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LEADING ARTICLE

Triazole Resistance in *Aspergillus* Species: An Emerging Problem

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Abstract *Aspergillus* species are ubiquitous fungal saprophytes found in diverse ecological niches worldwide. Among them, *Aspergillus fumigatus* is the most prevalent and is largely responsible for the increased incidence of invasive aspergillosis with high mortality rates in some immunocompromised hosts. Azoles are the first-line drugs in treating diseases caused by *Aspergillus* spp. However, increasing reports in *A. fumigatus* azole resistance, both in the clinical setting and in the environment, are threatening the effectiveness of clinical and agricultural azole drugs. The azole target is the 14-α sterol demethylase encoded by *cyp*51A gene and the main mechanisms of resistance involve the integration of tandem repeats in its promoter and/or single point mutations in this gene. In *A. fumigatus*, azole resistance can emerge in two different scenarios: a medical route in which azole resistance is generated during long periods of azole treatment in the clinical setting and a route of resistance derived from environmental origin due to extended use of demethylation inhibitors in agriculture. The understanding of *A. fumigatus* azole resistance development and its evolution is needed in order to prevent or minimize its impact. In this article, we review the current situation of azole resistance epidemiology and the predominant molecular mechanisms described based on the resistance acquisition routes. In addition, the clinical implications of *A. fumigatus* azole resistance and future research are discussed.

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1 Introduction

The incidence of fatal fungal diseases is escalating due to an increased population at risk in developed countries: patients who suffer from immuno-deficiencies or predisposing factors, such as hematological malignancies, solid organ transplant recipients, and those suffering from chronic obstructive pulmonary disease and receiving high-dose and continued corticosteroid therapy [1–5]. Patients in intensive care units and those infected with HIV are also sometimes classified as high risk [6, 7].

The genus *Aspergillus* is relatively unique among fungi in that they cause a wide range of infections such as chronic pulmonary and allergic pulmonary aspergillosis, saprophytic colonization, asthma with fungal sensitization and most severely invasive aspergillosis (IA) [3, 8–10]. Affected individuals will develop a specific form of aspergillosis depending on numerous host factors, but mainly based on underlying immune status [11]. In immunocompromised hosts, *A. fumigatus* represents a major cause of morbidity and mortality, in part because of the difficulty in diagnosis and late initiation of antifungal therapy.

Treatment options are limited to three antifungal drug classes: polyenes (amphotericin B), azole drugs and echinocandins. Among them, only three specific triazole agents (itraconazole, voriconazole and posaconazole) are recommended for the treatment and prophylaxis of aspergillosis [12, 13]. The antifungal action of azole drugs was first reported in 1944. Since then many azole compounds have been introduced including imidazoles, followed by triazoles [14]. Within the triazoles, fluconazole and itraconazole were introduced for clinical use during 1990s, followed by a second generation, including voriconazole (2002) and posaconazole (2006) [15]. More

recently, isavuconazole has been described as a new extended-spectrum triazole, and its activity against *Aspergillus* has been proven [16]. At present, voriconazole remains the primary treatment for IA and liposomal amphotericin B (L-AMB) is recommended as an alternative therapy [12, 13, 17]. Azoles are the only class of mold-active agents that can be administrated orally and intravenously so that they can be given in outpatient settings and they are used for both the evidence-based treatment and prevention of *Aspergillus* spp. infections [12, 13, 17]. However, azoles are generally fungistatic in vitro against yeast-like fungi and show species and strain-dependent fungicidal activity against *Aspergillus* species [18, 19].

Emerging resistance to existing antifungals is a current problem and a poor response to them has been described in infections caused by azole-resistant *A. fumigatus* [18, 20, 21]. Drug selection pressure due to the use of antifungal drugs, in medical centers and as pesticides in agriculture, is an important factor for resistance emergence and spread. Currently, the link between extensive use of azoles in the environment and the emergence of azole resistance among human fungal pathogens is subject of intensive research [22, 23]. Understanding the origin and development of *A. fumigatus* azole resistance is needed to minimize its global spread and to prolong the effectiveness of currently available antifungals [22].

2 Evolving Epidemiology

The incidence and prevalence of azole-resistant strains isolated from blood cultures, respiratory and deep tissue samples have been reviewed by several authors [20, 24–31]. Although the prevalence of mold isolation is low, the most frequently isolated genus is *Aspergillus* spp., ranging between 51–86% of the total number of isolates [25, 28, 30]. Among them, *A. fumigatus* is the most frequently isolated species, accounting for more than half of the total isolates in most of the studies [24, 26, 28, 30, 32]. Other species, in order of frequency, are *A. flavus* or *A. terreus*, *A. niger* and *A. tubingensis* [21, 24, 28, 30]. The cryptic *Aspergillus* species are probably underestimated, as their identification is more difficult; some studies have shown prevalence of up to 12% [28, 33]. This is an important issue as antifungal resistance among *Aspergillus* cryptic species can reach up to 40% of the isolates [28, 34–36].

Along with the expanding use of antifungal drugs globally, an increase in the number of *Aspergillus* triazole-resistant isolates has been reported. In the case of *A. fumigatus*, the prevalence of azole resistance is quite high within Europe with variable percentages observed in the rest of the world (Table 1). The prevalence of azole-resistant *A. fumigatus* infections also appears to vary between individual hospitals [37, 38]. Although surveillance of unselected clinical cultures provides resistance rates at a national level and offers information about the epidemiology of resistance mechanisms, recent studies show the need to determine the frequency of azole resistance at the hospital level and within different patient groups or departments [37, 38]. The high prevalence of resistance (20–30%) in clinical isolates of high-risk patients recently reported in the Netherlands and Germany highlights the need for rapid detection of azole resistance to initiate the appropriate therapy earlier [31, 39].

3 Azole Drugs Resistance Mechanisms

Triazole drugs inhibit the 14-α sterol demethylase (Cyp51) that catalyzes a key step in the ergosterol biosynthesis. This is a cytochrome P-450 enzyme containing a heme moiety in its active site and catalyzes the oxidative removal of the 14α-methyl group from cyclized sterol precursors (eburicol or lanosterol) by a three-step reaction [40]. Azole drugs act as competitive Cyp51 inhibitors through the interaction of the N-3 (imidazoles) or N-4 (triazoles) position of the aromatic ring with the iron atom of the heme moiety, which prevents oxygen activation, necessary for lanosterol/eburicol demethylation [41]. The basic heterocyclic nitrogen coordinates to the P450 heme iron, sharing its lone pair of electrons and blocking binding of molecular oxygen, whereas the non-ligated portion of the inhibitor molecule forms multiple contacts with the protein moiety, shaping the protein-ligand surface interface that largely defines the strength of the inhibition [41]. This interaction leads to the accumulation of various 14-α methyl sterols and ergosterol depletion that alters the fungal membrane and affects the cell wall integrity with consequent fungal growth impairment [42]. Specificity of azole compounds depends on the interaction between side groups of the azole compound and the Cyp51 protein [43, 44].

Aspergillus fumigatus has two Cyp51 isoenzymes, Cyp51A and Cyp51B [45], and both can fulfill the role of 14-α sterol demethylase in vitro with no significant differences [46, 47]. Growth is suppressed in the absence of both isoenzymes, but not in the absence of only one of them [46, 47]. Some research has indicated that *A. fumigatus* Cyp51A confers intrinsic fluconazole resistance [46]. Also, most studies conclude that mutations in *cyp*51A gene (promoter, coding region or both) are responsible for the great majority of the described azole resistance mechanisms [48]. In contrast, the role of Cyp51B in the susceptibility to azoles is still unclear [49].

Aspergillus fumigatus has different azole resistance mechanisms that can be classified in different categories;

Table 1 Triazole resistance rates of clinical *Aspergillus fumigatus* isolates with integration in the *cyp*51A promoter

Country	No. isolates	Overall resistance rate (%)	Resistance mechanisms[a]		References
			TR_{34}	TR_{46}	
Europe					
Belgium	192	5.7	54.5	9.1	[167]
Denmark	413	4.5	33.3	0	[25]
	1162	2.6	57.0		[32]
France	118	0.85	0	0	[64]
	125	4.6	16.6	0	[26]
	85	8	55.6	0	[168]
Germany	527	3.2	35.3	0	[67]
	119	5	66.7	16.7	[30]
	27	30	71.4	28.5	[31]
Portugal	159	0	0	0	[169]
Spain	156	0.6	0	0	[28]
	362	1.8	0	0	[29]
The Netherlands	170	1.7	–	–	[170]
	1912	1.7–6	94	0	[22]
	209	1.9	100	0	[171]
	2062	4.6	90.2	0	[21]
	1315	6.8	74.6	20.6	[78]
	105	20	62.0	14.3	[39]
	2941	3.2	48.9	6.4	[172]
Turkey	746	10.2	86.8	0	[148]
UK	519	6.6	5.9	0	[20]
Asia					
China	72	5.5	75	0	[173]
India	103	1.94	100	0	[174]
	685	1.75	83.3	0	[72]
Iran	124	3.2	75	0	[175]
	172	3.5	100	0	[176]
Japan	196	11.2	0	0	[27]
	171	1.75	–	–	[177]
Kuwait	16	12.5	100	0	[178]
Taiwan	40	5	–	–	[179]
America					
USA	181	0.55	–	–	[24]
	220	9.1	10	10	[89]
Oceania					
Australia	418	2.1	22.2	0	[73]

[a] Percentage of resistance mechanisms among resistant isolates: TR_{34}/L98H and TR_{46}/Y121F/T289A

the most commonly described are (1) target (*cyp*51A) modifications, although recently (2) *cyp*51A-independent mechanisms have been described.

3.1 *Aspergillus fumigatus* Azole Target (*cyp*51) Modifications

Azole-resistant *A. fumigatus* isolates of clinical origin exhibit different mutations that are responsible for the increase in minimum inhibitory concentrations (MIC) to one, or more triazoles [48]. The main mechanisms accounting for triazole resistance in *A. fumigatus* are point mutations in the *cyp*51A gene. About 30 different mutations have been described in *cyp*51A gene, although not all mutations are responsible for an azole-resistant phenotype [5, 44, 50]. Based on *cyp*51A modifications, azole resistance mechanisms can be classified.

3.1.1 Hot-Spot Single Point Mutations

The three most commonly described single-point mutations that appear in *A. fumigatus* are Cyp51A amino acid substitutions at G54, M220 and G448, and all are thought to have arisen in the clinical setting where azoles are used as therapy (Fig. 1).

One mutation is located at position glycine 54, including amino acid changes G54E, G54V, G54R, and G54W. Clinical strains with these mutations show resistance to itraconazole (ITC) and yield high posaconazole (POS) MICs but not voriconazole (VCZ) MICs [51, 52]. The second important single-point mutation is at methionine 220, including amino acid changes M220V, M220K, M220T and M220I [53, 54], which yield resistance to ITC and reduced susceptibility to POS and VCZ [53]. A third resistant mechanism is G448S with resistance to VCZ and reduced susceptibility to ITC and POS [55, 56]. This mutation has also been correlated with in vivo azole resistance [57].

Other less common mutations have also been described. The G138R substitution was described in an azole-resistant mutant generated in the laboratory [58] and afterwards, a multi-azole-resistant strain with a G138C substitution was reported from a patient under azole treatment showing high MICs to all azoles [59]. However, the mechanism involved in this multi-azole resistance phenotype remains to be fully clarified [60].

Protein structure modeling is an important tool in the study of drug action and resistance and has been used to create 3D homology models derived from Cyp51A protein sequences of azole-resistant *A. fumigatus* isolates. These models have been built to assess direct or indirect mutation effects on azole access or binding to the protein and conclude that M220 and G54 mutations have clear potential to block access to the azole entry channel, while G448S, located on the opposite side of the protein and near the heme group, is thought to disrupt its position within the protein [61]. This mutation reduces the ability of the azole to bind to the heme effectively and allows replacement by the substrate [61, 62].

In some cases, other single-point mutations (N22D, F165L, P216L, F219C, F219I, D262Y, A284T, Y431C, G432S and G434C, T440A, N479D, Y491H) have occasionally been described as related to azole resistance or associated with a reduced azole susceptibility profile, but

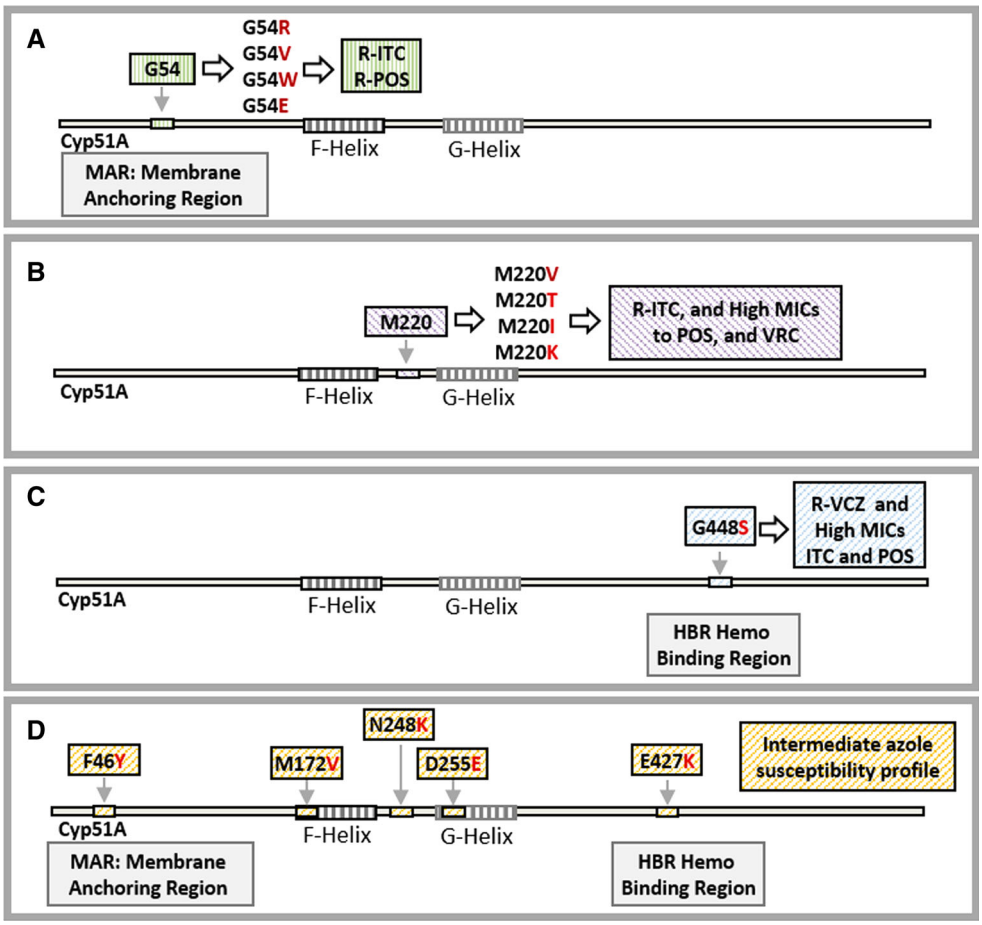

Fig. 1 Azole target (*cyp*51A) modifications and susceptibility profiles. Cyp51A hot spot single point mutations (**a**–**c**) and Cyp51A multiple point mutations (**d**). *MICs* minimum inhibitory concentrations, *ITC* itraconazole, *POS* posaconazole, *VCZ* voriconazole

further research needs to be done in order to confirm, or to exclude, their role in azole drug resistance [20, 25, 50, 60, 63–67].

3.1.2 *Cyp51A Multiple Point Mutations*

A combination of Cyp51A amino acid substitutions is frequently described among clinical strains isolated from patients who have been undergoing azole treatment. Basically, there are two combinations of amino acid substitutions: (1) a group of three (F46Y, M172V, and D255E) (2) or of five that included the three former ones (F46Y, M172V, N248T, D255E and E427K) (Fig. 1d). Both groups have been described with different profiles of azole susceptibility as azole susceptible or resistant, but in all cases, they have remarkably higher azole MICs than *A. fumigatus* wild-type strains [20, 21, 50, 65, 68–74]. Some authors described these substitutions as playing no role in azole resistance [65, 75]. However, a *cyp*51A deletion mutant showed an azole hyper-susceptible profile, suggesting that some of these changes could be responsible for the increased azole MICs of these strains [76], although their role in azole susceptibility in vivo remains to be clarified.

Finally, some substitutions have been described together because they are usually detected along other point mutations, such as H147Y with G448S [20], P394L with G54R [63], S393S with G54R [63], and S297T with F495I [22].

3.1.3 *Multiple cyp51A Modifications Involving cyp51A Overexpression*

Triazole resistance in *Aspergillus* spp. can evolve during therapy, but resistant isolates are also being detected in azole-naive patients, with evidence to suggest acquisition of resistant isolates from the environment. These isolates are characterized by having a particular genetic alteration consisting of a 34 bp tandem repeat (TR_{34}) in the promoter, together with a point mutation L98H in the *cyp*51A target gene conferring multi-azole resistance [77] (Fig. 2a). First reported in Europe, this mechanism is now being described across the world (Table 1). This issue is further complicated by the emergence of a new resistance mechanism, TR_{46}/Y121F/T289A in the *cyp*51A gene, responsible for VCZ resistance (Fig. 2b). First detected in 2009 in a Dutch patient [78] it has recently been reported in clinical and environmental isolates from Belgium, France, Denmark, Germany, Spain, China, India, Japan, USA, Colombia, Tanzania and Thailand [67, 79–89]. Interestingly, an environmental *A. fumigatus* strain harboring only the Y121F substitution has been reported recently. However, this mutation is responsible for high VCZ MICs and does not confer resistance to all azoles [90]. Another less common duplication in the *cyp*51A promoter (TR_{53}) without other substitutions in the *cyp*51A gene has also been described [86, 91] (Fig. 2c).

3.2 *Aspergillus fumigatus* Azole Resistance Mechanisms *cyp*51A-Independent

Other *cyp*51A-independent mechanisms have been reported as contributing to azole resistance. One important resistance mechanism is the reduction of the intracellular concentration of azole by active efflux systems, such as ATP-binding cassette (ABC) transporters and transporters of the major facilitator superfamily (MFS) [92]. The association between azole resistance and transporter upregulation is less clear in *A. fumigatus* than in yeast, such as *Candida albicans* [93, 94], *C. krusei* [95], and *C. glabrata* [96]. In *A. fumigatus*, there are 45 ABC proteins and 275 MFS proteins (http://www.membranetransport.org). Some studies have demonstrated correlation between ABC transporter expression and azole resistance [54, 97, 98]. However, a functional connection between a specific ABC transporter, RNA level and the development of azole resistance is still elusive. For example, the upregulation of the ABC transporter *atrF* has been described in a clinical strain of azole-resistant *A. fumigatus* after ITC treatment but its implication in the ITC resistance has not been confirmed [97]. The same applies to *AfuMDR4*, which was strongly upregulated in several ITC-resistant laboratory derived mutants, but further experiments need to be done to confirm its participation in azole resistance [54]. Also, transcriptome analysis of *A. fumigatus* exposed to VCZ revealed a number of transporter genes that were induced: five ABC transporter genes (*abcA-E*) and three MFS (*mfsA-C*) were upregulated [98]. Among these genes, only *abc*B, renamed *cdr1B*, has been demonstrated to have a direct role in *A. fumigatus* resistance [99].

In addition, some transcription factors such as SrbA are known to play a role in *A. fumigatus* azole resistance. SrbA is a transcriptional regulator belonging to the sterol regulatory element binding protein (SREBP) family and is important in *A. fumigatus* azole resistance. Disruption of *srb*A produces increased susceptibility to fluconazole, possibly due to the decreased expression of *cyp*51A [100]. The possibility that SrbA mutants are able to increase their activity and might elevate azole MICs has not been described at present [101]. Also, a P88L substitution in another transcription factor, HapE, leads to an increased azole resistance phenotype due to the induction of *cyp*51A expression in the mutant strains [102]. Just recently, the link between SrbA and HapE has been demonstrated. Gsaller et al. [103] have shown that the azole resistance exhibited by isolates with the HapEP88L modification is

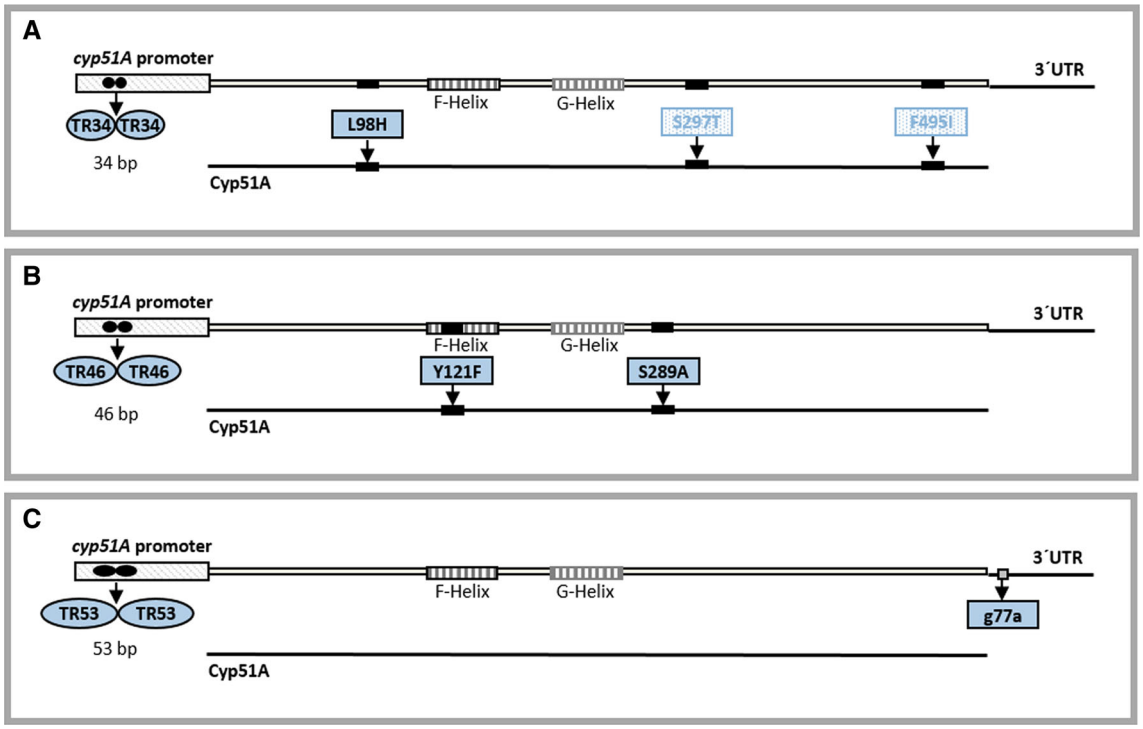

Fig. 2 Multiple *cyp*51A modifications involving *cyp*51A overexpression. Tandem repeats: 34 bp (**a**); 46 bp (**b**); 53 bp (**c**), and associated point mutations

linked to an inability of the modified CCAAT binding complex to bind effectively to its recognition site in the *cyp*51A promoter, leading to increased expression. Interestingly, the growth phenotype exhibited by a strain with the HapEP88L mutation is less severe than that of the HapE null, suggesting only a partial loss of function, all together will strongly suggest that targeting SrbA would provide an effective avenue for therapeutic intervention for resistant strains.

Recently, a probable role of mitochondrial complex I in fungal drug resistance via alteration in membrane dynamics or composition has been suggested. A mutation leading to an E180D amino acid change in the 29.9 KD subunit has been strongly associated with clinical *A. fumigatus* azole-resistant isolates [104]. Finally, the modification of AfYap1 (homolog of *Saccharomyces cerevisiae* Yap1), a basic region-leucine zipper transcription factor with nuclear location and regulated by oxidative stress [105], has been shown to increase resistance to VCZ but not to ITC [106].

3.3 Azole Resistance in Other *Aspergillus* Species

Although the main etiologic agent of invasive aspergillosis is *A. fumigatus*, there are increasing reports of fungal infections caused by other species of this genus [107].

Aspergillus section *Fumigati* has been studied using phylogenetic analysis of different targets (mainly β-tubulin gene) and is found to be composed of 63 species [108, 109]. Some of these species, usually called sibling or cryptic, have been reported to have higher MICs to a range of antifungal agents compared to *A. fumigatus* [28, 110–113]. Among them, *A. lentulus* shows high VCZ and ITC MICs, compared to those of POS [110, 112], and good response to isavuconazole [114]; these higher azole MICs have been linked to the amino acid sequence of the Cyp51A target [115, 116]. Other species within the section, such as *A. fumigatiaffinis* and *Neosartorya pseudofischeri,* have high triazoles MICs while *N. udagawae* exhibit higher VCZ MICs than ITC or POS [35, 110, 112, 117]. However, isavuconazole displays good activity against some of these species [114]. *Aspergillus viridinutans* has high VCZ and ITC MICs but lower POS MICs [35, 113] whereas, *A. hiratsukae* and *A. fumisynnematus* are susceptible to all drugs tested [110].

In *Aspergillus* section *Nigri*, *A. niger* and *A. tubingensis* are the most frequent species found in clinical settings, and have a variable susceptibility profiles depending on the isolate [112]. Within *Aspergillus* section *Flavi*, *A. flavus* and *A. alliaceus,* are the most remarkable species with variable azole susceptibility profiles [112]. In *Aspergillus* section *Terrei*, *A. terreus* has reduced susceptibility to azoles while *A. citrinoterreus* is more susceptible to them [118]. Finally, within *Aspergillus* section *Usti, A. ustus* and *A. calidoustus* are known for their high MICs for all

antifungals and are considered multi-resistant species [119, 120].

4 Development of Azole Resistance

Azoles are the only class of compounds that are used both in agriculture and in clinical medicine [121].

Generally, two different routes of azole resistance development in *A. fumigatus* have been described: (1) a medical route in which azole resistance is generated during long periods of azole treatment in clinical settings and (2) another route of resistance derived from environmental origin due to extended use of demethylation inhibitors (DMIs) in agriculture (http://www.frac.info/publications; FRAC Code List 2016) (Fig. 3). Although azole resistance is acquired by selective pressure in both cases, the result of this selection generates different resistance mechanisms and different azole susceptibility patterns.

1. In the first case, acquired azole resistance may develop in patients who have been treated long-term with prolonged azole exposure due to a chronic form of aspergillosis, for example patients with aspergilloma, cystic fibrosis or allergic bronchopulmonary aspergillosis. In these patients, isolation of resistant strains is almost always linked to previous azole exposure and the responsible mechanism can change over the course of infection [122]. Despite the fact that patient-to-patient transmission of resistant *Aspergillus* is uncommon, it would mean an important risk at patient level [32, 123]. These patients are initially infected by a susceptible *A. fumigatus* strain that evolves to a resistant phenotype under azole treatment pressure. Genotypic analysis of serial *A. fumigatus* isolates from patients with aspergillosis has revealed that the initial susceptible isolates and the later resistant ones had the same genotype [25, 124–126]. The underlying resistance mechanism normally involves single-point mutations in the *cyp*51A gene (G54, M220 and G448), which implies that the fungus is capable of rapidly adapting to azole drugs in patients exposed to long-term azole therapy [127].
2. In the agricultural setting, azoles are the most important group of fungicides due to their great efficiency in the field and the remarkable resistance of fungi to other classes of compounds [128]. Agricultural fungicides are used variably throughout the year, depending on the location, crop type and the risk of fungal infestation [128]. They are classified according to their biochemical mode of action as sterol biosynthesis inhibitors (SBIs) of class I, particularly DMIs. Some azoles used in crop protection (imidazoles and triazoles) have a similar molecule structure to medical triazoles and induction of cross-resistance between them has been demonstrated [129].

The resistance mechanisms associated with this route consist of tandem repeat integrations of different size in the *cyp*51A promoter followed by point mutations in the coding gene (TR_{34}/L98H and TR_{46}/Y121F/T289A). In plant-pathogenic molds treated with DMIs both mechanisms have been found but independently, either as integrations of different sequences in the *cyp*51A promoter and related to increased expression of the azole target or as point mutations in *cyp*51 and therefore related to a lack of

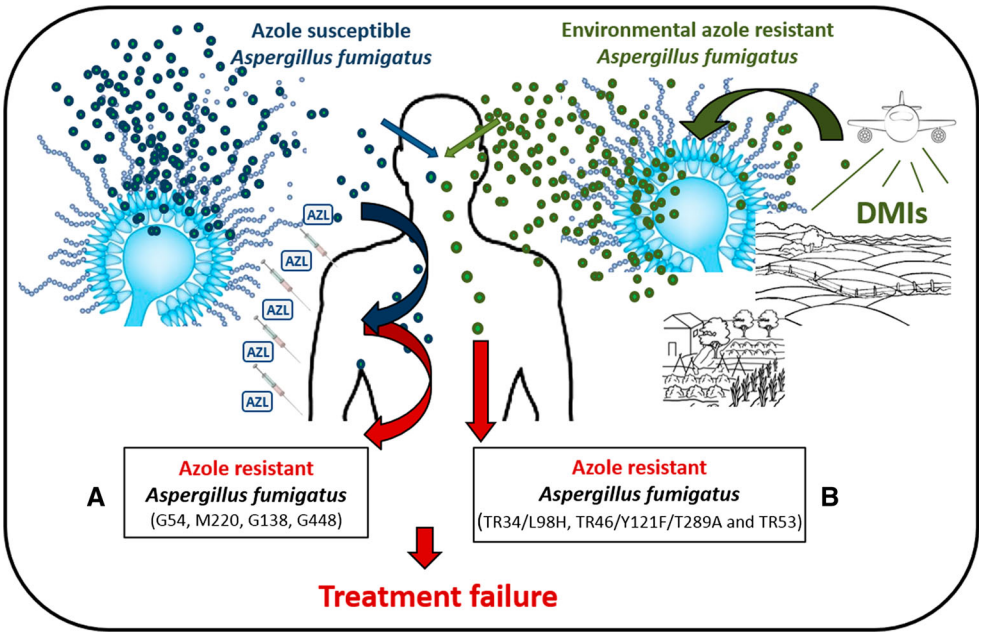

Fig. 3 Routes of *Aspergillus fumigatus* azole resistance acquisition. **a** Patient route: the patient is infected by *A. fumigatus* azole susceptible and after azole treatment there is a selection of *A. fumigatus* strains with point mutations at Cyp51A. **b** Environmental route: patients are infected by *A. fumigatus* azole resistant previously to treatment, and these strains have combined azole resistance mechanisms. *DMI* demethylation inhibitors

Table 2 Principal resistance mechanisms to azole fungicides found in plant pathogens

Plant pathogen	DMIs resistance	Cyp51 aa substitutions	*cyp*51-promoter alterations	Cyp51-increased expression	References
Pyrenopeziza brassicae	Four triazoles and prochloraz	S508T	151 bp insertion	Yes	[143]
Erysiphe necator	Myclobutanil	Y136F	–	Increased copy number	[166]
Venturia inaequalis	Myclobutanil	Non-detected	553 bp insertion	Yes	[132]
Penicillium digitatum	Triflumizole	Non-detected	126 bp (TR) (5 times)	Yes	[130]
	Imazalil	–	126 bp (TR) (5 times)	Yes	[131]
Blumeriella jaapii	Fenbuconazole	Non-detected	Truncated non-long terminal direct repeats	Yes	[133]
Monilinia fructicola	Propiconazole	–	65 bp insertion	Yes	[134]
Pyrenophora teres	Prochloraz	F489L	–	–	[142]
Erysiphe necator	Triadimenol	Y136F	–	–	[135]
Erysiphe graminis f. sp. *hordei*	Benzimidazol	Y136F	–	–	[136]
Ustilago maydis	Propiconazole	G464S	–	–	[137]
Blumeria graminis f. sp. *hordei*	Triadimenol	Y136F	–	–	[138]
	Propiconazole	K147Q	–	–	[138]
Mycosphaerella graminicola	Tebuconazole	I381V	–	No	[139]
	Triadimenol	F137Y	–	No	[140]
Puccinia triticina	Epoxiconazole	Y134F	No	–	[141]

appropriate competitive inhibition (Table 2). An example of the former is *Penicillium digitatum* with a 126 bp sequence tandemly repeated in the *cyp*51 promoter that has been directly related to a pattern of resistance to different DMIs [130, 131]. Similarly, *cyp*51 promoter insertions of different sizes have been described in other species, such as *Venturia inaequalis*, *Blumeriella japii* and *Monilinia fructicola* [132–134]. In other plant pathogens, many *cyp*51 single-point mutations have been implicated in DMI resistance (Table 2) [135–142]. However, the combination of both mechanisms has only recently been reported in the plant pathogen *Pyrenopeziza brassicae* in relation to resistance to DMIs used in crop protection [143].

Fungicides are applied recurrently over a long period of time and could thereby generate a persistent pressure of azole drugs on saprophytic fungi [144]. The existence of an environmental source of resistant *A. fumigatus* would be supported by the finding of primary IA caused by azole-resistant *A. fumigatus* in azole-naive patients [145]. These two resistance mechanisms (TR_{34}/L98H and TR_{46}/Y121F/T289A) have been reported in environmental isolates and also in azole-naive patients from the five continents [22, 72, 73, 78, 80, 82, 84–89, 146–150], strongly suggesting a primary acquisition of resistant isolates from the environment. Furthermore, genetic typing of the clinical *A. fumigatus* isolates revealed shorter genetic distances between TR_{34}/L98H azole-resistant isolates compared to wild-type isolates, suggesting a common source of resistance and a subsequent spreading phenomenon [22, 129, 146].

It seems quite clear that the extended use of azoles as fungicides is selecting resistant mutants in the environment, but it is unknown whether a specific type of DMI used for crop protection is responsible for the type of resistant mechanism selected. Alternatively, each pathogen, with their specific target-drug interaction, could select one specific type of mechanism; promoter insertions (TR), target point mutations, or a combination of both, as has been described with *A. fumigatus* and *P. brassicae*. In fact, each Cyp51A modification is responsible for a different pattern of triazole resistance, as described before.

5 Implications for Treatment and Treatment Options

The occurrence of azole-resistant *A. fumigatus,* in both patients and the environment, is a matter of global concern as azole resistance can seriously compromise treatment in patients with IA. Mortality rates in patients infected with azole-resistant strains are higher than those afflicted by azole susceptible microorganisms (88 vs. 30–50%) [21].

Reference antifungal susceptibility testing methods (Clinical and Laboratory Standards Institute and The European Committee on Antimicrobial Susceptibility Testing) together with molecular characterization of antifungal resistance mechanisms can provide useful information to optimize antifungal therapy and to detect emerging resistance [48]. However, the high mortality rates observed in patients with IA caused by azole-resistant *A. fumigatus* isolates poses a serious challenge with respect to timely resistance identification and appropriate therapeutic interventions [151].

The emergence of azole resistance raises concerns about first-line VCZ treatment in high-risk patients with suspected IA [152, 153]. Determination of azole resistance percentages at the hospital level, and within different patient groups or departments, will enable clinicians to decide whether reassessment of azole monotherapy as a primary treatment option is necessary [37, 38]. Recently, an international expert panel recommended that VCZ should be used as a first-line drug as long as the local resistance rate does not exceed 10% and a combination of VCZ with an echinocandin or L-AMB would be the therapeutic alternatives if the resistance rate is superior [17].

In the setting of azole resistance, alternative therapeutic options are limited: L-AMB is an important therapeutic option as no cross-resistance is described. Also, the combination of VCZ or POS with an echinocandin has been suggested as an alternative, although it is assumed that azoles will play a limited role in the treatment of azole-resistant invasive *Aspergillus* infections [154]. Anidulafungin is currently not approved for the treatment of IA, although combination therapy with VCZ is being explored as an alternative when drug resistance is suspected [155, 156].

6 Future Research Areas

The existence of several azole resistance mechanisms in *A. fumigatus* and the increasing number of affected patients emphasize the need for surveillance studies to determine each country's epidemiology, to discover the emergence of new mechanisms of azole resistance, and to assess the risk associated with treatment failure.

The need for systematic antifungal susceptibility testing, particularly in high-risk populations cannot be overstated, for assisting clinicians in selecting appropriate antifungal therapy as early as possible [157]. Additionally, techniques to identify resistance directly in clinical samples have the potential to overcome culture-based diagnostic limitations and should be further investigated [158, 159]. Meanwhile, investment in development of new (or repurposed) antifungal drug classes given the risk of losing azoles as first-line treatment is mandatory [160]. Future research should focus on evaluating the effectiveness of newer pharmaceutical agents that can be used as monotherapy or combinational treatments in order to avoid azole resistance. In addition, optimization of current drugs for the purpose of maximizing therapeutic effect while minimizing toxicities would be another important approach [161].

In agriculture, fungicides are applied recurrently over long periods of time and could thereby generate a persistent pressure of azole drugs on saprophytic fungi [144]. Environmental surveys are warranted to determine the prevalence of these resistance mechanisms in saprophytic fungi and to identify areas with a high burden of resistant *A. fumigatus* [162]. However, an indiscriminate reduction of fungicides used in agriculture would have a detrimental effect on food production and the economy [163]. Therefore, efforts should focus on unraveling the origin and spread of azole resistance in order to better inform guidelines and policy on the use of clinical and agricultural antifungals [164].

Next-generation sequencing (NGS) studies of *Aspergillus*-resistant strains would give insight into the dynamics of resistance. Moreover, with greater access to NGS technology, studying isolates without a known resistance mechanism would expand our knowledge of *A. fumigatus* azole resistance. In this respect, there are already some studies applying NGS to clinical *A. fumigatus* isolates that have reported both accumulation of mutations and genomic deletions that appeared to have occurred randomly in isolates recovered from aspergilloma [165]. This approach has already been used (including a genome and RNA-seq sequencing) in *Erysiphe necator*, finding a strong association between *cyp*51 gene copy number variation, Y136F mutation and fungicide treatment. This work suggests that the development of DMI resistance may be happening in two steps: the first, selection of isolates carrying the Y136F mutation and the second, structural rearrangements that increase the number of *cyp*51 copies carrying the Y136F [166].

In summary, conclusive evidence linking the use of DMIs to the emergence of *A. fumigatus* azole resistance is still lacking. A multidisciplinary approach to integrate epidemiological studies in the environment and among clinical isolates is required to track the development and spread of resistance mechanisms in *A. fumigatus* in order to prevent or minimize its impact [152, 153].

Compliance with Ethical Standards

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Conflict of Interest RGR, MCE and EM have no conflicts of interest to declare.

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