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Azole Resistance in *Aspergillus fumigatus* in Patients with Cystic Fibrosis: A Matter of Concern?

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Abstract *Aspergillus fumigatus* is the most frequent filamentous fungus isolated from respiratory specimens from patients with cystic fibrosis (CF). Triazoles are the most widely used antifungals in the treatment of allergic bronchopulmonary aspergillosis (ABPA) and invasive aspergillosis (IA) in CF patients. Treatment success could be severely compromised by the occurrence of azole-resistant *A. fumigatus* (ARAf), which is increasingly reported worldwide from both clinical samples and the environment. In previous studies, ARAf has been detected in up to 8% of CF patients. Isolates from CF patients requiring antifungal treatment should therefore be routinely subjected to antifungal susceptibility testing. The optimal treatment of ABPA or IA in CF patients with azole-resistant isolates has not been established; treatment options include liposomal amphotericin B i.v. and/or echinocandins i.v.

Keywords Azole resistance · *Aspergillus fumigatus* · *cyp51A* · Cystic fibrosis

Introduction

Aspergillus fumigatus is a ubiquitous mold that is distributed worldwide, and it is the most frequent filamentous fungus colonizing the airways of patients with cystic fibrosis (CF) [1]. While *A. fumigatus* is the cause of invasive aspergillosis (IA), a life-threatening disease that mostly occurs in severely immunocompromised patients, its detection in clinical specimen

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can also represent contamination or colonization. In patients with CF, *A. fumigatus* is often considered as a colonizer; however, CF patients who develop allergic bronchopulmonary aspergillosis (ABPA) or who underwent lung transplantation are frequently treated with triazoles, e.g., itraconazole or voriconazole. Recently, the success of triazole treatment has been hampered by the increasing prevalence of azole-resistant isolates. In this article, azole resistance in *A. fumigatus* is reviewed in the context of CF.

Epidemiology of *Aspergillus* spp. in Cystic Fibrosis

Aspergillus fumigatus is the most frequent filamentous fungus recovered from the sputum of patients with CF with prevalence rates ranging from 6 to 60% [2]. Although *A. fumigatus* is now considered as a species complex, *A. fumigatus* sensu stricto still remains the predominant species in CF patients. Cryptic species of the section *Fumigati* (e.g., *A. lentulus*, *A. pseudofischeri*) are only uncommonly detected in this population [3, 4]. Prevalence of *A. fumigatus* increases with the age of the patients. In a French study, the mean age at first isolation of *A. fumigatus* from respiratory samples was 12.3 years [5]. In recent years, important progress in molecular typing has led to a better understanding of the colonization dynamic of fungi in the respiratory tract of CF patients. CF patients can be transiently or chronically colonized with *A. fumigatus.* It is also now widely accepted that distinct patterns of colonization exist: from chronic colonization by a single genotype or multiple genotypes to occurrence of distinct genotypes over time in the same patient [6, 7]. Interestingly these patterns of colonization are not restricted to *A. fumigatus* but can also be observed for other species as recently shown for *A. terreus* [8].

Pathophysiological Role of *Aspergillus* spp. in CF

Some of the colonized patients develop *Aspergillus* sensitization or ABPA. *Aspergillus* sensitization occurs in 39.1% of CF patients and is defined by the presence of immediate skin test positivity to *Aspergillus* antigens or elevated serum IgE-levels against *Aspergillus* [9]. The disease is associated with poorer lung function and increased airflow obstruction compared to those without *Aspergillus* sensitization [10]. ABPA is a complex hypersensitivity reaction of the airways and its pathogenesis still remains only partially understood. The prevalence is about 9% in CF [11]. It is pathologically characterized by mucoid impaction of the bronchi, eosinophilic pneumonia and bronchocentric granulomatosis. Clinically, there are no features specific for ABPA. Symptoms can range from recurrent exacerbations with cough, wheeze and shortness of breath to systemic signs with fever, anorexia and malaise. The diagnosis of ABPA is challenging, and diagnostic criteria have only been established in 2003 [12]. Recently, a new classification for *Aspergillus* disease in CF has been proposed [13]. In this classification, galactomannan antigen in sputum and DNA detection of *Aspergillus* by real-time PCR are included besides serological markers.

Very rare events in CF patients are the development of aspergilloma, invasive pulmonary aspergillosis (occurring mostly after lung transplantation) or *Aspergillus* bronchitis [11, 14, 15].

Aspergillus in CF Lung Transplant Patients

End-stage CF lung disease is an indication for lung transplantation (LTx). LTx improves quality of life; however, a survival benefit of the transplantation has not been unequivocally demonstrated [16]. Overall, 16.4% of all LTx patients have CF as underlying disease [17]. Lung transplant patients represent a particular population of CF patients, because of immunosuppression related to antirejection treatments. As a consequence, these patients are at high risk for invasive aspergillosis and should be closely monitored for *Aspergillus* colonization. Aspergillosis of the anastomotic site generally occurs early after LTx, while the manifestation of invasive aspergillosis is generally later [18]. Hence, antifungal therapy should be considered in the early posttransplantation period in patient having *Aspergillus* colonization before transplantation according to recent guidelines [19].

Azole Resistance in *A. fumigatus*

Definition of Azole Resistance

For a reliable classification of isolates as azole susceptible or azole resistant, it is recommended to determine minimal inhibitory concentrations (MIC) of

antifungals, preferentially by a reference method (broth microdilution, according to either Clinical Laboratory Standards Institute (CLSI) [20] or European Committee on Antimicrobial Susceptibility Testing (EUCAST) [21]).

Clinical breakpoints (CBs) for MIC interpretation/categorization of *Aspergillus* isolates as susceptible, intermediate or resistant are available from EUCAST (no breakpoints for *Aspergillus* spp. have been established by CLSI so far). A clinical isolate with an MIC above the CB is considered resistant suggesting a high likelihood of therapeutic failure (http://eucast.org). EUCAST CBs are currently defined for itraconazole, voriconazole, posaconazole, isavuconazole and amphotericin B for some, but not all *Aspergillus* spp. (Table 1) [22]. There are currently no breakpoints for any echinocandin regarding *Aspergillus* spp.

In vitro resistance has been shown to be clinically relevant: Among patients infected with an azole-resistant isolate, a high mortality rate is observed in the hematological/oncological setting [23, 24]. Therefore, a precise identification to the species level and antifungal susceptibility testing is recommended for the therapeutic management of such patients [25]. However, it has to be taken into account that the currently available CBs have been set based on data mostly from patients with invasive aspergillosis in severely immunocompromised patients. If these breakpoints are valid in the CF context with patients who have a different degree of immunosuppression and likely display a different pulmonary distribution of antifungals because of an altered mucus composition has not been established so far. Recently, susceptibility testing of *A. fumigatus* on media mimicking CF sputum has demonstrated considerable differences compared to testing with standard medium [26]. However, whether the results of these susceptibility tests correlate better with the outcome of antifungal treatment in CF patients has not yet been demonstrated. Clinical studies are required to assess which susceptibility testing results are most useful in predicting outcome.

Molecular Basis of Azole Resistance

On a molecular level, azole resistance phenotypes can be categorized into *cyp51A*-mediated, efflux-based and the somewhat unsatisfying "unknown" group. The *cyp51A* gene encodes lanosterol 14α-demethylase, the target of azole antifungals. Mutations in *cyp51A* can lead to amino acid substitutions altering drug interactions, resulting in reduced affinity and efficacy of azoles. Especially amino acid substitutions along the ligand-binding channel (e.g., at G54, G138, M220, Y431 or G432) reduce docking potentials of drugs [27, 28], or limit access to the drug binding sites [28]. Additionally, changes in the promotor of *cyp51A* (e.g., tandem repeats, such as TR_{34} or TR_{46}) may increase its transcription [29, 30], leading to therapeutically less favorable inhibitor–target ratios. Among *cyp51A* mutations, the TR_{34}/L98H is the most common mutation in azole-resistant *A. fumigatus* (ARAf) reported from most studies worldwide [23].

In some ARAf isolates of clinical origin, no mutations within the *cyp51A* locus are detected [e.g., 31, 32] highlighting the existence of alternative, *cyp51A* unrelated, resistance mechanisms. So far,

Table 1 Clinical breakpoints (CB) defined by EUCAST for the main *Aspergillus* species (version 8.1, http://www.eucast.org/clinical_breakpoints) [22]

Antifungal agent	MIC breakpoint (mg/L)									
	A. flavus		*A. fumigatus*		*A. nidulans*		*A. niger*		*A. terreus*	
	S≤	*R*>	*S*≤	*R*>	*S*≤	*R*>	*S*≤	*R*>	*S*≤	*R*>
Amphotericin B			1	2			1	2	–	–
Isavuconazole			1	1	0.25	0.25			1	1
Itraconazole	1	2	1	2	1	2			1	2
Posaconazole			0.12	0.25					0.12	0.25
Voriconazole			1	2						

"–" Indicates that susceptibility testing is not recommended; isolates may be reported resistant without prior testing

only two other genes have been implicated in azole drug resistance: *hapE*, encoding a member of the HAP complex which is a central transcriptional regulator binding, and *cdr1B*, encoding the ABC transporter CDR1B [32–34]. Additional transporters up-regulated in clinical isolates have been identified in *A. fumigatus*, but their correlation with azole resistance is not clarified yet [32].

Epidemiology of Azole Resistance in CF

Azole resistance in *A. fumigatus* clinical isolates has been described in patients receiving azole therapy since the 1990s [35, 36] and increasingly explored since the description of an environmental acquisition of resistant isolates. In CF patients, azole resistance has been investigated in few studies only, even though CF patients are regularly exposed to azole antifungals. Patients are sometimes treated with azoles for extended periods, for various *Aspergillus* diseases such as bronchitis, ABPA or receive azoles as chemoprophylaxis in the setting of lung transplantation. In vivo selection and/or de novo acquisition of ARAf isolates through inhalation from the environment is therefore likely to occur in these patients. As expected, the first international prospective surveillance study of azole resistance in *A. fumigatus* involving 22 centers from 19 countries highlighted CF as the second most common underlying disease after chronic lung disease [37].

Until now six studies, all from European countries (Denmark, France, Germany, Italy and Portugal), have been conducted to determine the burden of azole resistance in CF patients, including a total of 664 patients [38–42, 76]. Even though the isolation of ARAf has now been reported from all continents [23], there are no studies from CF patients outside of Europe, until now. The studies which have been conducted are highly heterogeneous and differ in inclusion criteria (two studies focusing on patients with *A. fumigatus* colonization only), patient population, period of study, local use of antifungal prophylaxis and in vitro susceptibility testing methods, which could have an impact on the prevalence of ARAf; results are therefore difficult to compare. In addition, data from CF patients undergoing lung transplantation are lacking. In the three studies assessing the prevalence of *A. fumigatus* colonization in the general CF population, 383/757 (50.6%) patients were colonized (range 46.3–53.8%, Table 2) [38–40]. The prevalence of ARAf in CF patients varied between centers, ranging from 0 to 8% (28/664 patients). Prior mold-active exposure was noted in three studies for most patients (83.3–100%) with azole-resistant *A. fumigatus* [38, 40, 41], but not in the study by Fischer et al. [39], in which only 25% of ARAf patients had previously received an azole. The observed differences likely reflect different practices in the general use of azoles in different centers/countries. Molecular investigation of the *cyp51A* gene and its promoter highlighted the presence of mutations previously associated with azole resistance in other populations. TR_{34}/L98H was the most frequently detected mutation in all studies (10/20 patients, 50%), followed by mutations at amino acid M220 (7/20 patients, 35%).

While both TR_{34}/L98H and TR_{46}/Y121F/T289A mutations are likely of environmental origin resulting from fungicide use, mutations at residue M220 have been mostly associated with the long-term therapeutic use of azoles [23]. In line with this, both azole-exposed and azole-naïve patients can be at risk to acquire de novo ARAf isolates from the environment [43]. Interestingly, both colonization by multiple ARAf isolates and long-term colonization by a single ARAf isolate have been described in some studies [39–41].

Whether antifungal exposure contributes to the persistence/maintenance of ARAf and more generally the dynamics of fungal colonization in the respiratory tract of CF patients is unknown, but could represent an important threat for patients awaiting lung transplantation. However, there are currently few data on the prevalence of ARAF in LTx patients and even less in CF-LTx patients. In one prospective study including 22 LTx patients with *Aspergillus* colonization or infection, no resistant isolate was detected [44]. In a recent international multicenter prospective study, two among 28 patients with documented *Aspergillus* infection due to ARAf were LTx patients [37].

Epidemiology of Azole Resistance in the Environment

As outlined earlier, azole resistance in *A. fumigatus* in CF patients can be the result of resistance development in vivo or by acquisition of ARAf from the environment. For the latter, it has been postulated that azole resistance in *A. fumigatus* is a result of fungicide use in

Table 2 Studies investigating azole resistance in *A. fumigatus* in CF

Authors, reference	Amorim et al. [42][a]	Burgel et al. [38]	Fischer et al. [39]	Morio et al. [40][a]	Mortensen et al. [41]	Prigitano et al. [76][a]	All studies
Study period	2005–2009	2010–2011	2010–2013	2010–2011	2007–2009	2013–2015	
Country	Portugal	France	Germany	France	Denmark	Italy	
Design	Retrospective, single center	Prospective, single center	Retrospective, single center	Retrospective, single center	Retrospective, single center	Prospective, two centers	
No. of patients with positive cultures for Af/total number of patients	[11/11]	131/249 (52.6%)	119/221 (53.8%)	[50/50]	133/287 (46.3%)	[220/220]	383/757 (50.6%)
No. of Af isolates	159	285	526	85	1176	423	2654
No. of patients with ARAf/total number of patients with *A. fumigatus* tested (%)	0/11 (0%)	6/131 (4.6%)	4/119 (3.4%)	4/50 (8%)	6/133 (4.5%)	8/220 (3.6%)	28/664 (4.2%)
Prior mold-active azole exposure in patients with ARAf (*n*, %)	NA	5/6 (83.3%)	1/4(25%)	4/4 (100%)	6/6 (100%)	4/8 (50%)	20/28 (71.4%)
No. mutations in *cyp51A*							
TR_{34}/L98H	–	2	3	3	2 (TR_{34}/L98H-S297T-F495I, TR_{34}/L98H)	7	17 (60.7%)
TR_{46}/Y121F/T289A	–	–	1	–	–	–	1 (3.6%)
M220	–	2 (M220I, M220R)	1 (M220L)	2 (M220T)	2 (M220 K, M220I)	–	7 (25%)
Other *cyp51A* mutation	–	1 (G54E)	–	1 (G54R)	1 (Y431C)	1 (F219I)	4 (14.3%)
Non-*cyp51A* mutation	–	1	–	–	1	–	2 (7.1%)
Total number of patients with ARAf isolates	0	6	4	4	6	8	28 (100%)

NA not available, *Af Aspergillus fumigatus*, *ARAf* azole-resistant *A. fumigatus*

[a] These studies only focused on patients having positive cultures for *A. fumigatus*

the environment [45], since some azole fungicides [also called sterol demethylation inhibitors (DMIs)] and the medical triazoles share the same molecule scaffolds.

ARAf has been isolated from different biotopes including air samples, compost, plant seed and soil samples from flower beds, crop cultures, hospital surroundings and patients' homes [46–52]. The most common mechanism of environmental triazole resistance has been linked to the TR_{34}/L98H alteration, which is also the most common alteration reported in CF patients. First reported in the Netherlands [50], TR_{34}/L98H isolates have been later recovered from the environment in Denmark, Italy, Germany, the UK and France [46–49] as well as outside Europe, in Africa (Tanzania), Asia (Iran, India, China), Oceania (Australia) and lately the Americas (USA and Colombia) [reviewed in 23].

Also commonly found in the environment and in patient samples are isolates with the TR_{46}/Y121F/T289A genotype. TR_{46}/Y121F/T289A has first been described in Belgium and the Netherlands [51, 53] and later in other European countries [46, 47, 53], but is probably distributed worldwide.

The presence of environmental strains harboring the M220I or G54A substitutions (which are normally isolated from patients after prolonged azole exposure) could indicate that these substitutions also arise as a result of fungicide use in agriculture [46].

Current Techniques to Detect ARAf in the Microbiology Laboratory

The detection of molds by culture is currently the gold standard and is necessary for subsequent species identification and susceptibility testing. Culturing of *A. fumigatus* from respiratory specimens from CF patients should be performed after homogenization of the specimen and plating on standard mycological media (e.g., Sabouraud dextrose or malt extract agar) [54].

Ideally, identification of *Aspergillus* spp. should be performed to the species level as some cryptic species such as members of the section *Fumigati* (e.g., *A. lentulus*, *A. pseudofischeri*) have intrinsically elevated MICs for azoles [55]. Unfortunately, differentiation based on morphology within this section is difficult. MALDI-TOF mass spectrometry (MS) is a cost-effective and promising approach for a precise identification, at least to the species complex level [56]. Species belonging to the same species complex cannot be reliably distinguished by most commercially available databases at the moment. However, with the continuous enhancement of databases with more species this problem will likely be solved in the future.

The gold standard for identification to the species level therefore remains DNA sequencing, preferentially of a part of the β-tubulin gene which is highly discriminatory within the genus *Aspergillus*, in contrast to sequencing of the internal transcribed spacer (ITS) regions of ribosomal DNA [57, 58].

Drug susceptibility testing of *A. fumigatus* isolates should preferentially be performed with broth microdilution according to EUCAST [21] or CLSI [20]. It is recommended to test up to five different colonies from one plate to cover all potential different phenotypes that might be present in a single culture [25].

Depending on the individual amino acid substitution in the Cyp51A protein, MIC values for azoles differ substantially. The highest MICs are usually recorded for itraconazole, with lower MICs for voriconazole (for some isolates even in the susceptible range) and posaconazole. TR_{34}/L98H isolates usually show MICs of >8 mg/L for itraconazole, between 1 and 16 mg/L for voriconazole and 0.25–2 mg/L for posaconazole [24, 39, 40, 51]. In contrast, isolates with the TR_{46}/Y121F/T289A genotype often have lower MICs for itraconazole (≤2 mg/L) and posaconazole (≤2 mg/L), but higher MICs for voriconazole (>8 mg/L) [39, 51, 53]. We therefore recommend testing at least itraconazole and voriconazole before reporting an isolate to be susceptible.

Commercial techniques, such as Etest, have been evaluated for antifungal susceptibility of *Aspergillus* spp. with promising results [59]. However, the ability of Etest to reliably identify azole resistance should be further evaluated [60]. If the laboratory is not able to perform susceptibility testing, the isolate should be referred to a reference laboratory.

Currently, there are no clinical data available which demonstrate that resistance against azoles correlates with treatment failure in the CF setting. Therefore, it is an unresolved question whether *Aspergillus* isolates from CF sputa should routinely be subjected to susceptibility testing.

The culture-based detection of ARAf could potentially be improved by inoculation of sputum samples directly on azole-containing agars. However, there are no studies investigating whether additional agars containing an azole may be useful in CF. Furthermore, such agar plates have to be prepared in house as there are currently no commercial media available.

An alternative to culture-based assays is the detection of mutations mediating azole resistance directly in patient samples (e.g., bronchoalveolar lavage) with in-house [61–64] or recently launched commercially available PCR assays [65, 66]. These assays have been developed for the rapid detection of ARAf in patients at risk for invasive aspergillosis. However, in case of CF patients, the utility of a rapid detection of resistance can be discussed; firstly, recovering *Aspergillus* from CF patients by culture is less difficult than in hematological/oncological patients who often produce no or little sputum. Secondly, the rapid diagnosis of azole-resistant ABPA/*Aspergillus* sensitization or *Aspergillus* colonization is not critical because it is not a life-threatening condition, unless the patient is a lung transplant recipient or is immunocompromised for other reasons.

Since many laboratories do not perform susceptibility testing of filamentous fungi, the molecular detection of resistance-mediating mutations from cultures can be an alternative. If any of the common amino acid exchanges are detected (TR_{34}/L98H, TR_{46}/Y121F/T289A, M220 or G54), resistance can be assumed and an alternative treatment can be initiated. However, it has to be emphasized that the current assays only detect *cyp51A* mutations (and some commercial assays only the most frequent mutations like the TR_{34}/L98H). Therefore, azole resistance caused by other mechanisms will remain undetected with these assays and azole susceptibility cannot be reliably predicted by the absence of the common mutations.

Treatment of CF Patients with ARAf

The treatment of CF patients with isolation of *Aspergillus* spp. from a respiratory specimen remains a controversial issue. Most physicians do not treat with antifungals, unless there is evidence of ABPA.

One randomized, placebo-controlled study of itraconazole for 24 weeks in CF patients with *Aspergillus* spp. in sputum but without ABPA did not show a benefit for itraconazole [67]. It is important to note that this study had several limitations, including that 43% of patients did not reach therapeutic levels of itraconazole in serum.

In contrast, patients with exacerbation of ABPA usually receive corticosteroids, often in combination with an antifungal agent. Even though antifungal treatment has been shown to be beneficial in some patients with ABPA [68, 69], this has not been clearly demonstrated in the CF population. In a Cochrane review from 2016, it was pointed out that there are no appropriate randomized controlled trials available investigating the benefit of antifungal therapy for the treatment of ABPA in CF [70]. In contrast, a systematic review by Moreira et al. [71] included also observational studies and the authors concluded that antifungal treatment for ABPA in CF patients showed potential benefits in terms of clinical outcomes (frequency of exacerbations, lung function, inflammatory biomarkers, radiological findings and steroid-sparing effect). In most of the included studies, antifungal therapy was based on oral triazoles.

Immunosuppressed patients with azole-resistant invasive aspergillosis (IA) have been reported to have a poor treatment outcome [24, 72]. However, up to now there are no studies or case reports about treatment failure due to azole-resistant *A. fumigatus* causing ABPA or sensitization in CF patients. Based on the clinical data from IA and from pharmacological considerations, it can only be assumed that an azole therapy in CF patients with azole-resistant ABPA likely is ineffective. Furthermore, the effect of the altered mucus of CF patients on antifungals has not been systematically investigated and could further influence the outcome.

Recently, an international expert panel discussed the management of IA caused by ARAf and recommended to use liposomal amphotericin B or voriconazole in combination with an echinocandin [25]. These recommendations cannot simply be adapted one-to-one to CF patients because they usually do not suffer from an invasive infection (unless there is additional immunosuppression, e.g., after lung transplantation). The treatment of CF patients is often performed in the outpatient setting and azoles are the only drugs with *Aspergillus* activity which can be given orally. In case of azole resistance, patients have to be treated with i.v. formulations of amphotericin B or an echinocandin.

The increasing azole resistance in *Aspergillus* spp. could therefore result in a higher number of hospital admissions of CF patients.

An alternative treatment option could be the inhalation of antifungals. A positive effect of aerosolized amphotericin B on exacerbations of ABPA has been reported in several case reports and a small randomized controlled trial from India [73, 74]. A recent study on nebulized amphotericin B has demonstrated clinical benefits in only few patients with ABPA (non-CF), but a high rate of side effects (mostly bronchospasm) [75].

Larger studies are needed to evaluate the effect of antifungals on ABPA in CF patients in general and on ABPA by azole-resistant *Aspergillus* spp. specifically.

In conclusion, there are currently no clinical data available for guiding therapeutic considerations in case of azole-resistant *Aspergillus* disease in CF patients. More epidemiological surveillance projects, studies on the relevance of azole-resistant *Aspergillus* in CF and on different treatment options are urgently needed.

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