

High Prevalence of Azole-Resistant *Aspergillus fumigatus* in Adults with Cystic Fibrosis Exposed to Itraconazole

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Aspergillus fumigatus is the most frequent fungus found in the sputum of cystic fibrosis (CF) subjects. Itraconazole is prescribed for allergic bronchopulmonary aspergillosis (ABPA) or *Aspergillus* bronchitis in CF subjects. We hypothesized that *A. fumigatus* isolates in the sputum of CF subjects with previous exposure to itraconazole was associated with higher prevalence of azole resistance. From June 2010 to April 2011, sputum samples from adult CF subjects at Cochin University Hospital (France) were examined systematically for the detection of *A. fumigatus*. MICs of *A. fumigatus* isolates against azoles were screened using Etest, and reduced susceptibility to azoles was confirmed using the CLSI broth microdilution method. *A. fumigatus* was isolated from the sputum of 131/249 (52.6%) adult CF subjects, and 47/131 (35.9%) subjects had received previous treatment with itraconazole. Reduced *A. fumigatus* susceptibility to itraconazole (MIC, ≥ 2 mg/liter) was confirmed in 6/131 (4.6%) subjects. All 6 isolates also had reduced susceptibility to posaconazole (MIC, ≥ 0.5 mg/liter), and 3/6 isolates had reduced susceptibility to voriconazole (MIC, ≥ 2 mg/liter). Mutations in the *cyp51A* gene were detected at positions previously implicated to cause resistance in 5 isolates. Azole-resistant *A. fumigatus* isolates were found in 5/25 (20%) subjects exposed to itraconazole within the previous 3 years. High rates of azole-resistant *A. fumigatus* isolates were present in adult CF subjects and were associated with recent itraconazole exposure. Although the clinical implications of these findings will require further studies, the cautious use of itraconazole in adult CF subjects can be recommended.

Aspergillus fumigatus is the most frequently found fungus in the sputum of cystic fibrosis (CF) subjects (18, 19). It has been isolated from sputum samples from 56% of patients at our CF center (19). *A. fumigatus* colonization has been associated with allergic bronchopulmonary aspergillosis (ABPA) that occurred in up to 15% of CF subjects (24). Furthermore, selected CF subjects with *Aspergillus* colonization showed respiratory exacerbations, which improved with antifungal therapy (22). Because the latter subjects had no ABPA, this condition was referred to as *Aspergillus* bronchitis (22). Although *A. fumigatus* colonization has not consistently been associated with clinical manifestations in all CF subjects (10), a recent report suggested that persistent *A. fumigatus* infection was an independent risk factor for hospital admissions (4). Thus, the interest on the impact of *A. fumigatus* on respiratory manifestations and use of antifungal therapy in CF subjects is growing (13).

Azoles (including itraconazole, voriconazole, and posaconazole) are the only orally active therapies against *A. fumigatus*. These antifungal agents have been proposed as an adjunctive treatment to oral steroids in CF subjects with ABPA (12, 24), and their use also was suggested for CF subjects with *Aspergillus* bronchitis (22). During the past 10 years, azole resistance has emerged in *A. fumigatus* isolates (14) and has been associated with treatment failure in subjects with aspergillosis (15). This increase in azole-resistant *A. fumigatus* isolates was suggested to occur as a consequence of increasing therapeutic or environmental exposure to azoles (27). The frequency of azole-resistant *A. fumigatus* isolates varied considerably among countries and diseases. For example, studies in the United Kingdom (15) and the Netherlands (23)

have described high frequencies (5 to 6%) of azole resistance, especially in subjects with chronic aspergillosis (15). However, a recent study in France identified a very low prevalence of resistance to azoles in *A. fumigatus* collected from patients with invasive aspergillosis who had been treated for hematological malignancies (2).

Only limited data are available regarding the prevalence of azole-resistant *A. fumigatus* isolates in CF subjects and their relationship to previous azole exposure. In a study performed in Portugal, the authors tested 159 *A. fumigatus* isolates cultured from the sputum of 11 CF patients (7 who never received antifungal treatment and 4 who received azoles) and found no azole-resistant isolates, leading the authors to conclude that antifungal resistance appeared to be rare among CF patients (5). Another recent study, which was performed in a large cohort of Danish CF subjects ($n = 133$), reported that 4.5% of *A. fumigatus* isolates were azole resistant (18). The authors underlined that all 6 of these patients previously had been exposed to azoles, and they concluded that the emergence of azole resistance in the *A. fumigatus* isolates of CF patients was of concern (18). Thus, there is conflicting evidence

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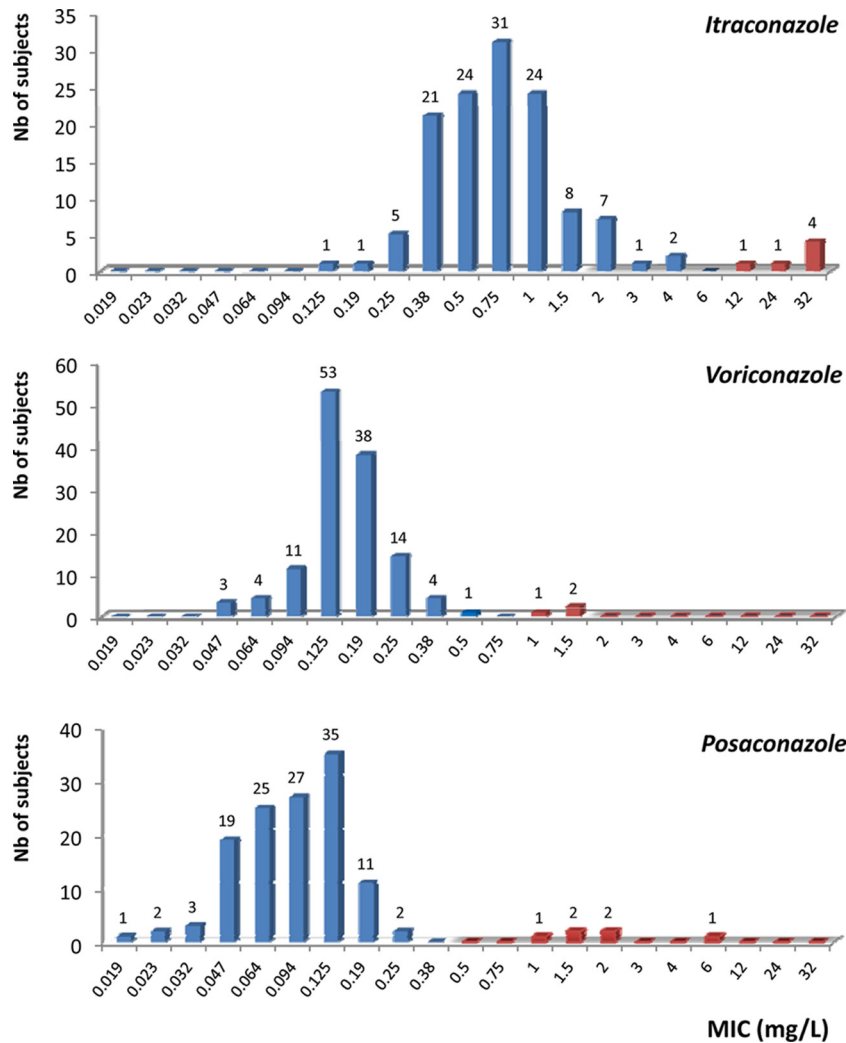


FIG 1 MICs of itraconazole, voriconazole, and posaconazole against *A. fumigatus* isolates obtained from the sputum of 131 CF subjects. Blue bars indicate azole-susceptible isolates. Red bars indicate isolates in which resistance was confirmed by the CLSI broth dilution technique.

on the prevalence of azole-resistant *A. fumigatus* in CF subjects. Furthermore, the impact of azole therapies on *A. fumigatus* resistance to azole has not been established in CF subjects.

In the present study, we prospectively evaluated the azole susceptibility of *A. fumigatus* isolates in our cohort of adult CF subjects. Our objectives were to evaluate the prevalence of *A. fumigatus* isolates with reduced susceptibility to azoles and to examine the impact of previous treatment with itraconazole on the azole susceptibility of *A. fumigatus* isolates.

MATERIALS AND METHODS

Patients. From 1 June 2010 to 1 April 2011, all adult subjects attending the CF center at Cochin Hospital (Paris, France) were recruited in this prospective observational study. CF was diagnosed on the basis of clinical manifestations with a sweat chloride concentration exceeding 60 mM and/or two disease-causing mutations in the *cystic fibrosis transmembrane conductance regulator* (*CFTR*) gene (11). Clinical information was obtained using our CF center database. Clinical data included age, gender, age at diagnosis of CF, *CFTR* mutations, pancreatic status, chronic airway colonization with *Pseudomonas aeruginosa*, body mass index, and spirometry. Chronic *A. fumigatus* colonization

was defined as the presence of two or more positive sputum cultures during the time of the study (4). Intermittent colonization was defined as the presence of one positive sputum culture and at least one negative culture during the time of the study. ABPA was defined according to consensus criteria (24); subjects received itraconazole according to international guidelines. In brief, *Aspergillus* colonization was treated if it was symptomatic and no response was achieved with antibacterial therapy (22). For subjects with ABPA, azole therapy was prescribed in combination with systemic steroid therapy (24). Azole exposure was considered present when a subject was treated for more than 2 weeks with itraconazole, voriconazole, or posaconazole. We defined subjects with recent exposure to itraconazole as subjects who received this treatment (for at least 2 weeks) within the previous 3 years. Subjects who received itraconazole before the previous 3 years were defined as subjects with past itraconazole exposure. The study was approved by the Institutional Review Board on Medical Research (CCTIRS 08-370).

Culture and identification. Respiratory samples were cultured on Sabouraud-chloramphenicol-gentamicin agar (bioMérieux, Marne la Coquette, France) and incubated for 10 days at 27 and 37°C to recover filamentous fungi, and cultures were inspected every day. *A. fumigatus* was identified on the basis of macroscopic and microscopic morphology.

TABLE 1 Comparison of Etest and CLSI methods for determination of azole MICs in 16 isolates with Etest itraconazole MICs of ≥ 2 mg/liter^a

Patient no.	MIC (mg/liter) and test method						<i>cyp51A</i> mutation
	Itraconazole		Voriconazole		Posaconazole		
	Etest	CLSI	Etest	CLSI	Etest	CLSI	
1	>32	>16	1.5	>16	2	8	Unknown
2	>32	16	0.047	0.25	1.5	1	G54E
3	>32	>16	0.5	0.5	6	0.5	M220R
4	>32	>16	0.25	2	2	0.5	M220I
5	24	>16	1.5	8	1	1	TR/L98H
6	12	>16	1	8	1.5	1	TR/L98H
7	4	1	0.38	1	0.125	0.25	
8	4	1	0.25	1	0.19	0.25	
9	3	0.25	0.38	0.5	0.25	0.125	
10	2	0.5	0.19	0.5	0.094	0.125	
11	2	0.5	0.125	0.5	0.19	0.25	
12	2	0.5	0.25	0.5	0.125	0.125	
13	2	0.5	0.19	0.5	0.125	0.125	
14	2	0.5	0.25	0.5	0.125	0.125	
15	2	0.5	0.19	0.5	0.094	0.125	
16	2	0.25	0.19	0.5	0.19	0.125	

^a Gray shading corresponds to isolates with reduced azole susceptibility. *cyp51A* mutations were assessed in the 6 isolates with CLSI-confirmed reduced susceptibility.

Aspergillus fumigatus colonies showed blue-green-gray surface pigmentation, and microscopic examination showed septate hyphae with conical heads, which usually were columnar and uniseriate. Recently, novel *Aspergillus* species with reduced azole susceptibilities and morphological features resembling those of *A. fumigatus* have been described (3). To confirm that *Aspergillus* isolates with reduced azole susceptibilities had not been mistaken with other *Aspergillus* section *Fumigati* with primary resistance to itraconazole (e.g., *A. lentulus* or *A. viridinutans*), we further identified all isolates using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (1). This analysis confirmed that all isolates were *A. fumigatus*.

Susceptibility testing for azole resistance in *A. fumigatus* isolates.

Itraconazole, voriconazole, and posaconazole susceptibilities were determined initially using Etest strips according to the manufacturer's instructions (AB Biodisk, bioMérieux, France) after 24 h of incubation at 37°C. When more than one sputum samples had been tested for *A. fumigatus* azole susceptibility in a single patient, we described the sample with the highest itraconazole MIC. Isolates with itraconazole MICs of ≥ 2 mg/liter by Etest were further analyzed for MICs to itraconazole, voriconazole, and posaconazole using the CLSI M38-A2 broth microdilution method (8). We considered reduced susceptibilities to itraconazole and voriconazole for MICs of ≥ 2 mg/liter and to posaconazole for MICs of ≥ 0.5 mg/liter (20, 21, 26).

Mixed-format real-time PCR assay to detect *A. fumigatus* mutations. All of the *A. fumigatus* isolates for which the CLSI itraconazole MIC was ≥ 2 mg/liter were subjected to a mixed-format real-time PCR assay, as described previously for the detection of mutations at positions in the *cyp51A* gene known to cause triazole resistance in *A. fumigatus* (17).

Statistics. Continuous variables were reported as medians and interquartile ranges (IQR) and were compared using the Wilcoxon rank-sum or Kruskal-Wallis test, as appropriate. Categorical variables were reported as counts and percentages and were compared using Fisher's exact tests. Statistical analyses were two sided, and $P < 0.05$ was considered to have statistical significance. Analyses were performed using Stata 11.0 (StataCorp LP, TX).

RESULTS

From 1 June 2010 to 1 April 2011, 570 sputum samples were prospectively collected from 249 adult CF subjects. *A. fumigatus* was present in 285/570 (50.0%) samples, corresponding to 131/249

(52.6%) individual CF subjects. During the study period, 97/131 (74%) subjects met criteria for chronic *Aspergillus* colonization (≥ 2 *A. fumigatus*-positive sputum samples), and 19/131 (15%) subjects had intermittent colonization. The remaining 15/131 (11%) had only one (positive) sample processed during the study period.

Distribution of azole MICs, as determined by Etest, in subjects with *A. fumigatus* isolates is provided in Fig. 1. *A. fumigatus* isolates with Etest itraconazole MICs of ≥ 2 mg/liter were found in 16/131 (12.2%) subjects. These 16 isolates were further analyzed using the CLSI reference broth microdilution, and reduced susceptibility to itraconazole was confirmed in 6/131 (4.6%) subjects. A comparison of azole MICs between Etest and CLSI methods is provided in Table 1. The 6 isolates with Etest MICs of >4 mg/liter also had elevated CLSI MICs to itraconazole, whereas isolates with Etest MICs of ≤ 4 mg/liter had CLSI MICs to itraconazole of <2 mg/liter. *A. fumigatus* isolates with reduced susceptibilities to posaconazole (Etest MICs of ≥ 0.5 mg/liter) were found in 6/131 (4.6%) subjects, and all of these isolates were confirmed to be resistant to posaconazole and itraconazole using the CLSI method. Furthermore, 3 of these 6 isolates had reduced susceptibility to voriconazole.

Characteristics of the 131 CF subjects positive for *A. fumigatus* isolates are provided in Table 2. When comparing subjects harboring isolates with CLSI-confirmed reduced susceptibility to itraconazole ($n = 6$) to subjects with itraconazole-susceptible isolates ($n = 125$), no significant clinical or demographic difference was found between groups. However, previous exposure to itraconazole was found in 42/125 (33.6%) subjects with CLSI itraconazole MICs of <2 mg/liter versus 5/6 (83.3%) subjects with CLSI itraconazole MICs of ≥ 2 mg/liter ($P = 0.02$). One isolate from an itraconazole-nonexposed patient had the TR/L98H mutation. The other 5 resistant isolates were from itraconazole-exposed patients and involved G54E ($n = 1$), M220I ($n = 1$), M220R ($n = 1$), and TR/L98H ($n = 1$); no known *cyp51A* mutation was identified in 1 patient.

TABLE 2 Characteristics of patients according to itraconazole MICs of *A. fumigatus* isolates

Variable ^a	Results for:			P values
	All subjects (n = 131)	Subjects with itraconazole-susceptible isolates (n = 125)	Subjects with itraconazole-resistant isolates (n = 6)	
Age (yr [IQR])	27 [23; 35]	27 [23; 34]	23 [21; 36]	0.37
Age at CF diagnosis (yr [IQR])	1.0 [0.2; 6.0]	1.5 [0.2; 6.0]	0.6 [0.2; 1.0]	0.53
CFTR mutation (no. [%])				
F508del/F508del	56 (42.7)	53 (42.4)	3 (50.0)	0.89
F508del/other	56 (42.7)	54 (43.2)	2 (33.3)	
Other/other	19 (14.6)	18 (14.4)	1 (16.7)	
Male (no. [%])	77 (58.8)	72 (57.6)	5 (83.3)	0.40
Pancreatic insufficiency (no. [%])	112 (85.5)	107 (85.6)	5 (83.3)	1.00
FEV ₁ (% predicted [IQR])	54 [39; 74]	55 [40; 73]	44 [21; 78]	0.36
FVC (% predicted [IQR])	71 [55; 89]	72 [55; 89]	64 [34; 79]	0.28
Chronic <i>P. aeruginosa</i> (no. [%])	75 (57.3)	70 (56.0)	5 (83.3)	1.00
Chronic <i>A. fumigatus</i> (no. [%])	97 (74%)	93 (74.4)	4 (66.7)	0.65
ABPA (no. [%])	28 (21.4)	25 (20.0)	3 (50.0)	0.11
Previous itraconazole therapy ^b (no. [%])	47 (35.9)	42 (33.6)	5 (83.3)	0.02
Previous itraconazole therapy ^c (no. [%])				
None	84 (64.1)	83 (66.4)	1 (16.7)	0.003
Past	22 (16.8)	22 (17.6)	0 (0)	
Recent	25 (19.1)	20 (16.0)	5 (83.3)	
Previous voriconazole therapy ^b (no. [%])	7 (5.3)	5 (4.0)	2 (33.3)	0.20
Previous posaconazole therapy ^b (no. [%])	0	0	0	
Sputum samples/patient (median [IQR])	3.0 [2.0; 4.0]	3.0 [2.0; 4.0]	3.5 [1.0; 5.0]	0.81
<i>A. fumigatus</i> -positive sputum samples/patient (median [IQR])	2.0 [1.0; 3.0]	2.0 [1.0; 3.0]	3.5 [1.0; 5.0]	0.34
Itraconazole MIC				
Median (IQR) by Etest	0.75 [0.50; 1.00]	0.75 [0.50; 1.00]	32.00 [24.00; 32.00]	<0.001
MIC of ≥ 2 mg/liter by CLSI (no. [%])	6 (4.6)	0 (0)	6 (100)	
Voriconazole MIC				
Median (IQR) by Etest	0.125 [0.125; 0.190]	0.125 [0.125; 0.190]	0.750 [0.250; 1.500]	0.005
MIC of ≥ 2 mg/liter by CLSI (no. [%])	3 (2.3)	0 (0)	3 (50)	<0.0001
Posaconazole MIC				
Median (IQR) by Etest	0.094 [0.064; 0.125]	0.094 [0.064; 0.125]	1.75 [1.5; 2.0]	<0.0001
MIC of ≥ 0.5 mg/liter by CLSI (no. [%])	6 (4.6)	0 (0)	6 (100)	<0.0001

^a FEV₁, forced expiratory volume in 1 s. FVC, forced vital capacity.

^b At any time point.

^c Recent exposure, within the previous 3 years; past exposure, before the previous 3 years.

We next examined the prevalence of azole-resistant *A. fumigatus* in CF subjects, taking into account previous exposure to itraconazole. Forty-seven (35.9%) subjects had previously received itraconazole therapy, 24 (51.0%) subjects for ABPA and 23 (49.0%) subjects for *Aspergillus* bronchitis. The median (IQR) duration of itraconazole therapy was 25.0 (11.0; 44.3) months, and most subjects received 400 mg/day itraconazole. Because we hypothesized that recent itraconazole therapy was associated with the increased prevalence of azole-resistant isolates, we compared subjects with recent (within the previous 3 years) itraconazole treatment to subjects with past itraconazole treatment (but no treatment within the previous 3 years) and to subjects who had never been treated with itraconazole (Table 3). Isolates with reduced susceptibilities to itraconazole (CLSI MICs of ≥ 2 mg/liter) were found in 1/84 (1.2%) and 0/22 (0%) subjects without itraconazole treatment or with past itraconazole treatment, respectively. However, isolates with reduced susceptibilities to itraconazole and to posaconazole were found in 5/25 (20%) of subjects with recent itraconazole treatment ($P = 0.003$) (Table 3). No significant association was found

between the number of months on itraconazole or daily dosage of itraconazole and reduced susceptibilities to itraconazole and posaconazole (not shown).

DISCUSSION

In the present study, we prospectively examined azole susceptibilities of *A. fumigatus* isolates obtained from the sputum of adult CF subjects. In this single-center cohort, we found that 4.6% of subjects harboring *A. fumigatus* had isolates with reduced susceptibilities to itraconazole. All of the isolates with reduced susceptibilities to itraconazole also had reduced susceptibilities to posaconazole, and half of these isolates also were resistant to voriconazole. A strong association was found between itraconazole treatment within the previous 3 years and reduced susceptibilities to both itraconazole and posaconazole.

To the best of our knowledge, only three studies reported systematic screening for *A. fumigatus* azole susceptibilities in CF subjects. Mortensen et al., who studied azole susceptibilities using an itraconazole screening agar and the EUCAST method, found itraconazole MICs of ≥ 2 mg/liter in 6/133 (4.5%) CF subjects with *A.*

TABLE 3 Characteristics of patients according to itraconazole exposure

Variable ^a	Result according to itraconazole exposure			P values
	No exposure (n = 84)	Past exposure (n = 22)	Recent exposure (n = 25)	
Age (yr [IQR])	27 [23; 36]	30 [27; 34]	24 [21; 37]	0.01
Age at CF diagnosis (yr [IQR])	1.5 [0.2; 9.0]	1.4 [0.1; 7.0]	1.0 [0.2; 3.0]	0.39
Male (no. [%])	50 (59.5)	14 (63.6)	13 (52.0)	0.73
Pancreatic insufficiency (no. [%])	71 (84.5)	18 (81.8)	23 (92.0)	0.55
FEV ₁ (% predicted [IQR])	58 [42; 74]	57 [34; 66]	48 [36; 78]	0.29
FVC (% predicted [IQR])	75 [63; 92]	67 [54; 81]	62 [54; 83]	0.04
Chronic <i>P. aeruginosa</i> (no. [%])	49 (58.3)	11 (50.0)	15 (60.0)	0.76
ABPA (no. [%])	4 (4.8)	11 (50.0)	13 (52.0)	<0.001
Voriconazole therapy ^b (no. [%])	1 (1.2)	3 (13.6)	3 (12)	0.01
Posaconazole therapy ^b (no. [%])	0	0	0	
Sputum samples/patient (median [IQR])	3.0 [2.0; 4.0]	3.0 [2.0; 4.0]	4.0 [3.0; 5.0]	0.008
<i>A. fumigatus</i> -positive sputum samples/patient (median [IQR])	2.0 [1.0; 3.0]	2.5 [1.0; 4.0]	3.0 [2.0; 4.0]	0.24
Itraconazole MIC				
Median (IQR) by Etest	0.750 [0.440; 1.000]	0.750 [0.500; 1.000]	1.000 [0.500; 2.000]	0.03
MIC of ≥ 2 mg/liter by CLSI (no. [%])	1 (1.2)	0	5 (20.0)	0.003
Voriconazole MIC				
Median (IQR) by Etest	0.125 [0.125; 0.190]	0.158 [0.125; 0.190]	0.190 [0.125; 0.250]	0.50
MIC of ≥ 2 mg/liter by CLSI (no. [%])	1 (1.2)	0	2 (8.0)	0.17
Posaconazole MIC				
Median (IQR) by Etest	0.094 [0.064; 0.125]	0.094 [0.064; 0.125]	0.125 [0.064; 0.19]	0.001
MIC of ≥ 0.5 mg/liter by CLSI (no. [%])	1 (1.2)	0	5 (20.0)	0.003

^a FEV₁, forced expiratory volume in 1 s. FVC, forced vital capacity.

^b At any time point.

fumigatus isolates (18). A recent Dutch study reported a prevalence of 4.2% azole resistance among 442 isolates after screening with a real-time PCR (25). Amorim et al. studied 159 *A. fumigatus* isolates collected from the sputa of 11 adult CF patients using the CLSI broth dilution method and reported no resistance to itraconazole or posaconazole (5). In the latter study, 4/11 patients had isolates with itraconazole MICs of 2 mg/liter, indicating intermediate susceptibility to itraconazole, and *A. fumigatus* also had intermediate susceptibility to posaconazole in one of these patients (5). Our findings confirmed that *A. fumigatus* isolates with reduced susceptibility to itraconazole were present among CF subjects and extended previous data by showing that cross-resistance to posaconazole and to voriconazole also was prevalent.

Subjects with reduced itraconazole susceptibility were more likely to have received itraconazole therapy within the previous 3 years. This finding is in line with the report by Mortensen et al., who found that the 6 subjects with reduced *A. fumigatus* susceptibility to itraconazole were previously exposed to itraconazole (18). However, no data were provided on the rates of azole resistance in subjects exposed to azole therapies (18). Here, we show that reduced itraconazole and posaconazole susceptibilities were present in 20% of subjects with recent itraconazole treatment. These data suggest a major role for itraconazole therapy in the selection of azole-resistant isolates.

Subjects with past treatment with itraconazole (but without treatment within the previous 3 years) had no evidence of decreased azole susceptibility. In a recent study, de Valk et al. performed the molecular typing of *A. fumigatus* isolates obtained during longitudinal follow-up in 36 adult CF subjects (9). The authors found that only 17% of the patients were chronically colonized with a single genotype, suggesting that in many subjects *A.*

fumigatus isolates were cleared but that recolonization with other genotypes of *A. fumigatus* occurred (9). We speculate that the clearance of *A. fumigatus* isolates, followed by the acquisition of novel isolates, explains the low rates of reduced susceptibility to itraconazole in CF subjects with past (but without recent) itraconazole treatment. It also is possible that azole resistance in *A. fumigatus* isolates results in the alteration of growth and virulence (loss of fitness), favoring their disappearance from CF airways (6). Further studies will be required to confirm these hypotheses.

The present study has several strengths. It was performed in a prospective design in consecutive adult CF subjects with various degrees of respiratory impairment. The size of the cohort allowed the assessment of the impact of itraconazole exposure on azole-resistant *A. fumigatus*. We also recognize limitations. Susceptibility to azoles was initially tested using Etest, which is not the reference method (8, 21). The confirmation of azole resistance was obtained using the reference CLSI broth dilution technique. Although isolates that had CLSI-confirmed azole resistance had the highest Etest MICs (Table 1), our data suggest that breakpoints or epidemiological cutoff values (ECVs) are method specific. The use of Etest had the advantage of being commercially available, and it can be routinely performed in most laboratories (14). However, the confirmation of resistance using a broth dilution technique and/or genetic analysis appears necessary until Etest-specific breakpoints are established. Only 13/47 (27.6%) subjects with itraconazole treatment had therapeutic drug monitoring, and itraconazole concentrations were lower than recommended in 4 subjects (data not shown). Thus, we were unable to examine the effect of long-term underdosing on azole susceptibility to *A. fumigatus*. Because azole plasma concentrations may be widely variable in CF subjects receiving similar doses of azole therapies (7),

the role of pharmacokinetic variability in the reduced azole susceptibility of *A. fumigatus* isolates will require further studies.

The molecular characterization of the azole-resistant isolates identified mutations in the *cyp51A* gene (26), including TR/L98H, and mutations at positions M220 and G54 (14). A variety of mutations at M220 and G54 have been previously implicated as causing azole resistance (including G54R, G54E, G54W, G54V, G54K, M220V, M220K, M220T, M220R, and M220I) and were associated with azole exposure. In contrast, the TR/L98H mutation also is described in azole-naïve patients and was present in one patient without previous therapeutic exposure to itraconazole, suggesting that it resulted from environmental exposure. One isolate, with cross-resistance to itraconazole, voriconazole, and posaconazole, had no mutation at any of the positions covered by the real-time PCR-based screening assay (also including G138) (17). Further studies will be necessary to determine the molecular basis of azole resistance in this isolate.

Itraconazole therapy has been advocated in subjects with *Aspergillus* bronchitis (22) and as an adjunctive treatment to oral steroids in subjects with ABPA (24). These attitudes are based on relatively short-term studies. However, many patients in our study were treated for long periods, and the median time of itraconazole therapy was 25 months. Because exposure to itraconazole is associated with a marked increase in *A. fumigatus* MICs to both itraconazole and posaconazole, we suggest that the benefits of long-term treatment with itraconazole are uncertain. For example, the *in vitro* resistance of *A. fumigatus* to oral azole therapies had been associated with treatment failure in subjects with aspergillosis (15). These findings may be particularly relevant for subjects with severe CF in whom lung transplantation is associated with a higher risk of invasive aspergillosis (16).

In conclusion, we found a prevalence of 4.6% azole-resistant *A. fumigatus* isolates in CF adults, which was similar to studies in Denmark and the Netherlands. Reduced susceptibilities to itraconazole and posaconazole were clearly associated with recent exposure to itraconazole. We suggest that the *in vitro* testing of *A. fumigatus* azole susceptibility should be performed systematically when considering azole therapy in CF subjects. Although further studies are clearly required, we speculate that a combination of limiting azole therapy to short periods of time and the monitoring of therapeutic drug concentrations will reduce the emergence of azole-resistant *Aspergillus* isolates.

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