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Azole Resistance in *Aspergillus fumigatus*: Can We Retain the Clinical Use of Mold-Active Antifungal Azoles?

Paul E. Verweij,[1] Anuradha Chowdhary,[2] Willem J. G. Melchers,[1] and Jacques F. Meis[1,3]

[1]Department of Medical Microbiology, Radboud University Medical Centre, Nijmegen, The Netherlands; [2]Department of Medical Mycology, Vallabhbhai Patel Chest Institute, University of Delhi, India; and [3]Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital, Nijmegen, The Netherlands

Azole resistance in *Aspergillus fumigatus* has emerged as a global health problem. Although the number of cases of azole-resistant aspergillosis is still limited, resistance mechanisms continue to emerge, thereby threatening the role of the azole class in the management of diseases caused by *Aspergillus*. The majority of cases of azole-resistant disease are due to resistant *A. fumigatus* originating from the environment. Patient management is difficult due to the absence of patient risk factors, delayed diagnosis, and limited treatment options, resulting in poor treatment outcome. International and collaborative efforts are required to understand how resistance develops in the environment to allow effective measures to be implemented aimed at retaining the use of azoles both for food production and human medicine.

Keywords. emergence of azole resistance; azole fungicides; aspergilloma; invasive aspergillosis; chronic pulmonary aspergillosis.

Aspergillus fumigatus is a saprophytic mold that causes allergic, chronic, and acute invasive diseases in humans and animals [1]. The fungus is ubiquitous due to an abundant asexual reproduction cycle, producing many billions of spores, and its ability to survive in very different environments. The fungus is thermotolerant, able to resist temperatures as high as 60°C, and is important for the degradation of organic matter. Although *A. fumigatus* is not a primary pathogen for living animals or plants, it has evolved as an important cause of opportunistic fungal diseases in humans. Several decades ago, invasive aspergillosis was a much-feared complication of immunosuppressive treatments as the disease was associated with high morbidity and mortality [2–4]. The survival rates of immunocompromised patients with invasive aspergillosis have improved dramatically due to many factors, one of which is the availability of azole antifungal drugs. This class comprises a number of agents with activity against aspergilli, including itraconazole (available for clinical use since 1997), voriconazole (since 2002), posaconazole (since 2006), and, most recently, isavuconazole [5]. Each of these agents has proved beneficial for the treatment of acute invasive and chronic pulmonary aspergillosis, the prevention of invasive aspergillosis, and for difficult-to-treat disease, such as central nervous system *Aspergillus* disease [6, 7]. Recent studies also show that there is a role for azole therapy in patients with severe asthma with fungal sensitization as its use improved their quality of life and pulmonary function [8]. Moreover, azole drugs are the only anti-*Aspergillus* agents that are orally available, and therefore play an important role in long-term or ambulatory therapy such as for chronic pulmonary aspergillosis [9].

However, the clinical advances that have been made possible through the use of azole drugs might be threatened by the emergence of azole resistance in *A. fumigatus* [10–12]. We aim to describe the epidemiology and spread of azole resistance in *A. fumigatus*, the clinical implications, and directions of research that will help to understand and possibly contain this problem.

RESISTANCE DEVELOPMENT IN *A. FUMIGATUS*

Generally, 2 routes of resistance development are distinguished: through long-term azole patient therapy and via the application of azole compounds in the environment [13, 14]. Although the clinical characteristics of these routes are very different (Table 1), the fundamental prerequisites for azole resistance development are the same: Any setting that brings together actively reproducing *Aspergillus* and azole compounds has a risk of mutations developing that confer resistance to azole compounds [14, 17]. Such conditions could be present in a patient with an aspergilloma receiving azole therapy. Within the pulmonary cavity, asexual reproduction of *A. fumigatus* occurs and spores are produced abundantly, many of which may harbor azole resistance mutations. Genetic analysis of *A. fumigatus* from dissected aspergillomas and clinical cultures from patients with aspergilloma indeed confirm that *A. fumigatus* undergoes multiple genetic changes during infection, including those

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Correspondence: J. F. Meis, Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital, PO Box 9015, 6500GS Nijmegen, The Netherlands (jacques.meis@gmail.com).

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Table 1. Characteristics of Patient-Acquired Resistance and Environmental Resistance in *Aspergillus fumigatus*

Patient-Acquired Resistance	Environmental Resistance
Chronic pulmonary aspergillosis with cavitary lesion or aspergilloma	All *Aspergillus* diseases, including allergic bronchopulmonary aspergillosis, acute invasive aspergillosis, chronic colonization in cystic fibrosis
Previous or ongoing azole therapy in all patients	Two-thirds of patients have no history of azole therapy
Clinical failures to azole therapy	Clinical failures to azole therapy
Multiple resistance mutations may be present in a single clinical sample	Only 1 azole resistance mechanism present in most patients
Both azole-susceptible and azole-resistant phenotypes simultaneously present in culture	Both azole-susceptible and azole-resistant phenotypes simultaneously present in culture
Multiazole and panazole resistance phenotypes	Multiazole and panazole resistance phenotypes
Point mutations in the *Cyp51A* gene, including substitutions at G54, P216, F219, M220, G138, Y431, and G448 non-*Cyp51A*-mediated resistance mechanisms: HapE unknown resistance mechanisms	Mutations in the *Cyp51A* gene in combination with a transcriptional enhancer (tandem repeat) in the promoter region of the gene: TR_{34}/L98H, TR_{53}, and TR_{46}/Y121F/T289A[a]
High genetic diversity between azole-resistant isolates from unrelated patients	Low genetic diversity between azole-resistant isolates from unrelated patients
Aspergillus fumigatus colonies may show an abnormal colony morphology, lack of sporulation or reduced growth rate	No apparent fitness cost

[a] Recently the presence of 2 point mutations was reported in the environment: G54 and M220 [15, 16].

conferring azole resistance [14]. This may be reflected in diagnostic specimens as multiple azole resistance mechanisms may be present in culture, concomitant with azole-susceptible colonies [17]. Although *A. fumigatus* is a eukaryotic microorganism, the complex cellular composition does not preclude rapid resistance development in response to antimicrobial exposure, as seen with bacterial pathogens. However, horizontal gene transfer, which is common in the spread of bacterial resistance, is not commonly seen in fungi. Acquired resistance has been exclusively described in patients with a cavity or aspergilloma [14, 17]. Resistance mechanisms that are recovered in culture are characterized by point mutations in the *Cyp51A* gene, which is the target of the antifungal azoles (Table 1). However, although the *Cyp51A* gene is considered a hot-spot for resistance mutations, many isolates with an azole-resistant phenotype are found to have no mutations in this gene, which suggests that other resistance mechanisms are present, some of which have been identified but many of which remain unknown [18].

Resistance mutations are also believed to develop in the environment when the fungus is exposed to azole compounds that exhibit anti-*Aspergillus* activity [18]. Although *A. fumigatus* is not a phytopathogen, many azole fungicides were found to have activity against *A. fumigatus* isolates [19, 20]. Some of the azole fungicides are of the triazole class and have a similar molecule structure to the medical triazoles [20]. It was hypothesized that *A. fumigatus* develops resistance due to use of azole fungicides to combat phytopathogens for crop protection. Because of the molecule similarity of fungicides with medical triazoles, the latter also lose activity. In addition to abundant asexual reproduction, parasexual and sexual reproduction probably also occurs in the environment, thereby increasing the fungus's ability to undergo genetic recombination and thus overcome cellular stress caused by fungicide exposure. Azole fungicides have a broad range of applications, including plant and crop protection, prevention of postharvest spoilage, and preservation of materials. Azole fungicides are used globally, thus creating an environment where azole-resistant *A. fumigatus* can thrive. In contrast to the United States, where environmental azole resistance in *A. fumigatus* appears to be uncommon [21], health authorities in Europe have been called to action. The European Centre for Disease Prevention and Control brought together experts from agricultural, veterinary, and medical fields to discuss the problem of emerging azole resistance in *Aspergillus* [22].

Clinically, environmental resistance is characterized by a complete lack of patient risk factors. Only residency in or visiting of a geographic area with known environmental resistance can be considered a risk.

EPIDEMIOLOGY

In vitro susceptibility testing of *A. fumigatus* isolates is not routinely performed in most clinical microbiology laboratories, thus underestimating the true prevalence of resistance. Studies that had investigated the frequency of azole resistance in *Aspergillus* culture collections report finding the first resistant isolates up to 17 years earlier [23]. TR_{34}/L98H was first found in the Netherlands in 1998 [13], and recently a TR_{34}/L98H isolate was reported from Italy, also originating from 1998 [23]. The TR_{46}/Y121F/T289A resistance mechanism was also first reported in the Netherlands in 2009 [24], but a recent study reported the recovery of TR_{46}/Y121F/T289A from a patient in the United States already in 2008 (Table 2) [25]. Surveillance studies and case series suggest the global presence of azole resistance in *A. fumigatus* [15, 16, 25–39], including reports from Europe, the Middle East, Asia, Africa, Australia and, most recently, North and South America (Figure 1) [25, 35].

It remains unclear when and where these resistance mechanisms first emerged, although genotyping of epidemiologically and geographically unrelated strains shows a lower genetic diversity among isolates harboring TR_{34}/L98H and TR_{46}/Y121F/T289A compared with wild-type isolates, which suggests that each mutation might have originated from a common ancestor [24, 40, 41]. Our current understanding is that resistance traits can migrate rapidly. Isolates harboring TR_{46}/Y121F/T289A from the Netherlands were found to be genetically highly related to resistant isolates from India [30]. Whole-genome sequencing and population analysis indicated that azole-resistant alleles are segregating into diverse genetic backgrounds,

Table 2. Country and Year of First Recovery of TR_{34}/L98H and TR_{46}/Y121F/T289A Resistance Mechanisms in *Aspergillus fumigatus* and Year of Publication[a]

TR_{34}/L98H				TR_{46}/Y121F/T289A			
Country	First Case	Type of Isolate	Year of Publication [Reference]	Country	First Case	Type of Isolate	Year of Publication [Reference]
Netherlands	1998	C + E	2008 [13]	United States	2008	C	2015 [25]
Italy	1998	C + E	2015 [23]	Netherlands	2009	C + E	2013 [24]
Turkey	2000	C	2015 [26]	Belgium	2012	C + E	2012 [12, 27]
Spain	2003	C	2013 [12, 28]	Germany	2012	C + E	2015 [12, 15]
Australia	2004	C	2015 [29]	India	2012	E	2014 [30]
Iran	2005	C + E	2013 [12, 30]	France	2013	C + E	2015 [31]
Belgium	2006	C + E	2012 [12, 27]	Tanzania	2013	E	2014 [32]
Denmark	2007	C + E	2010/2011 [12, 33]	Denmark	2014	C	2015 [33]
China	2008–2009	C	2011 [30, 34]	Spain	2014	C	2015 [28]
India	2008	C + E	2012 [12, 30]	Colombia	2015	E	2015 [35]
United Kingdom	2009–2011	C + E	2009 [12, 36]				
France	2010	C + E	2012 [12]				
United States	2010	C	2015 [25]				
Germany	2012	C + E	2012 [12, 15]				
Taiwan	2011	C	2015 [37]				
Kuwait	2013	C + E	2015 [30, 38]				
Poland	2006–2014	C	2015 [39]				
Colombia	2015	E	2015 [35]				

Abbreviations: C, clinical; E, environmental.

[a] Due to space restriction, we were not able to include all individual publications. We have cited reviews, which included reports from individual countries over the years.

which will result in increasing genetic diversity over time [42]. As far as we know, azole resistance, due to mutations in the *Cyp51A* gene, is not associated with a fitness cost [43]. The consequence is that resistant isolates would be predicted to compete with wild-type isolates in the field and persist in the environment.

Surveillance studies have shown that between 64% and 71% of patients with *Aspergillus* disease due to environmental azole-resistant *A. fumigatus* had no history of prior azole therapy [24, 44]. Furthermore, azole resistance may occur in any *Aspergillus* disease, including acute invasive aspergillosis, chronic pulmonary aspergillosis, or allergic manifestations such as allergic

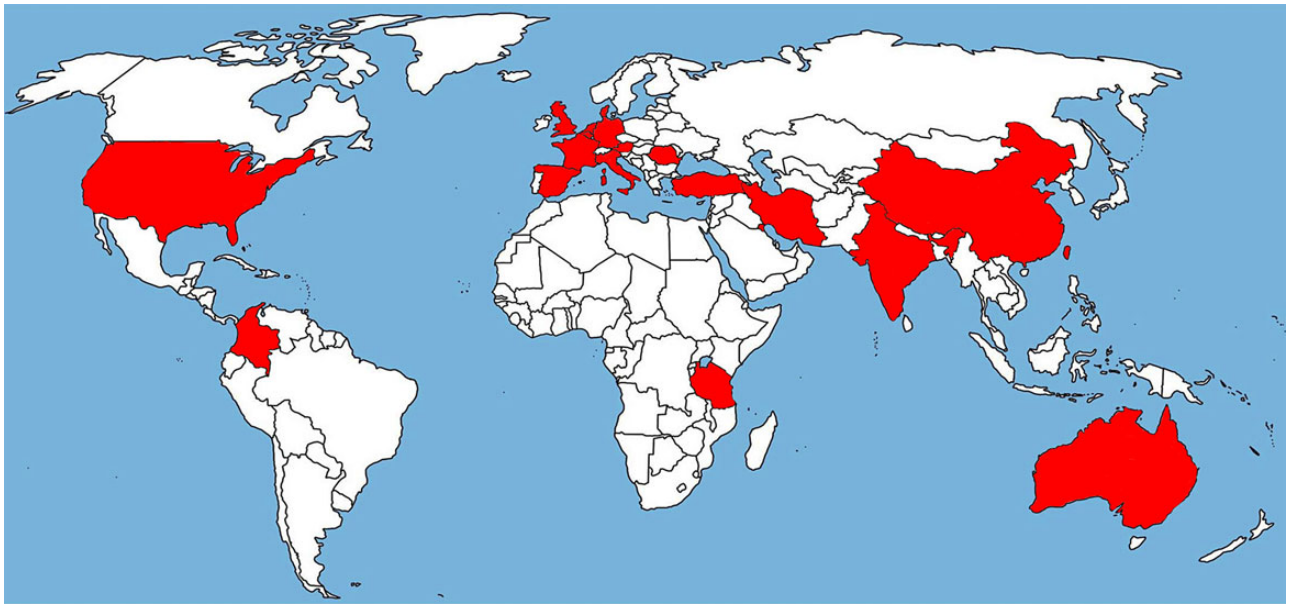

Figure 1. Shaded areas show countries that have reported the TR_{34}/L98H and TR_{46}/Y121F/T289A resistance mechanism in clinical or environmental *Aspergillus fumigatus* isolates.

bronchopulmonary aspergillosis. It is possible that azole prophylaxis or azole monotherapy provides a selective advantage for azole-resistant *A. fumigatus* and might increase the risk for azole-resistant breakthrough infections [45]. Unfortunately, the environmental resistance route was found to be the dominant route for resistance cases. In the Netherlands, between 82% and 89% of azole-resistant cases were due to TR_{34}/L98H and TR_{46}/Y121F/T289A (P. Verweij, personal communication), whereas this was the case in 64% of cases in Belgium [27] and 87% of cases in Turkey [26].

DETECTION OF AZOLE-RESISTANT DISEASE

Azole-resistant *Aspergillus* disease is difficult to diagnose, as *Aspergillus* cultures are negative in the majority of patients. Biomarkers based on *Aspergillus* cell wall components, such as galactomannan or 1,3-β-D-glucan, are unable to detect azole resistance. At best, circulating biomarkers may indicate treatment failure if they continue to increase during azole therapy. Several investigators have used in-house molecular tests to detect azole resistance mutations directly in patient samples, using both tissue and respiratory secretions [46, 47]. Recently, a commercial polymerase chain reaction (PCR)–based assay became available (AsperGenius, PathoNostics, Maastricht, the Netherlands) that enables the detection of several *Aspergillus* species and includes markers for the detection of the TR_{34}/L98H and TR_{46}/Y121F/T289A resistance mechanisms. Preliminary clinical validation studies indicate that this approach is feasible when bronchoalveolar lavage fluid is tested, although only very few *Aspergillus* culture-negative and resistance PCR-positive cases have been described [48]. The sensitivity of the resistance PCR might be a limiting factor, as only a single copy of the *Cyp51A* gene is present in each *Aspergillus* cell, in contrast with the multigene targets that are commonly used for detection of *Aspergillus* species. This is especially a concern when only serum is tested. A negative resistance PCR may be due to the high detection limit of the test rather than the absence of resistance mutations, and therefore it will prove difficult to rule out resistance. Furthermore, at present, only 2 resistance mutations are detected, while at least 15 *Cyp51A* gene–mediated resistance mechanisms have been described [18].

Azole resistance can be tested when *A. fumigatus* is recovered through culture. However, even in culture-positive patients, resistance may be missed due to concomitant presence of azole-susceptible and azole-resistant colonies [38]. Furthermore, positive cultures may have to be sent to reference laboratories, due to limited availability or experience with fungal resistance testing on site, thus causing delay of effective therapy.

MANAGEMENT OF AZOLE-RESISTANT *ASPERGILLUS* DISEASES

All studies to date show that azole resistance is associated with treatment failure [24, 36, 44, 45]. Mortality rates in case series of patients with culture-positive azole-resistant invasive aspergillosis varied between 50% and 100% [24, 44, 45]. Preclinical experimental models also indicate that an elevated azole minimum inhibitory concentration (MIC) significantly reduces the efficacy of azole monotherapy [49], but controlled trials that compare azole-resistant with azole-susceptible cases in relation to treatment success have not been performed. Nevertheless, it seems important to identify patients with azole-resistant *Aspergillus* disease as early as possible to initiate effective therapy. Furthermore, azole resistance mechanisms generally reduce the activity of all azoles. In vitro susceptibility testing of TR_{34}/L98H isolates showed that 99.6% of isolates were resistant to itraconazole, 92.4% to voriconazole, and 97.8% to posaconazole. For TR_{46}/Y121F/T289A 100% of isolates were resistant to voriconazole, whereas 82.7% were resistant to itraconazole and 94.9% to posaconazole [50]. The recently introduced new azole isavuconazole also had high MICs in strains with reduced susceptibilities to other triazoles, mirroring changes in voriconazole susceptibility [5]. These results indicate that the clinical role of azoles in azole-resistant aspergillosis will, at best, be very limited.

In the absence of management guidelines, an expert panel recently discussed the approach they would use in patients with documented azole-resistant *Aspergillus* disease, or in regions where azole resistance has been reported [51]. As clinical evidence is generally lacking, the panel members relied on anecdotal experience, preclinical studies, and expert opinion with respect to treatment decisions. Most experts recommended moving away from azole monotherapy in patients in whom azole resistance was documented, switching to liposomal amphotericin B or voriconazole in combination with an echinocandin. In areas with confirmed environmental resistance, the threshold at which first-line therapy with azole monotherapy should be avoided was the subject of much debate, but most experts would consider moving away from azole monotherapy when resistance rates exceeded 10%. In that situation, azole-echinocandin combination therapy or liposomal amphotericin B was deemed an appropriate alternative choice [51]. It is therefore important to determine if azole resistance is present in a hospital by regular resistance testing of (stored) clinical *A. fumigatus* isolates. Most surveillance studies indicate that the frequency of azole resistance is still below the 10% threshold [52]. These studies relied on screening of unselected clinical *A. fumigatus* isolates, through, for instance, the use of agar plates supplemented with different azoles [52]. Although this approach is useful to determine the frequency of resistance, the role of environmental mutations, and trends over time, 2 recent Dutch studies indicated that the frequency of resistance may vary considerably between departments or risk groups within the same hospital. In one study, a resistance rate of 26% was found in *A. fumigatus* culture-positive patients in the intensive care unit, which was higher than in all other departments in the hospital (14%; $P = .06$) [53]. The authors suggested that patients with (undiagnosed) azole-resistant invasive aspergillosis

Table 3. Reported Mortality Rates in Patients With Invasive Aspergillosis in Different Time Periods

	Aspergillus Disease			
Era	IA	Comment	CNS IA	Comment
c-AmB era	65% [2]	122 of 187 patients receiving c-AmB died.	95%–100% [3]	Literature review
	71.6% [55]	187 of 261 patients with IA died.	99% [56]	Review of 141 cases of CNS IA in immunocompromised patients, of whom 140 died.
Azole era	27.5% [57]	9-wk mortality: 39 of 142 patients receiving voriconazole monotherapy.	45.6% [7]	Retrospective analysis of 81 patients with CNS IA treated with voriconazole
	28.5% [58]	Population-based study analyzing 8563 aspergillosis cases in France.	35.4% [59]	Literature review: 4 of 11 patients with CNS IA who received voriconazole monotherapy.
Azole resistant	100% [44]	Culture-positive patients with proven and probable IPA treated with voriconazole (5/5)	86% [24, 44, 60]	7 cases of azole-resistant CNS IA have been reported, of which 6 were fatal.
	88% [45]	8 HSCT patients with culture-positive, azole-resistant IA, of whom 7 died.		
	100% [54]	ICU patients with culture-positive azole-resistant IA died (10/10), compared with 21 of 28 (75%) with azole-susceptible IA.		

Abbreviations: c-AmB, conventional amphotericin B; CNS, central nervous system; HSCT, hematopoietic stem cell transplant; IA, invasive aspergillosis; ICU, intensive care unit; IPA, invasive pulmonary aspergillosis.

might fail azole therapy while in the department. Subsequent clinical deterioration of the patient requires intensive care support where cultures become positive due to progressive disease. Another study reported the highest azole resistance rates in hematology patients, when primary *A. fumigatus* cultures were analyzed for resistance [54]. Therefore, general resistance surveillance might not reflect resistance rates in specific high-risk patient groups and detailed audits will be required to determine which primary treatment strategy would be appropriate.

A "POSTAZOLE" ERA?

Compared with antibacterial resistance, the looming problem of azole resistance in *A. fumigatus* may seem relatively insignificant as the number of patients affected is low and the question can be raised if drug resistance in an opportunistic pathogen is altogether a threat to public health. After all, *Aspergillus* diseases affect only specific patient groups with chronic lung disease or those with immunosuppression. Although the number of azole-resistant cases is still low, there is every reason to assume that new azole resistance mechanisms will continue to emerge in the environment and rapidly migrate across the world, as has been the case with TR_{34}/L98H and TR_{46}/Y121F/T289A [12, 13, 24]. Increasing azole resistance rates will challenge our current primary treatment recommendation (ie, voriconazole monotherapy), necessitating alternative treatment strategies such as azole-echinocandin combination therapy or liposomal amphotericin B in hospitals or wards where the 10% resistance threshold is exceeded [51]. In addition, the number of cases of breakthrough aspergillosis in patients on azole prophylaxis will increase and certain manifestations of invasive aspergillosis, such as central nervous system aspergillosis, will be virtually untreatable as the use of voriconazole will be precluded. The advances made with the clinical use of the azole class will be at least partly lost and, unless new drug targets are discovered, the overall mortality of *Aspergillus* diseases will increase (Table 3).

RETAINING THE AZOLE CLASS

In medicine we are confronted with the consequences of azole resistance selection in the environment, and given the prominent role of azole compounds both for management of fungal disease in humans and animals and for food production, the optimum strategy to overcome azole resistance would be to aim to retain the use of azoles for both applications. Measures that prohibit the use of specific azoles in the environment may severely compromise global food production and may not be effective. Although 5 azole fungicides were identified that might play a role in the emergence of resistance mutations [11, 20, 22], many azole fungicides show activity against *A. fumigatus* and thus may contribute to providing an environment with a selective advantage for azole-resistant strains, thus facilitating its persistence and spread.

An integrated approach focusing on clinical management, public health surveillance programs, and resistance selection in the environment is necessary to improve the survival of patients with azole-resistant aspergillosis, to track the emergence and spread of resistance mechanisms, and to understand how azole resistance develops in *A. fumigatus* in the environment.

Investigations in the environment should incorporate all applications of azoles including those in agriculture, biocides, and medicine. Recently, 2 *Cyp51A* point mutations, G54 and M220, were recovered from the environment in Germany [15], Romania, India, and Tanzania [16]. These mutations were previously considered to be associated with the patient route of resistance development, but the recovery from the environment suggests that these mutations either develop in the environment or

that through use of azoles in hospitals and veterinary practices these mutations migrate to the environment. By understanding how azole resistance develops and persists in the environment, effective measures can be designed and implemented that prevent resistance development. It was suggested that the application of azole fungicides is crucial for the risk of resistance selection in *A. fumigatus* rather than the volume of use [61]. If this is the case, changes in current practices may reduce the risk of resistance selection without losing the azole class as a whole. An integrated approach would require an international and multidisciplinary collaboration including healthcare professionals, epidemiologists, researchers from agricultural and veterinary medicine, mycologists, and experts in fungal genetics. Furthermore, governments and other policy makers should recognize that action is urgently warranted if we want to retain the clinical use of azoles and evade a "postazole" era. However, if we are successful in preventing azole resistance selection in the environment, only time will tell if the clinical burden of azole-resistant *Aspergillus* disease will also diminish.

Note

Potential conflicts of interest. P. E. V. has received research grants from Astellas, Basilea, F2G, Gilead Sciences, Merck, and Pfizer; has been a consultant to Basilea, F2G, Gilead Sciences, Merck, and Pfizer; and has received speaker's fees from Basilea, Gilead Sciences, and Merck. J. F. M. has received grants from Astellas, Basilea, and Merck; has been a consultant to Astellas, Basilea, and Merck; and has received speaker's fees from Merck, United Medical, and Gilead Sciences. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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