be cross reactivity to other fungi colonizing the respiratory tract in CF, as m3 is the crude *Af* antigen and shows cross reactivities to other opportunistic fungi (Fukutomi et al., 2016). In our cohort 85.3% were co-colonized with *Candida* spp. and 11% with other fungi (see **Table 1**). If this problem could be overcome by using recombinant antigens, is unclear, because so far, no investigation has been performed under this aspect. The studies conducted with recombinant antigens to date show vastly different results (Nikolaizik et al., 1995; Kurup, 2005; Fricker-Hidalgo et al., 2010; Alghamdi et al., 2019). Finally, in some patients in the cohort of acute *Af* diseases, Gm3 exceeds the measurement range of 200 mg/L. Quantification above 200 mg/L could therefore improve sensitivity and specificity.

The non-significant difference in median Gm3 levels between control and bronchitis group could be explained by a predominantly endobronchial host-pathogen interaction in *Af* bronchitis, which may less likely lead to IgG production than in the more invasive forms of aspergillosis. This may also explain why a significant proportion of the bronchitis group had Gm3 levels below the manufacturer`s cut-off. On the other hand, also a small but still significant proportion of the ABPA group had Gm3 levels below the manufacturer`s cut-off, which may be due to a predominant Type 1 hypersensitivity reaction in these cases.

Our results of a Gm3 cut-off-level of 78.05 mg/L with a sensitivity of 75% and a specificity of 74.6% in *Af* pneumonia and ABPA in CF differ substantially from those found in other populations. Agarwal et al. examined *Af* specific Gm3 in asthmatic patients with ABPA and *Af* sensitization without ABPA and found a sensitivity of 88% and specificity of 100% when using a cut-off level of 26.9 mg/L ImmunoCAP Gm3 (Agarwal et al., 2017). In chronic pulmonary aspergillosis in non-CF-patients, Sehgal et al. found a sensitivity and specificity of 95.6% and 100% using a Gm3 cut-off of 27.3 mg/L (Sehgal et al., 2018). For simple aspergilloma in pulmonary tuberculosis patients, the same working group found a sensitivity of 63.5% and a specificity of 98.3% using a cut-off of 27.3 mg/L. However, the Gm3 cut-off-level is still under discussion, as a recent multicentric study found a median Gm3 of 21.3 mg/L and a 90% quantile for Gm3 of 78 mg/L in a population of 121 healthy donors (Raulf et al., 2019). The working group suggested that IgG values higher than at least 90% of the healthy donor group indicate elevated levels, which is more consistent with our findings of a cut-off-level of 78.05 mg/L.

Our follow-up data, where some patients showed a rise in Gm3 at a second point of time after diagnosis are consistent with the data of Agarwal et al. in asthmatic patients with ABPA, who observed an increase of Gm3 in 23.1% of the patients and a decrease in 23.1% (Agarwal et al., 2017). However, immunoglobulins are often maintained long-time following antigen contact, and, so far, no longitudinal data exist for titer progression of Gm3. Considering the small number of cases in our cohort, no statement can be made if and after what time Gm3 is suitable as a follow-up marker. Due to the low prevalence of *Af* diseases in CF, the suitability of Gm3 as a screening and outcome parameter should be investigated in a multicenter study.

CONCLUSION

Af-specific IgG may be a useful biomarker for acute ABPA and *Af* pneumonia, but not for *Af* bronchitis in CF. However, due to the large interindividual variability of Gm3, it should only be interpreted alongside other biomarkers. Therefore, due to its broad availability, it could be suitable as a biomarker for ABPA and *Af* pneumonia in CF, if the results can be supported by a larger multicenter cohort. Standardized, longitudinal studies and a better knowledge of underlying immunological mechanisms may improve its usability.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethikkommission Charité Universitätsmedizin Berlin. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

PE, CG, and CS contributed to the conception and design of the study. PE and CG organized the database. CG performed the statistical analysis. PE, CG, and CS wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Azole Resistance in *Aspergillus fumigatus*: A Five-Year Follow Up Experience in a Tertiary Hospital With a Special Focus on Cystic Fibrosis

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Azole-resistant *Aspergillus fumigatus* (AR*Af*) has emerged worldwide during the last decades. Drug pressure after long term treatments of chronically infected patients and the propagation of environmental clones selected under the pressure of imidazoles fungicides used in agriculture and farming both account for this emergence. The objectives of this study were to determine the rate of azole resistance in *Aspergillus fumigatus* during a 5-year period, taking into account (i) differences between underlying diseases of the patients treated, (ii) cross-resistance between azoles, and (iii) focusing on the 5-year evolution of our center's cystic fibrosis cohort. Overall, the rates of voriconazole (VRC)-resistant and itraconazole (ITC)-resistant *A. fumigatus* isolates were 4.1% (38/927) and 14.5% (95/656), respectively, corresponding to 21/426 (4.9%) and 44/308 (14.3%) patients, respectively. Regarding cross-resistance, among VRC-R isolates tested for ITC, nearly all were R (20/21;95%), compared to only 27% (20/74) of VRC-R among ITC-R isolates. The level of azole resistance remained somewhat stable over years but greatly varied according to the azole drug, patient origin, and clinical setting. Whereas azole resistance during invasive aspergillosis was very scarce, patients with cystic fibrosis were infected with multiple strains and presented the highest rate of resistance: 5% (27/539) isolates were VRC-R and 17.9% (78/436) were ITC-R. These results underline that the interpretation of the azole resistance level in *Aspergilllus fumigatus* in a routine setting may consider the huge variability depending on the azole drug, the clinical setting, the patient background and the type of infection.

Keywords: azole resistance, *cyp51A*, *Aspergillus fumigatus*, cystic fibrosis, invasive aspergillosis, hematology, intensive care unit

INTRODUCTION

Aspergillus fumigatus, a ubiquitously distributed opportunistic pathogen, is the leading agent of aspergillosis, ranking first among fungal killers. In profoundly immunocompromised patients, the clinical picture rapidly evolves towards an acute angio-invasive form or invasive aspergillosis (IA) that accounts for one of the major severe invasive fungal diseases (Ullmann et al., 2018). In the immunocompetent individual, the infection is generally limited to cavitating, chronically evolving forms (such as aspergilloma), chronic fibrotic or immuno-allergic forms (allergic bronchopulmonary aspergillosis and severe asthma with *Aspergillus* sensitization) (Denning et al., 2016). During cystic fibrosis, abnormally viscous bronchial secretions prevent mucociliary clearance and promote the trapping and the proliferation of inhaled bacteria and fungal spores (Prigitano et al., 2017). *A. fumigatus* is the most frequent filamentous fungus colonizing the airways of patients with cystic fibrosis (CF) followed by *Scedosporium* sp. (Pihet et al., 2009; Felton and Simmonds, 2014; Hamprecht et al., 2018). Recent publications underlined that patients with *Aspergillus* in the airway have greater abnormalities on CT imaging, particularly in children, at the time of infection and in the following years, as shown in longitudinal studies (Breuer et al., 2019).

Voriconazole (VRC), isavuconazole (ISA), itraconazole (ITC), and posaconazole (POS) are four triazole antifungals recommended as first-line drugs in the treatment or prophylaxis of aspergillosis. They inhibit the lanosterol 14-α-demethylase enzyme (Cyp51A) encoded by the *cyp51A* gene, thereby inhibiting the ergosterol synthesis. Voriconazole and isavuconazole are highly recommended for the treatment of IA (Ullmann et al., 2018). Posaconazole shows a very wide spectrum of activity and its primary clinical indications are i) salvage therapy for patients with IA and ii) prophylaxis in patients with neutropenia and hematopoietic cell transplantation (Ullmann et al., 2018). It has also been reported as an alternative treatment for ABPA in CF patients (Periselneris et al., 2019). Itraconazole is the oldest but still robust triazole drug used in chronic and immuno-allergic aspergillosis and allows minimizing the use of corticosteroids (Denning et al., 2016). Azole resistance in *A. fumigatus* isolates is increasingly reported with variable prevalence in the six continents (Chowdhary et al., 2013; Meis et al., 2016; Wiederhold and Verweij, 2020). Two main origins of the emergence of azole resistant *A. fumigatus* (*ARAf*) are recognized: (i) long-term use of triazoles in patients with chronic *Aspergillus* infections and (ii) increased use of agricultural fungicides against plant-pathogenic moulds, such as *Fusarium, Mycosphaerella* and *A. flavus*, with cross-activity against *A. fumigatus* (Meis et al., 2016).

A global rate of resistance of *A. fumigatus* is however a metric with little significance, especially if we do not take into account variability between patients and drugs. During CF, particularly-high rates of AR*Af* have been reported (Stevens et al., 2003; Mortensen et al., 2011; Burgel et al., 2012; Morio et al., 2012; Meis et al., 2016; Prigitano et al., 2017; Risum et al., 2020; Wiederhold and Verweij, 2020) while azoles are recommended in the guidelines for the management of allergic bronchopulmonary aspergillosis (ABPA) (Stevens et al., 2003).

The aim of this prospective study was to estimate the frequency of azole resistance in *A. fumigatus* isolated from patients in our tertiary care University hospital, over a 5-year period. In particular, we analyzed the prevalence of AR*Af* according to patient background and azole drug, the cross resistance between azoles, and the characteristics of the longitudinal carriage of *Aspergillus* in CF patients, and more particularly of AR*Af*.

METHODS

Patients

Data of antifungal susceptibility testing performed on *A. fumigatus* clinical isolates as a part of the routine lab procedure between January 2015 and December 2019 were retrospectively collected. A total of 929 A. *fumigatus* isolates from 426 patients were tested for susceptibility to at least one azole. Among them, 595 isolates were obtained from sputum samples or throat swabs from 123 CF patients included in the cohorts of adult (N = 87) and pediatric (N = 36) "Centres de Ressources et de Compétences de la Mucoviscidose" at Rennes University Hospital (France). The remaining strains were isolated from patients hospitalized in ICU (N = 57), Pulmonology (N = 159), Hematology (N = 13) or other clinical wards (N = 105), and used for comparison to isolates from CF patients.

Mycological Examination

(i) Culture and identification. All isolates of *Aspergillus fumigatus* were obtained after a culture of respiratory samples, using two Sabouraud dextrose agar slants supplemented with 0.5% chloramphenicol incubated at 30°C and 37°C for up to 7 days. Depending on the date of isolation, identification was performed using macroscopic and microscopic examinations of cultures, combined to MALDI-TOF mass spectrometry (MALDI Biotyper, Brucker France, Marne-la-Vallée) or molecular sequencing since 2018.

(ii) *In vitro* susceptibility to azoles. Itraconazole (ITC), voriconazole (VRC) and posaconazole (POS) minimum inhibitory concentrations (MICs) were determined using Etest® strips and RPMI medium (Biomérieux, Marcy-L'Etoile, France) according to the manufacturer recommendations. MICs were determined following a 48 h incubation at 37°C. Strains with MIC ≥2 mg/L for ITC and VRC, and ≥0.25 mg/L for POS, were considered resistant (R), according to recent EUCAST breakpoints for fungi (European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2020). Of note, isolates with MICs at 2 mg/L for ITC and VRC and 0.25 mg/L for POS (graded in ATU group, Area of Technical Uncertainty) remain suitable drugs for treatment depending on the clinical setting,

following latest EUCAST recommendations (European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2020). All other MICs classified isolates as susceptible (S), and therapeutic advice was given with the recommendation to use the tested drug.

(iii) Molecular characterization of *cyp51A* gene. While not performed routinely, the promoter and the whole *cyp51A* gene were sequenced in both strands from *A. fumigatus* isolates in some patients with repeated high MICs and/or clinical failure, using five sets of primers: PA5 and PA7 (Mellado et al., 2001), AF306F and AF855R, AF766F and AF1330R, AF1179F and AF1709R, and AF1426F and AF2025R (Alanio et al., 2011).

The PCR mixture contained 5 µl of DNA extract and 20 µl of a mix composed of 0.625 U of GoTaq® Hot Start Polymerase (Promega, Charbonnières-les-Bains), 1x Colorless GoTaq®Flexi Buffer (Promega), 2mM of MgCl2 (Promega), 0.8mM of dNTP mix (Eurobio, Les Ulis), and 0.2 µM of each primer. The amplification program consisted of 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 58°C, and 1 min at 72°C, followed by a final step of 10 min at 72°C. Sequences of resistant strains were compared to the wild-type *A. fumigatus* sequence CM 237 (GenBank accession number AF338659), at http://blast.ncbi.nlm.nih.gov/Blast.cgi.

Ethics and Statistics

According to the French Public Health Laws (Code de la Santé Publique, 2017), protocols of this type do not require approval from an ethics committee and are exempt from the requirement of formal informed consent. MIC values were expressed as median and interquartile range. Data analysis was performed using the GraphPad PRISM® v.5.02 software. For comparison of proportion, the Fisher's test was used. A p-value of 0.05 was considered statistically significant.

RESULTS

Global Prevalence of Resistance in Azoles

Before 2015, *A. fumigatus* isolated in our center were considered as *a priori* susceptible to azoles and resistance monitoring with MICs was only performed in case of treatment failure. The growing number of treatment failures stressed the need to perform a systematic MIC determination to VRC and/or ITC, for each *A. fumigatus* isolated from 01/2015 to 01/2020. A total of 927 and 656 strains were evaluated for VRC and ITC susceptibility, respectively. Susceptibility to POS was tested in case of MIC ≥2 mg/L for VRC and ITC, or when the patient had a history of AR*Af* carriage.

From 2015 to 2019, the rates of VRC-resistant and ITC-resistant *A. fumigatus* isolates were 4.1% (38/927) and 14.5% (95/656), respectively; they were recovered from 21/426 (4.9%) and 44/308 (14.3%) patients, respectively. During the last 4 years with an exhaustive dataset of VRC MIC determination (2016–2019), the rate of VRC-resistant *A. fumigatus* remained stable, with 3.8% (8/183), 4.8% (10/209), 3.0% (6/201), and 3.6% (8/223) (ns), with an overall median MIC at 4 [2; 8] mg/L (**Figure 1**). An exhaustive dataset was obtained during the last 3 years for ITC and showed a consistent rate at 9.7% (20/206) in 2017, increasing to 16.8% (34/201), and 17% (30/176) in 2018 and 2019, respectively (ns). The median MIC of ITC-resistant isolates was 14 [3; 32] mg/L. Among these resistant strains, 20/95 (21%) isolates and 10/38 (26.3%) had ITC MICs and VRC

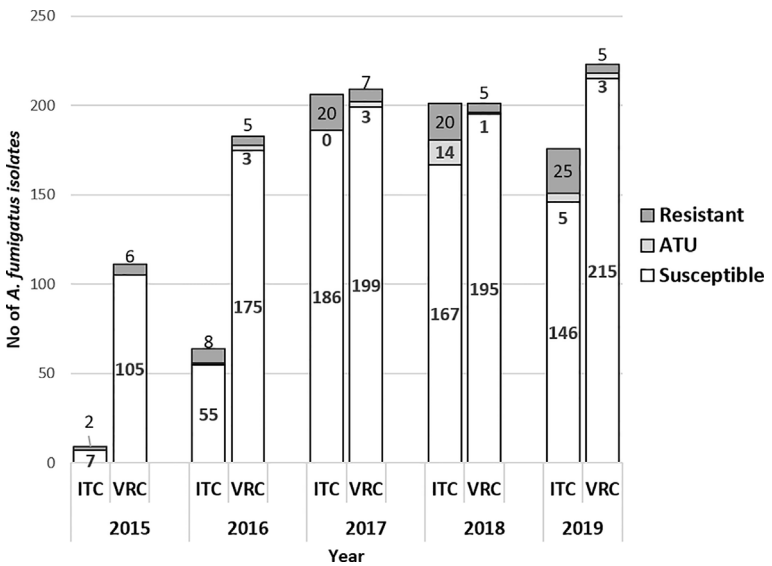

FIGURE 1 | Number of *A. fumigatus* strains susceptible, ATU and resistant to ITC and VRC per year (2015–2019, N = 929) (VRC, n = 927); (ITC, n = 656). White bars indicate susceptible isolates; dark grey bars indicate resistant isolates with MIC >2mg/L and light grey bars represent isolates resistant isolates in ATU group (MIC = 2 mg/L). ITC, itraconazole; VRC, voriconazole; ATU, Area of Technical Uncertainty.

MICs in the ATU range, respectively, corresponding to 20/656 (3.0%) and 10/927 (1.1%) of all tested isolates for ITC and VRC, respectively (**Figure 1**).

2. Prevalence of Azole Resistance According to the Clinical Setting

Patients From the Cystic Fibrosis Cohort

CF patients are managed in special units. They are sometimes hospitalized in other Pulmonology units, but we have reclassified them as "CF patients" because this cohort of patients is followed individually. A total of 595 A. *fumigatus* isolates were collected from respiratory samples of 123 CF patients and screened for VRC and/or ITC and/or POS *in vitro* susceptibility, of whom 34 patients presented with at least one isolate resistant to one drug. The rate of isolates resistant to at least one azole reached 90/595 (15.1%) over the period 2015–2019, among which three isolates were only POS-R. Regarding VRC, the global frequency of resistant *A. fumigatus* strains from 2016 to 2019 was 5% (27/539), recovered from 13 out of 119 patients (10.9%). The rate of VRC-R isolates was relatively steady from 2016 to 2019 (**Figure 2**). Over the same period (2016–2019), the rate of ITC-R isolates was higher than that of VRC-R, with 78/436 (17.9%) resistant isolates, recovered from 29/111 (26.1%) patients. A tendency to increase was noticed, with rates of AR*Af* detection from 14 to 24% from 2017 to 2019, respectively.

Pulmonology

A total of 159 isolates from 143 patients hospitalized in pulmonology, excluding CF patients, were screened for azole susceptibility between 2015 and 2019. Only four isolates from four patients were resistant to ITC (including one ATU isolate), of which one was also resistant to VRC (**Table 1**). Another isolate was ATU to VRC only.

Intensive Care Units

Fifty-seven isolates from 50 patients were screened for azole resistance from 2015 to 2019. Six patients harbored an ITC-R isolate (of which 4 were ATU) and two isolates from one patient were VRC-R, none of them with azole cross resistance (**Table 1**). None of these patients were CF. A few patients with

TABLE 1 | Itraconazole (ITC) and voriconazole (VRC) resistance of *A. fumigatus* isolates from patients from pulmonology (excluding CF), intensive care unit, and other units.

Year	Number of *A. fumigatus* isolates resistant to antifungals* (n/N)					
	Pulmonology		ICU		Other	
	ITC	VRC	ITC	VRC	ITC	VRC
2015	0/1	0/30	nd	0/9	0/4	1/18
2016	0/2	0/21	0/2	0/6	0/5	0/21
2017	0/35	0/35	1/9	0/10	0/25	0/27
2018	4/31	1/31	4/15	0/15	1/15	0/15
2019	0/35	1/42	1/11	2/17	4/26	1/37
Total	4[1]/104	2[2]/159	6[3]/37	2/57	5[4]/75	2[2]/118[$]
%	3.8%	1.3%	16.2%	3.5%	6.6%	1.7%

ICU, intensive care unit; ITC, itraconazole; VRC, voriconazole; nd, not determined
**MIC ≥ 2 mg/L.*
[$]Among them, 13 isolates were from hematology patients; 0/13 were resistant.
[1]Among them, 1/4 was ATU.
[2]Among them, 1/2 was ATU.
[3]Among them, 4/6 were ATU.
[4]Among them, 1/5 was ATU.

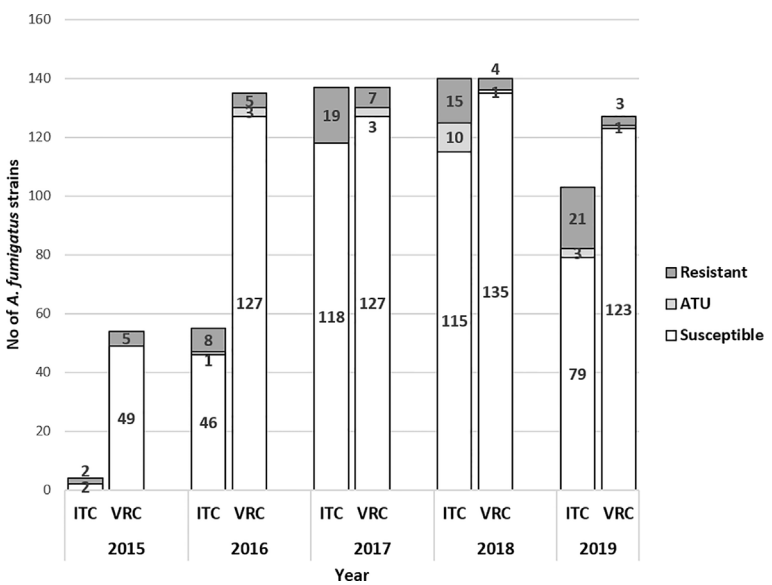

FIGURE 2 | Number of *A. fumigatus* strains susceptible, ATU and resistant to itraconazole (ITC) and voriconazole (VRC) per year in CF patients (2015–2019, N = 595 (VRC, n = 593); (ITC, n = 440). White bars indicate susceptible isolates; dark grey bars indicate resistant isolates with MIC >2mg/L and light grey bars represent isolates resistant isolates in ATU group (MIC = 2 mg/L). ITC, itraconazole; VRC, voriconazole; ATU, Area of Technical Uncertainty.

hematological malignancies have been occasionally hospitalized in ICU. We keep them as "ICU patients" as the specific patient management in ICU (mechanical ventilation for example) is different and can contribute to modify the epidemiology.

TABLE 2 | Prevalence of azole resistance in *A. fumigatus* isolates tested simultaneously for the three azole antifungals (N = 56).

Susceptibility profile	Azole antifungal			Number
	ITC	VRC	POS	
No azole R	S	S	S	20
R to 1 azole	**R**	S	S	2
	S	**R**	S	1
	S	S	**R**	3
R to 2 azoles	**R**	S	**R**	14
	S	**R**	**R**	1
	R	**R**	S	1
R to 3 azoles	**R**	**R**	**R**	14

R, resistance; S, susceptibility.

Other Wards

Cultural analysis in Hematology patients remains rare for the diagnosis of IA compared to galactomannan determination in the serum used for screening in high-risk patients. Only 13 A. *fumigatus* were isolated and tested in patients with hematological malignancies since 2015 and no azole resistance was detected. These patients are grouped with patients from other units in **Table 1**. In patients with miscellaneous clinical backgrounds (other units), five were infected with an ITC-R strain (co-resistant to VRC in one case), of which one was an ATU isolate.

Cross-Resistance Between Azoles

We then analyzed whether there was cross-resistance between the main azole antifungals used routinely, in order to help for the decision of doctors excluding ATU isolates. Among the 83 A. *fumigatus* strains with high MIC for ITC and/or VRC (> 2 mg/L), 20 (24%) were resistant to both ITC and VRC. However, the proportions of ITC-R/VRC-S and ITC-S/VRC-R differed greatly. Among VRC-R isolates tested for ITC, nearly all were R to ITC (20/21;95%), while only 27% (20/74) of ITC-R isolates were VRC-R.

TABLE 3 | Characteristics of isolates obtained from CF patients with at least one resistance to VRC or ITC over the study period (2015–2019).

No	No ofisolates	No of ITC-Risolates	MIC range of ITC-R isolates	No of VRC-R isolates	MIC range of VRC-R isolates	Mean time between isolates (days)	Preexposure to azole
1	1	ND	ND	1	4	NA	ND
2	18	11*	2;>32	10**#	2;>32	84	yes
3	16	4*	2;>32	1	16	101	yes
4	3	1	2	0	NA	458	yes
5	6	4	4;16	0	NA	74	yes
6	4	1	4	0	NA	86	no
7	14	1	2	1#	2	73	no
8	13	1	3	0	NA	104	yes
9	3	1	>32	1	> 32	89	yes
10	9	1	2	0	NA	185	no
11	3	2	>32	1	3	77	yes
12	11	1	2	0	NA	135	no
13	1	1	>32	0	NA	NA	no
14	6	1	24	0	NA	115	ND
15	7	3*	2;12	0	NA	160	yes
16	2	1	16	0	NA	730	ND
17	10	3	3;>32	3*	2;>32	146	yes
18	2	1	8	0	NA	1267	yes
19	3	1	8	0	NA	109	no
20	3	1	16	2	3;12	119	no
21	13	1	2	0	AN	125	ND
22	5	1	8	0	NA	195	yes
23	5	5	8;32	1	4	210	yes
24	14	9	2;>32	0	NA	114	yes
25	1	1	16	1	3	128	yes
26	9	5	6;>32	2*	2;8	169	yes
27	4	2*	2;3	0	NA	54	no
28	8	0		1	2	169	no
29	3	1	2	0	NA	476	no
30	12	4*	2;16	0	NA	123	no
31	13	9*	2;>32	4**	2;3	111	yes
32	12	0		1	3	138	no
33	2	1	32	1	32	1320	no
34	7	1*	2	0	NA	187	no

**Indicates the presence of 1 ATU among R isolates.*

***Indicates the presence of 2 ATU among R isolates.*

#ATU differed from the ITC-ATU isolate.

NA, not applicable; ND, not determined; MIC, minimal inhibitory concentration (mg/L).

In our routine setting, POS is only tested in case of azole-resistance history or clinical failure after VRC or ITC treatment. Of the 56 isolates tested simultaneously for ITC, VRC and POS, 20 (36%) were susceptible to all of them (**Table 2**). A cross-resistance was observed in 54% (30/56) (**Table 2**). Two isolates had a decreased susceptibility to ITC only (MIC at 2 and 3 mg/L, respectively), and one isolate had an increased MIC for VRC only (at 2 mg/L). Among the 31 ITC-R isolates, 28 (90.3%) were POS-R whereas only 15 isolates (48.4%) were VRC-R. Overall, 76 isolates benefited from MIC evaluation to POS, of which 37 (48.7%) were POS-R. Thirty-four of the 37 (91.9%) isolates POS-R were also resistant to ITC and/or VRC, of which 32 were observed in CF patients.

Phenotypic and Genotypic Characterization of Isolates from CF Patients

During the five-year follow-up, 34 out of 123 patients (27.6%) (90/595 isolates, 15.1%) presented at least once with an ARA*f* in induced sputum samples. About half of them (18/34) harbored a single ARA*f* among several *Aspergillus* isolates (2 to 18 consecutive isolates), of which seven were ATU (**Table 3**), whereas three patients had a single positive sputum with ARA*f* and no further positive *Aspergillus* cultures (patients #1, #13, and #25, **Table 3**). As seen in **Table 3**, the range of ITC and VRC MIC for different ARA*f* from a same patient was sometimes huge, suggesting that some patients are colonized with a multiplicity of strains over time. The MIC distribution of all isolates collected from patients with at least one ARA*f* is shown in **Figure 3**.

As an example of the multiplicity of strains, distribution of MICs of consecutive isolates from four CF patients with >10 isolates were recorded over time. **Figure 4** shows that for a given patient, MIC values fluctuated over time, with cultures of susceptible and resistant phenotypes, underlining the diversity of *A. fumigatus* strains colonizing the airways of CF patients.

Cyp51A genotyping of ARA*f* was performed in strains isolated from six CF patients, reflecting the diversity of genetic alterations related to triazole resistance (**Table 4**). Interestingly, *cyp51A* polymorphism were found in 4/6 isolates. While the multi-azole resistant isolate harbored the L98H mutation together with the tandem repeat TR34 (patient 9), the two ITC-R and POS-R ARA*f* displayed the substitutions M220K and G54R respectively (patients 2 and 24). Finally, the combined F46Y, M172V, and E427K amino acid changes were simultaneously found in the ITC-R isolate. Triazole resistance of isolates from patients #3 and #26 was not linked to *cyp51A* polymorphism (wild type genotype) supporting the existence of other biological mechanisms responsible for triazole resistance.

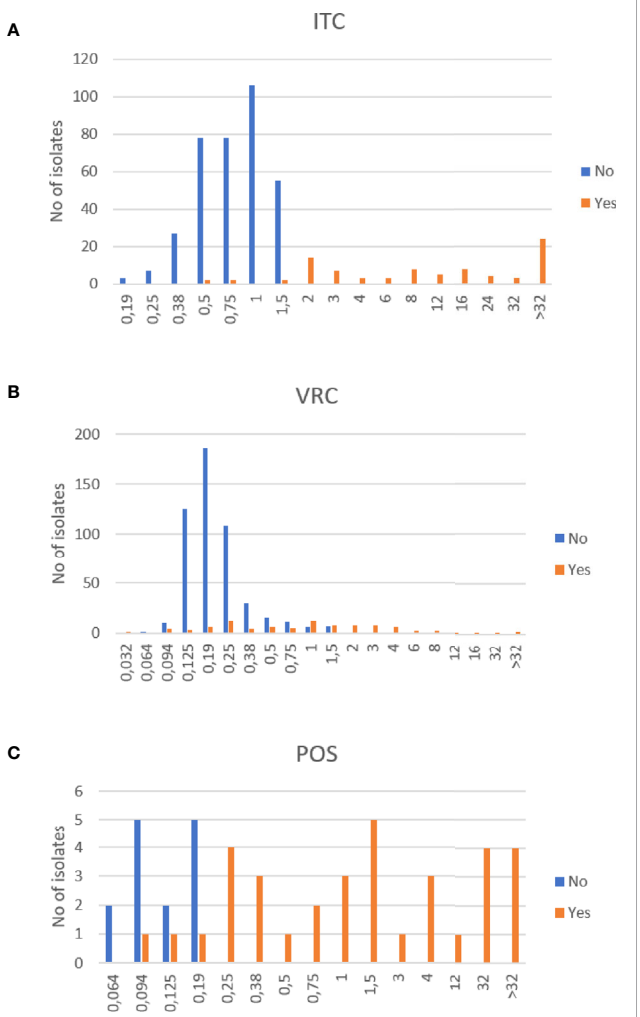

FIGURE 3 | Distribution of azole MICs of *Aspergillus fumigatus* isolates from CF patients. Itraconazole (ITC) MICs **(A)**, voriconazole (VRC) MICs **(B)** and posaconazole (POS) MICs **(C)** of isolates with at least one resistance to one azole (Yes) or with no resistance to any azole (No). Unit: mg/L.

DISCUSSION

The burden of fungal diseases due to *Aspergillus* remains high in France, with multiple forms, from chronic or allergic to invasive aspergillosis (Gangneux et al., 2016). Furthermore, the emergence *Aspergillus fumigatus* isolates resistant to azoles led international experts to recommend the *in vitro* susceptibility screening of isolates and to shift from azole to amphotericin B as first-line treatment of IA when resistance is higher than 10% (Verweij et al., 2015; Ullmann et al., 2018).

Here we analyzed the results of *in vitro* susceptibility of *A. fumigatus* during a 5-year survey in a routine practice. Because the micro-dilution method recommended by EUCAST is difficult to use in routine when numerous isolates are tested per day, we use Etest® strips and RPMI medium in our routine practice as in many hospitals. The global level of resistance of isolates to voriconazole and itraconazole was 4.1 and 14.5%, respectively. This level remained somewhat stable over years. Interestingly, a recent publication from the Netherlands showed that the VRC-resistance frequency was 34% lower in 2018 than in 2013 ($p = 0.0001$) (Lestrade et al., 2020). However, our results show that the level of resistance greatly varied according to azole drug, patient origin and clinical setting. Chronic respiratory diseases and particularly cystic fibrosis are favorable to ARA*f* emergence because of recurrent treatment with azoles. Besides,

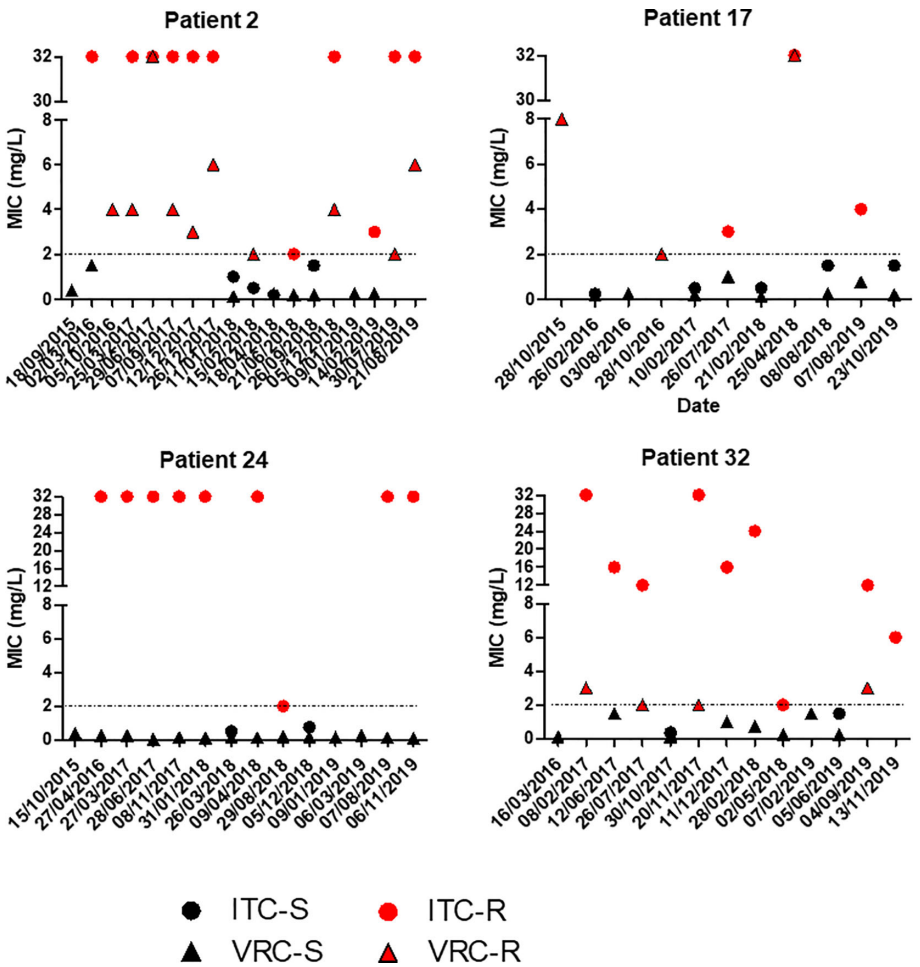

FIGURE 4 | Evolution of itraconazole (ITC) and voriconazole (VRC) MIC values of *A. fumigatus* isolates from four patients in the time. For clarity, isolates with ATU MIC values were gathered with resistant isolates.

TABLE 4 | Profile of azole resistance in CF patients with *cyp51A*-genotyped AR*Af*.

No of patient	ITC MIC	VRC MIC	POS MIC	*cyp51A* mutations
2	>32	1.5	>32	M220K
3	32	16	0.094	none
9	>32	>32	>32	TR34/L98H
24	>32	0.25	>32	G54R
26	32	8	4	none
30	2	0.75	ND	F46Y, M172V, E427K

ND, not determined; MIC, minimal inhibitory concentration (mg/L).

we detected only a very low number of AR*Af* during invasive pulmonary aspergillosis.

Regarding the management of IPA, a few other French papers from Paris area reported similar low levels of ARAf detection (Alanio et al., 2011; Alanio et al., 2016). At the time of hospitalization in Hematology, none of the patients had an AR*Af* isolated, probably linked to the short course of chemoprophylaxis with posaconazole. These results comfort us in the use of azoles as first-line curative drugs (VRC and ISA) or prophylaxis (POS).

Regarding CF patients during 2015–2019, we observed a higher frequency of AR*Af* (11%) than in other French studies. Our center is one of the two tertiary hospitals of the Brittany region which is mainly rural with an intense agricultural and farming activity that could account for high azole resistance rate, and furthermore 16/30 patients with AR*Af* had a pre-exposure to azoles. In the West of France near Brittany, Morio et al., (2012) reported a 8% prevalence of azole resistance in 2010–2011, while this rate was 6.8% four years later, mainly due to a genotypic mutation TR34/L98H (Lavergne et al., 2019). In a cohort from Paris, 4.8% of resistant isolates were detected (Burgel et al., 2012), whereas in a mixed cohort of CF and immunocompromised patients from the North of Paris, only 1.8% of AR*Af* were detected (Choukri et al., 2015). European studies showed that 5.3% (101/2888) of *A. fumigatus* isolates were azole-resistant in a prospective multicenter study in Germany (Seufert et al., 2018). The frequency was 7.1% in the

Netherlands, with TR34/L98H being the dominant resistance mechanism (Engel et al., 2019). In a bicentric Italian study, no resistance was detected in one center, while the frequency of resistance was 8.2% in the other (Prigitano et al., 2017). In Denmark, Risum et al. recently reported the occurrence of AR*Af* strains in 7.3% (10/137) CF patients (Risum et al., 2020).

The issue of cross-resistance remains moderate in our center even if azole exposure is frequent, as VRC remains usable for 73% of ITC-R isolates, and a triple-resistance to azole was very scarce. Importantly, isolates that were both ITC-R and VRC-R were POS-R except in one case. Thus, we decided to stop POS *in vitro* susceptibility testing to the benefit of ISA susceptibility testing. Data obtained during this survey incited us to adopt new rules for *in vitro* testing strategy:

(i) When VRC is considered as the first-line treatment: we perform VRC and ISA susceptibility testing and recommend to switch for liposomal amphotericin B when VRC-R and ISA-R. Such situation is possible during invasive, allergic and chronic aspergillosis.

(ii) When ITC is considered as the first-line treatment: we perform ITC, VRC and ISA susceptibility testing and first recommend to change for VRC or ISA in case of ITC-R, and to consider liposomal amphotericin B in case of pan-azole-R. Such situation is possible mainly during allergic and chronic aspergillosis.

Finally, during CF, we confirmed the high diversity of *Aspergillus* isolates. Indeed, CF patients may be colonized with a wide succession of different phenotypes, either susceptible or resistant. This finding could be confirmed by a genotypic characterization of isolates, since it has been described that different genotype, until 5 to 10, could be isolated in a same patients (Neuvéglise et al., 1997; Amorim et al., 2010). Here again, this result made us change our routine procedure to a systematic antifungal susceptibility testing on various colonies from the same samples, as recommended by international guidelines (Ullmann et al., 2018). And as shown by Lestrade et al. (2020), the monitoring of genotypes may also be combined to *in vitro* susceptibility determination since they reported that the mean VRC MIC of TR34/L98H isolates decreased from 8 mg/L (2013) to 2 mg/L (2018) in the Dutch experience.

Overall, this survey underlines a huge variability of azole resistance level depending on the azole drug, the patient origin and the clinical setting, and also variability within a patient. In addition to epidemiological data, this study made us changing our routine procedure of azole *in vitro* testing. The limits of this field and practical study are its monocentric design, some incomplete data for some patients, and the absence of *in vitro* data on isavuconazole that was only recently introduced in our centre.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité d'éthique de Rennes, France. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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