Update and recent advances on azole resistance mechanisms in *Aspergillus*

Alba Pérez-Cantero [1], Loida López-Fernández [1], Josep Guarro-Artigas [1], Javier Capilla[1]

[1]Author affiliations: Unitat de Microbiologia, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili and Institut d'Investigació Sanitària Pere Virgili (IISPV). Reus, Tarragona, Spain

Correspondence: Javier Capilla. Unitat de Microbiologia, Facultat de Medicina, Universitat Rovira i Virgili. Carrer Sant Llorenç, 21, 43201 Reus, Spain.

Tel : +34 977759359

Fax : +34 977759322.

Email : javier.capilla@urv.cat

ABSTRACT

Aspergillus fumigatus is the main causal agent of invasive aspergillosis (IA) although other species of the genus can also be the cause of IA, such as *A. flavus, A. terreus, A. niger* and related cryptic species. This infectious disease mainly affects immunosuppressed patients and it is linked to elevated mortality rates. Being voriconazole (VRC) the treatment of choice for this condition, the relevant increase in the number of azole resistant isolates in recent years has gathered alarming attention, as it translates into a clinical failure increment as well.

In this review, we summarize and discuss the azole resistance molecular data described to date in the most clinically prevalent sections of *Aspergillus*, comprising mechanisms that involve target proteins Cyp51 and ATP Binding Cassette (ABC) or Major Facilitator Superfamily (MFS) efflux pumps. Other resistance mechanisms proposed but not fully-characterized yet are also discussed.

Keywords: Aspergillosis, triazoles, antifungal resistance, cyp51, efflux pumps

1. INTRODUCTION

Aspergillus is a broad fungal genus that comprises more than 300 different species ubiquitously distributed worldwide. Several species of the genus are biotechnologically used by their ability to produce important metabolites in the medical and industrial fields. However, *Aspergillus* is also responsible of important economic losses as it can negatively affect crops. The genus is currently organized in different subgenera and sections [1,2], being sections *Fumigati, Flavi, Terrei* and *Nigri* the most clinically relevant due to their great impact as human opportunistic pathogens [1].

Aspergillus infections show a wide range of clinical manifestations, including infections that drive to hypersensitive responses, such allergic *Aspergillus* sinusitis [3] or allergic bronchopulmonary aspergillosis [4]; infections through skin that result in cutaneous aspergillosis [5]; aspergilloma and chronic conditions as chronic pulmonary aspergillosis [6]. The most problematic pathology in terms of patient outcome and disease management is invasive aspergillosis (IA), a life-threatening condition that, although has been reported to affect immunocompetent patients in rare occasions [7,8], mainly affects immunocompromised patients such those suffering from cancer, hematological malignancies, or those subjected to chemotherapy, corticosteroid treatment and transplants [9,10]. The presentation of the disease generally differs between neutropenic and non-neutropenic patients. IA in non-neutropenic patients is characterized by few symptoms such as fever, cough and chest pain, while pneumonia and higher fatality rates are found in neutropenic patients suffering IA [11].

Scarce data is available on IA epidemiology and, although *A. fumigatus* continues to be considered the most frequent causal agent of the condition [12], rates of infection due to other emerging species are uncertain and probably underestimated as a result of inaccurate molecular identification. Nonetheless, it has been reported that cryptic species belonging to sections *Flavi*, *Nigri* and *Terrei* are nowadays frequently isolated from IA patients as well [12–15]. In general, about 200,000 cases of IA are estimated to occur every year worldwide, which seems to account for approximately only a half of the actual cases, as a direct consequence of misdiagnosis. In fact, lack of accuracy in diagnosis or treatment strategy makes IA mortality rates vary from 50 % to 100 % [16].

As currently stated in the aspergillosis management guidelines, the recommended treatment for IA is voriconazole (VRC) and alternative treatments consist of liposomal amphotericin B or isavuconazole (ISA). Other treatments used as salvage therapies are amphotericin B lipid complex, caspofungin,

81 micafungin, posaconazole (PSC) and itraconazole (ITC). Additionally, PSC or VRC constitute the
82 prophylactic recommended measures to prevent aspergillosis [10].
83 Specifically, azoles have been described to target the cytochrome P450 sterol 14 α-demethylase
84 enzyme (Cyp51) by non-competitive binding, causing the inhibition of the demethylation of ergosterol
85 precursors and thus blocking ergosterol biosynthesis [17]. However, a few studies suggest that azoles
86 act as competitive inhibitors of Cyp51 instead, as their interactions consist of reversible and competitive
87 binding to iron of the heme-group and residues from its close proximity [18,19]. In any case, the result
88 is the accumulation of 14-methylated sterols that cause alterations in membrane fluidity and its final
89 disruption, which in turn reduces the activity of membrane-bound enzymes and leads to the inhibition of
90 cell growth and proliferation. Hence, it is believed that azoles may disrupt the ergosterol stimulating role
91 on growth and proliferation [17,18,20,21].
92 Nevertheless, the incidence of azole-resistant *Aspergillus* isolates has alarmingly increased in recent
93 years, which is thought to develop from the use of azoles in both the clinical and agricultural settings,
94 and directly contributes to therapeutic failure [22–26].
95 The aim of this review is to compile azole resistance data described to date in the most prevalent
96 sections of *Aspergillus.*

97 2. AZOLE RESISTANCE

98 Azole resistance is considered the ability of fungal strains to overcome doses of azole drugs that exert
99 good antifungal activity in other susceptible isolates. The threshold values that distinguish resistant from
100 susceptible strains are stablished through determination of the Minimal Inhibitory Concentration (MIC)
101 of a drug against a broad range of strains. Specifically, MICs distribution together with pharmacokinetic
102 and pharmacodynamics profiles allow to establish Clinical Break Points (CBPs), which are used as
103 predictors to anticipate treatment effectiveness in patients.
104 In the case of *Aspergillus* species, different reference methods have been stablished to characterize
105 isolates in basis of their antifungal susceptibility, such the ones developed by the European Committee
106 on Antimicrobial Susceptibility Testing (EUCAST) and by the Clinical Laboratory Standards Institute
107 (CLSI) [27–30] .

Whereas some CBPs have been stablished for a few antifungals in *Aspergillus* species by the EUCAST method [31], there are no available CBPs set by the CLSI method, although it does provide Epidemiological Cutoff Values (ECVs) for *Aspergillus* instead [32,33]. ECVs categorize isolates into wild-type (wt) and non-wild type (non-wt), being the latter indicative of decreased susceptibility towards a particular antifungal. This susceptibility reduction can be linked to potential acquired resistance mechanisms, although it cannot be considered a predictor of patient's response to therapy as CBPs are [34].

To decipher which are the mechanisms responsible for antifungal resistance, it is essential to consider the effect of the drug within the cell. Specifically, azoles exert an inherent antifungal potency by binding the Cyp51 protein. Nevertheless, the precise physiological effects derived from the Cyp51 inhibition on the fungal cell biology remain unclear, and the specifics on the fungicidal effect of azoles in *Aspergillus* is currently under study [35].

Traditionally, researchers have considered two fundamental aspects regarding the nature of azole resistance in *Aspergillus*: the relevance of polymorphisms in Cyp51 proteins, which is related to azole affinity decrease, and the transcriptional response of the fungus, which seems to be crucial for the fungal adaptation towards azole stress [36]. On this basis, the most studied molecular mechanisms that contribute to the appearance of resistant phenotypes in *Aspergillus* can be classified in: (i) alterations in the Cyp51 protein that reduce the affinity between the azole drug and its target, (ii) overexpression of the target enzyme, which increases the necessary azole levels to inhibit fungal growth and (iii) upregulation of the efflux pumps system to decrease intracellular drug concentration. However, other mechanisms consisting of biofilm formation, cellular stress response, drug enzymatic degradation and activation of alternative pathways in order to bypass the drug effects, have also been proposed to contribute to antimicrobial resistance [25,37] (Fig. 1).

2.1. *cyp51* GENES

Among all the studied resistance mechanisms in *Aspergillus*, the Cyp51 enzyme encoded by the *cyp51* gene (*ERG11* in yeasts), is the major candidate for azole resistance mediation at the molecular level. Remarkably, many filamentous ascomycetes have suffered gene duplication as occurred in *Aspergillus*,

which contains varying number of *cyp51* paralogs among species located in different chromosomes throughout the genome. While some species, such *A. fumigatus, A. nidulans* and *A. niger* present two paralogs (*cyp51A* and *cyp51B),* others as *A. flavus*, and *A. oryzae* carry three paralogs (*cyp51A*, *cyp51B* and *cyp51C*) [20,25]. Additionally, *A. terreus* and section *Nigri* species *A. carbonarius* seem to display three *cyp51* paralogs as well, which has not been reported so far to our knowledge (accession numbers of these proteins are XP_001218650 and OOF93749, respectively) (Fig. 2).

Very interestingly, Cyp51s share a common ancient origin, and assuming that the different *cyp51* versions diverged upon evolution, we find similar sequence identities among them in *Aspergillus*. However, they are evolutionarily differentiated in two lineages of paralogous proteins (Cyp51A and Cyp51B). The third one (Cyp51C), appears to have originated from duplications of both genes, *cyp51A* or *cyp51B* depending on the species (Fig.2). In this sense, some authors have proposed that *cyp51* duplications derive from evolutionary mechanisms that govern azole toxicity adaptation [38,39]. As gene duplications are a great source of genetic adaptive potential, if selection favored cells with additional copies of *cyp51* to have better growth under selective conditions, we should expect those strains with two or more copies of the gene to have greater tolerance to azoles than those with only one copy. Nevertheless, this hypothesis will need to be corroborated by future studies.

Moreover, functional analyses have demonstrated that both enzymes share the same substrate and display comparable functions. This has been observed by heterologous expression of *A. fumigatus cyp51A* and *cyp51B* in a *Saccharomyces cerevisiae cyp51* defective mutant, which resulted in effective complementation in terms of ergosterol content and azole tolerance [40]. In the specific case of *cyp51A,* its heterologous expression in a Cyp51 defective strain of *S. cerevisiae* caused a decrease in azole susceptibility, although not for all the azoles tested [40]. These findings have been reinforced by further siRNA silencing studies in which *cyp51A* silencing has been seen to increase azole susceptibility in a non-wt *A. fumigatus* strain [41]. By contrast, in the case of *cyp51B* neither its deletion nor its heterologous expression carries any effects on azole susceptibility [40,42] (Table 1).

Whether it has been demonstrated that neither of the two *cyp51* forms is individually essential in *A. fumigatus*, the lack of both genes has a lethal result in this organism [42]. Curiously, deletion of *cyp51A* increases azole susceptibility without altering *cyp51B* expression levels [43], which means that *cyp51* redundancy does not lead to genetic compensation by transcriptional adaptation.

As reviewed elsewhere, for those species carrying an additional *cyp51C* copy of the gene, *cyp51A* characteristics could be displayed by *cyp51C* instead [25,39]. However, a recent study performed on section *Flavi,* seems to indicate that this does not occur in this section [44]. Accordingly, further studies are needed in order to characterize the real involvement of each Cyp51 paralog on azole response in these species.

Taken together, the features displayed by these genes imply that *cyp51A* (or *cyp51C* in some species) encodes major enzymatic activity and gathers greater influence in terms of azole response at the same time. As for *cyp51B,* it constitutes a functional redundant enzyme that could have potential alternative functions yet to be defined [45].

2.1.1. Cyp51 protein sequence variations

At structural level, Cyp51s are widely conserved proteins due to their essential role in ergosterol biosynthesis and its narrow substrate specificity. Therefore, alterations in the *cyp51* gene sequences may occur in regions that do not compromise their functional activity [46]. However, polymorphisms in azole-binding amino acids that do not compromise Cyp51 activity could decrease the affinity of the enzyme towards these drugs [47]. Consequently, single point mutations in the *cyp51A* gene causing amino acid substitutions within the Cyp51A protein have emerged as the major resistance-mediating mechanism in *Aspergillus*. The presence of such mutations might alter the structure, stability and functionality of Cyp51, thus hindering substrate recognition and eventually leading to different azole resistance patterns [45,48]. The screening of specific variants of the *cyp51* gene associated with increased resistance to azoles has led to extensive genotyping studies, in which some *Aspergillus* species (mostly *A. fumigatus*) have been characterized in the search of potential mutations that could explain resistant phenotypes. It is worth mentioning, though, that some non-synonymous mutations within the *cyp51A* gene initially associated with resistance, have been later reported in wt strains as well [49,50], which suggests that some association studies can lead to errors due to the low number of strains analyzed.

So far, Cyp51A polymorphisms in *A. fumigatus* consisting of amino acid substitutions in positions G54, Y121, G138, P216, F219, M220, A284, Y431, G432, G434 and G448 have been reliably correlated with

reduced azole susceptibility (45–59) (Table 2 and Fig. 3). In contrast, no polymorphisms in Cyp51B seem to contribute to azole resistance in this species [25,39].

While some point mutations in *A. fumigatus* Cyp51A have been validated as an important resistance mechanism, their role in azole susceptibility in other species of the genus remains unclear and poorly studied. In fact, reports on non-wt *Aspergillus* spp. isolates lacking *cyp51* mutations are abundant [54,66–68]. In this regard, a mutation (S240A) in the *A. flavus* Cyp51C protein initially reported to reduce VRC susceptibility [69] was later found in both, wt and non-wt strains, thus leading to the hypothesis that it represented a geographical variation, instead [70]. Furthermore, several mutations in the three Cyp51 enzymes (A, B and C) have been reported in *A. flavus* isolates with reduced VRC susceptibility, although more studies are needed in order to validate them. This is the case of amino acid changes R450S, K197N or the combinations K197N / D282E / M288L and Y132N / T469S, which have been found in the Cyp51A enzyme, while in the case of the Cyp51B protein only the variation Q354K has been reported. Finally, Y319H and the combination S196F / A324P / N423D / V465M have been identified in Cyp51C, which is the responsible protein for the major enzymatic activity in *A. flavus* [66,70,71].

Doubts on the involvement of Cyp51 mutations in azole resistance arise in the case of section *Nigri*, as elevated azole MIC values cannot be explained by polymorphisms in numerous cases regarding this section [72]. Reported Cyp51A mutations in *A. niger* and *A. tubingensis* seem to mostly account for reduced ITC and/or VRC susceptibility. Particularly, the reported amino acid substitutions in *A. niger* consist of V104I, H382R and the combination I377V / S507I / L511M. In the case of *A. tubingensis* L21F, T321A or the combination A9V / L21F / A140V / P413S / D505E or A185G / T321A / 327S / V422I / L492M / I503F / Q504P have been reported [73,74].

Studies on the azole resistance of section *Terrei* are scarce, and to date only amino acid changes in Cyp51A position M217 have been described to have effects on ITC and PSC susceptibility [75,76].

The reported amino acid changes to date with effects on azole susceptibility are summarized in Table 2 and represented in Fig. 3.

2.1.2. *cyp51* overexpression

Another major mechanism hypothesized to be responsible for azole resistance acquisition in *Aspergillus* consists of *cyp51* overexpression. It is reasonable to believe that if azoles bind the Cyp51 enzyme, greater abundance of these proteins within the cell could rescue the fungus from the inhibitory effect of the drugs. Therefore, differences in the expression profile of both genes could be a key aspect to understand azole tolerance.

Despite the great similarity displayed by Cyp51 proteins, both isoenzymes diverge in their transcriptional regulation in *Aspergillus.* In general terms, while *cyp51A* expression seems inducible by azoles, *cyp51B* gene maintains a constitutive pattern of expression in *A. fumigatus* [18]. Furthermore, the importance of *cyp51A* expression in azole resistance was proven by heterologous expression assays, in which a decrease on *A. fumigatus* ITC susceptibility was conferred by the introduction of extra copies of the *A. nidulans pdmA* (*cyp51A*) gene [77]. Still, it remains to be determined if that same effect occurs for the *cyp51B* gene.

On this basis, one would expect a clear correlation between *cyp51A* expression and resistance. However, numerous studies present patterns of expression that do not fit this hypothesis. For instance, in a study where *A. fumigatus* clinical isolates didn't show either *cyp51A* point mutations nor *cyp51A* overexpression, *cyp51B* was overexpressed in one of the non-wt strains tested [78], suggesting that *cyp51B* is not constitutively expressed in all strains and that it could be, unusually, involved in azole response as well.

Regarding other species of the genus, *cyp51A* and *cyp51B* expression levels in *A. flavus* do not seem to be related to VRC resistance. As a matter of fact, the expression profiles of these genes barely vary among wt and non-wt strains, even after exposure to azole [44,69]. Moreover, *cyp51C* displays very low expression levels compared to its paralogous, and its expression is not induced by VRC [44]. This leads us to think that sterol 14α-demethylase activity in this species is mainly due to the expression of *cyp51A* and *cyp51B* genes*,* even though *cyp51C* had been initially accepted to account for this [25,39]. Similarly, it has been shown that transcript levels of *cyp51A* and *cyp51B* cannot explain azole resistance in species from section *Nigri*. Specifically, *cyp51* basal expression does not appear to be related to azole susceptibility [74,79] and, despite *cyp51A* is up-regulated after azole exposure, *cyp51B* displayes a low constitutive expression in contrast [79]. This seems to indicate that Cyp51A is the major player on azole

response in this section, even though expression profiles do not show correlation with azole susceptibility.

In addition, there are specific cases in which overexpression of *cyp51* occurs along with other factors also associated with resistance, which makes it difficult to establish reliable associations. In fact, overexpression of *cyp51A* gene was detected in 4 out of 5 non-wt isolates of *A. flavus* [80] that also displayed increased *mdr1* (multidrug efflux pump) transcript levels, making difficult to distinguish the degree of involvement in azole resistance of *cyp51A* or *mdr1* by themselves. Similar results have been also reported in a recent study on *A. flavus*, despite in this case overexpression of *cyp51A, cyp51B and cyp51C* along with *mdr1, mdr2, atrF and mfs1* (multidrug efflux pumps) was found in a single non-wt isolate [71].

The transcriptional upregulation of *cyp51* is the result of an efficient response to azole toxicity carried by transcription factors and transcriptional activators regulation. In certain cases where there are tandem repeats (TRs) of 34 bp (TR_{34}), 46 bp (TR_{46}) or 53 bp (TR_{53}) in the promoter region of *A. fumigatus cyp51A,* the expression of this gene is enhanced [81–84]. TRs have been observed in strains that display ITC and VRC reduced susceptibility [84]. However, they are frequently found in combination with amino acid substitutions (TR_{34} / L98H, TR_{46} / Y121F and TR_{46} / T289A) in the *cyp51A* of non-wt strains [81,85,86] with the exception of TR_{53} , which has not been linked to any other *cyp51A* modification [87]. It is hard to distinguish whether TRs or point mutations are responsible for the resistance phenotypes in the case of TR_{34} and TR_{46}, and so, mutant strains harboring TR_{34} or TR_{46} alone or in combination with different amino acid substitutions were generated and characterized. Results show that strains with TRs display increased expression of *cyp51A*, suggesting that these sequences could act as transcriptional enhancers indeed. However, the contribution of these TRs to azole resistance seems to be insignificant [82,83].

As sterol biosynthesis is highly regulated, many genes coding for enzymes of this pathway harbor sterol regulatory binding elements in their promoter regions [88]. Regarding transcription factors (TF) that regulate *Aspergillus cyp51* expression relatively little is known. Recent studies have identified TFs that bind to *A. fumigatus cyp51* promoters and regulate their expression, such SrbA, HapE and AtrR. In particular, SrbA is a transcriptional regulator that belongs to the sterol binding protein (SREBP) family. This regulator has been described to participate in fungal growth during hypoxia conditions, sterol

biosynthesis, cell polarity, hyphal morphogenesis, *A. fumigatus* virulence and *cyp51* transcriptional regulation, as its deletion increases azole susceptibility [89,90]. HapE is a CCAAT-binding transcription factor complex subunit that regulates negatively the transcription of *cyp51A*. A single mutation in the amino acid position 88 of this negative regulator as well as its deletion cause overexpression of *cyp51A,* with a decrease on azole susceptibility as a result [91]. Additionally, recent investigations towards the role of the Zinc finger AtrR have revealed that this TF regulates *cyp51* expression among many other genes, and its deletion in *A. fumigatus* not only reduces *cyp51A* and *cyp51B* transcription, but also virulence, indicating the essential role of this TF in fungal pathogenesis and resistance [92,93].

Additionally, cytochrome b_5 CybE has been also found to regulate *cyp51A* transcription levels in *A. fumigatus*. In this particular case, deletion of *cybE* resulted in the compensatory upregulation of *cyp51A;* however, its deletion also caused an increase in the VRC susceptibility and accumulation of the ergosterol precursor eburicol [94].

2.2. MULTIDRUG EFFLUX PUMPS

Multidrug efflux pumps consist of transmembrane proteins that mediate active extrusion of antimicrobial molecules or toxic compounds and endogenous metabolites to the extracellular space [95]. Thus, efflux activity constitutes a determinant factor to be considered in drug resistance and fungal survival. Considering the great number of efflux–pump protein coding genes present in the *Aspergillus* genome, functional reduncancy could be expected in terms of antifungal transport [96].

Currently, two types of efflux superfamilies are known to modulate azole extrusion from the fungal cell: the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) transporters. Both the structure and the mechanisms of action differ between the two types of proteins. ABC transporters are constituted by two transmembrane and two cytoplasmic nucleotide-binding domains and use the energy derived from ATP hydrolysis to extrude the substrate across the membrane, while MFS transporters contain 12-14 transmembrane domains and in almost all cases use proton-motive force to accomplish drug efflux [97,98]. There are 45 ABC and 275 MFS transporters identified in the *A. fumigatus* genome [99], although only a few of them have been identified as drug transporters, which are also referred as multi-drug resistance (MDR) or pleiotropic drug-resistance (PDR) proteins [100]. To elucidate the contribution of efflux pumps into azole resistance, intensive research by means of expression analysis

and site-directed mutagenesis has been performed in *A. fumigatus* and, to a lesser extend, in other *Aspergillus* species. In most cases, the overexpression of these transporters could cause the intracellular drug levels not to reach the necessary levels to be effective against the fungal cell [97]. However, their expression does not show a clear correlation with azole MIC values (Table 1).

ABC transporters investigated in terms of azole resistance so far include *cdr1B, mdr1, mdr2, mdr3, mdr4, abcD, abcE, atrI, atrB, atrC* and *atrF.* In the case of *A. fumigatus, cdr1B* (*abcB)* has been reported to contribute to azole resistance since its disruption largely increases azole susceptibility [68,101,102], demonstrating its importance in azole resistance. Nonetheless, despite its clear upregulation upon VRC exposure [103], its relevance in the underlying resistance mechanisms remains uncertain. This transporter has been also found to be important in *A. flavus* VRC resistance, as it was found to be more expressed than other efflux pumps investigated (*mdr2, mdr4, mfs1* and *atrF*) in basal conditions and upregulated upon azole exposure [44]. However, this observation is not completely reliable considering the limited number of analyzed strains and the fact that only a few non-wt strains exhibited overexpression of these transporters.

In addition, deletion of the ABC transporters *mdr1* (*abcA*), *mdr2*, *mdr3* and *mdr4* in *A. fumigatus* does not have any clear effect in azole susceptibility, although *mdr1* expression is highly induced by ITC and VRC. Moreover, non-wt isolates have been reported to show upregulation of theses efflux pumps under azole exposure on *A. fumigatus* and other *Aspergillus* species, although when overexpressed, azole susceptibility was not always reduced [52,68,71,80,101,103–105]. Similarly, less studied transporters *abcD, abcE, atrI* and *atrF* also showed patterns of upregulation under azole induction in *A. fumigatus* and *A. flavus*, being their expression commonly higher in non-wt strains [99,103–106].

With regard to the MFS transporters, there are fewer published studies and those analyzed so far only include *mdrA, mfs56, mfs1, mfsA, mfsB* and *mfsC.* Among them just *mdrA* and *mfs56* have been studied through deletion experiments in *A. fumigatus* resulting in an increase of ITC and VRC susceptibility in Δ*mdrA* mutant [99], while the disruption of *mfs56* did not affect azole susceptibility [68], thus indicating its minor importance in azole resistance events. Regarding gene expression responses during azole exposure, only *mfsA, mfsB* and *mfsC* have been confirmed to be upregulated in *A. fumigatus*. [103] In the case of *A. flavus,* transporter *mfs1* expression has been studied under VRC exposure, showing no correlation with azole susceptibility [44].

An important limitation of these studies, though, lies in the few strains tested in every case (in some cases, analyses are limited to only one strain), thus not allowing generalization and extrapolation of results. Further studies are needed in order to better understand the real involvement of efflux pumps in azole resistance in *Aspergillus.*

2.3. OTHER RESISTANCE MECHANISMS

Even though it is widely accepted that azole-related ergosterol depletion is the main cause of fungal viability and growth inhibition, little is known about the exact mechanism triggered by azoles to exert their fungicidal effect on *Aspergillus.* In this context, it has been reported that resistant events, although proven to be mediated by Cyp51, could result from the cellular stress responses displayed by the fungal cell or other molecular mechanisms. As a matter of fact, the cell wall integrity pathway as well as other intracellular pathways have been recently identified by several investigations to play a role in *Aspergillus* azole response and resistance, as will be further explained.

2.3.1. Amino acid substitutions in HMG-CoA

Ergosterol biosynthesis takes place in a complex pathway that seems to be specific to fungal taxa and includes about 20 different enzymes [107]. The catalysis of the first step in this metabolic pathway is carried by the 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA) protein [88], encoded by the *hmg1* gene in *A. fumigatus.* This enzyme, contains a sterol-sensing domain, which is involved in the negative regulation of its own reductase activity through direct interaction with sterols [108] and recently, mutations located at the beginning of this sensing domain have been described as candidate mediators of azole resistance. As a matter of fact, in a recent study, 52% of the clinical *A. fumigatus* non-wt isolates tested were found to contain mutations in this protein. Specifically, mutations F262del, S305P, P309L and I412S notably increased VRC, PSC, ITC and ISA MICs leading to an accumulation of ergosterol precursors without altering *cyp51* gene expression [108]. Moreover, introduction of three of these mutations (F262del, S305P and I412S) in a wt genetic background resulted in a reduction in the triazole susceptibility and the correction of these substitutions restored susceptibility [108]. Mutations in the *hmg1* gene have also been studied in another study, however the substitution S269F (potentially

involved in azole resistance), did not have any effects in azole susceptibility when introduced in awt isolate, thus discarding any role in azole resistance [109].

2.3.2. Stress response

One of the effects resulting from azole exposure is the induction of mitochondrial reactive oxygen species (ROS) production, which seems to contribute to inhibit fungal growth. As azoles promote the accumulation of ROS, the involvement of the mitochondrial complex constitutes a field of interest in terms of azole resistance. Inhibition of mitochondrial complex I has been shown to abolish the release of deleterious ROS in an azole-exposed *A. fumigatus* [110]. In addition, amino acid substitution E180D in the mitochondrial complex I subunit 29.9 kD has been found in azole-resistant *A. fumigatus* isolates, suggesting that resistance could arise from the complex I activity loss. Moreover, inhibition of this mitochondrial complex led to azole resistance in *A. fumigatus* [111].

The importance of mitochondria in azole response has also been described in other studies. As a matter of fact, it has been recently reported that VRC induces a sequence of events that include a cytoplasm sudden expulsive release, arrest of mitochondrial dynamics, mitochondrial fragmentation and final lysis of the mitochondria, which eventually leads to fungal death in *A. fumigatus* [35]. This clearly suggests an essential involvement of mitochondria in azole response, though not-fully characterized yet.

The connection between oxidative stress adaptation and azole resistance is reinforced by previous studies on Yap1, a transcription factor that regulates defense mechanism against ROS and azole antifungal drugs in *A. fumigatus* [112,113]. When truncated, this version of the *yap1* gene has been observed to confer attenuated susceptibility to VRC [112]. Yap1 has also been considered to participate in VRC resistance in *A. flavus*, since a point mutation causing the amino acid substitution L558W in this factor led to ATP transporter *atrF* upregulation, which in turn is correlated to low VRC susceptibility [114].

Recently, many signaling pathways have been also shown to mediate responses to azole toxicity and play key roles in azole tolerance. For instance, it has been proven that compromising Hsp90 function, one of the most studied stress-related proteins, enhances the activity of many antifungal drugs. This protein is a molecular chaperone that interacts with a diverse amount of proteins by which gene

391 expression is controlled during stress conditions. Among others, Hsp90 activates phosphatase
392 calcineurin by interacting with its catalytic subunit to regulate stress response, including azole-induced
393 stress in *A. fumigatus*. Inhibition of Hsp90 (with geldanamycin) or calcineurin (with FK506) increases
394 azole susceptibility, proving the involvement of these proteins in azole resistance [115,116].
395 Likewise, the Damage Resistance Protein (Dap) family, which comprise DapA, DapB and DapC,
396 responds to azole stress and controls ergosterol biosynthesis by SrbA regulation. Specifically, deletion
397 of *dapA* increases azole susceptibility, therefore demonstrating its potential involvement in azole
398 resistance [117,118].
399 The calcium-signaling pathway also constitutes another example of this kind. While the role of the Ca^{2+}-
400 mediated signal transduction in growth, development, proliferation, secretion, transportation and stress-
401 response is well recognized, adaptation to drug toxicity could also involve this signaling pathway in fungi
402 [119]. After ITC exposure, induction of calcium signaling is achieved, thus proving the role of the pathway
403 in the azole-inducing stress response. Moreover, deletion of genes of this pathway *cnaA* or *crzA*
404 increase azole susceptibility [120].
405 An additional example in this field is the case of the stress-related cell wall integrity pathway.
406 Interestingly, a recent study has provided details into the cellular chain of events promoted by azoles
407 that lead to *A. fumigatus* death. Curiously, they seem to be related to increased *β*-1,3-glucan synthesis
408 in patches all along the hyphae walls that deform the cell membrane and trigger cell wall stress. After
409 this, the cell wall salvage system is activated, leading to fungal cell integrity failure and death [35].
410 Furthermore, the disruption of the *A. fumigatus* Mkk2 signaling kinase, which is a central modulator of
411 the cell wall biosynthesis and organization, results in an increase of the susceptibility towards PSC and
412 VRC as well as a reduction in the virulence and the adherence of this species [121]. Also in the context
413 of *A. fumigatus* cell wall integrity, loss of the endoplasmic reticulum localized protein PerA is associated
414 to impaired cell wall integrity and increased susceptibility to triazoles [122], showing evidence of the cell
415 wall integrity pathway importance in azole response.
416

2.3.3. Biofilm formation

Biofilms are structured microbial cells populations attached onto surfaces and embedded in a self-produced extracellular cell matrix (ECM) made of polysaccharides. Fungal biofilms display reduced susceptibility to the host immune system as well as antifungal drugs in comparison to planktonic cells, which is thought to arise from the own biofilm structural complexity, the protection provided by ECM and the up-regulation of efflux pump genes [123,124].

In this context, there are some proposals on how biofilm takes part in *A. fumigatus* azole resistance. It seems that the cell density reached in a mature biofilm might hinder drug penetration, a similar effect hypothesized in the case of the ECM role in azole resistance, as its hydrophobic nature cohesively binds hyphae [125]. Additionally, up-regulation of the ABC transporter *mdr4* has also been observed in *A. fumigatus* biofilm, which could contribute to azole resistance [95].

2.3.4. Cholesterol import

Another mechanism that has been suggested to be involved in *Aspergillus* azole resistance consists of exogenous cholesterol import [126]. It has been demonstrated that exogenous cholesterol import in *A. fumigatus* is a very regulated process linked to enhanced fungal growth. Interestingly, this import is enhanced by the presence of azoles and attenuates their effects, probably due to the fact that although imported cholesterol seems to be stored in lipid particles, some of it is also incorporated into the fungal membrane. This suggests a possible compensation of fungal ergosterol depletion, which in turns seems to be related to negative consequences in terms of azoles efficacy [127].

3. CONCLUSION

Although in the last years genetic and molecular studies have shed light on our knowledge of different mechanisms contributing to azole resistance, it is clear that azole resistance is a complex and multifactorial event that requires several elements. To date, resistance mechanisms are not fully characterized in *A. fumigatus,* which is the most prevalent IA-causing species, and there is much to explore in the emergent species *A. flavus, A. niger* and cryptic species. In this sense, more studies

445 comprising a higher number of isolates are necessary to determine the real importance of every one of
446 these factors as well as to clarify controversy around the most studied mechanisms, such *cyp51*
447 overexpression and the contribution of multidrug efflux pumps. Another limitation of major concern
448 regarding Cyp51 amino acid substitutions is the fact that although there are studies that carry out deep
449 analyses on amino acid substitutions that evidence azole binding impairment by structural protein
450 changes, many other studies do not conduct considerations on the location of the mutations (*i.e.* near
451 the heme group or the azole binding residues), resulting in unreliable associations between amino acid
452 substitutions and azole resistance.
453 Addressing these limitations will be of major significance to really understand the molecular mechanisms
454 of azole resistance in *Aspergillus.* In addition, multiple lines of evidence converge into the hypothesis
455 that intracellular stress response constitutes another key factor in azole tolerance, corroborating the
456 complexity of drug resistance. It will be essential to go in depth in the study of these pathways, as well
457 as potential other mechanisms, as drug degradation or activation of alternative pathways to bypass the
458 drug effects. To do so, as elsewhere proposed [128], further analysis based on forward genetics, by
459 generation of drug-resistant mutants and genetic screening on wt and non-wt strains, will reveal the
460 gene networks mediating resistance to azoles in *Aspergillus.*
461

462 # DECLARATIONS

463 **Funding:** APC is the recipient of a FI fellowship from Generalitat de Catalunya (Spain).
464 **Competing Interests**: The author reports no conflicts of interest in this work.
465 **Ethical Approval:** Not required
466
467
468
469
470
471

REFERENCES

[1] Samson RA, Visagie CM, Houbraken J, Hong S-B, Hubka V, Klaassen CHW, et al. Phylogeny, identification and nomenclature of the genus Aspergillus. Stud Mycol 2014;78:141–73. doi:10.1016/j.simyco.2014.07.004.

[2] Frisvad JC. Taxonomy, chemodiversity, and chemoconsistency of Aspergillus, Penicillium, and Talaromyces species. Front Microbiol 2015;5:773. doi:10.3389/fmicb.2014.00773.

[3] Chakrabarti A, Kaur H. Allergic Aspergillus Rhinosinusitis. J Fungi (Basel, Switzerland) 2016;2. doi:10.3390/jof2040032.

[4] Greenberger PA, Bush RK, Demain JG, Luong A, Slavin RG, Knutsen AP. Allergic bronchopulmonary aspergillosis. J Allergy Clin Immunol Pract 2014;2:703–8. doi:10.1016/j.jaip.2014.08.007.

[5] Tatara AM, Mikos AG, Kontoyiannis DP. Factors affecting patient outcome in primary cutaneous aspergillosis. Medicine (Baltimore) 2016;95:e3747. doi:10.1097/MD.0000000000003747.

[6] Kosmidis C, Denning DW. The clinical spectrum of pulmonary aspergillosis. Thorax 2015;70:270–7. doi:10.1136/thoraxjnl-2014-206291.

[7] Mohammed AP, Dhunputh P, Chiluka R, Umakanth S. An unusual case of invasive aspergillosis in an immunocompetent individual. BMJ Case Rep 2015;2015. doi:10.1136/bcr-2015-210381.

[8] Cheon S, Yang MK, Kim C-J, Kim TS, Song K-H, Woo SJ, et al. Disseminated Aspergillosis in the Immunocompetent Host: A Case Report and Literature Review. Mycopathologia 2015;180:217–22. doi:10.1007/s11046-015-9903-4.

[9] Desoubeaux G, Bailly É, Chandenier J. Diagnosis of invasive pulmonary aspergillosis: Updates and recommendations. Médecine Mal Infect 2014;44:89–101. doi:10.1016/j.medmal.2013.11.006.

[10] Patterson TF, Thompson GR, Denning DW, Fishman JA, Hadley S, Herbrecht R, et al. Practice guidelines for the diagnosis and management of aspergillosis: 2016 update by the infectious diseases society of America. Clin Infect Dis 2016;63:e1–60. doi:10.1093/cid/ciw326.

[11] Lass-Flörl C. The changing face of epidemiology of invasive fungal disease in Europe.

501 Mycoses 2009;52:197–205.
502 [12] Lass-Flörl C, Cuenca-Estrella M. Changes in the epidemiological landscape of invasive mould
503 infections and disease. J Antimicrob Chemother 2017;72:i5–11. doi:10.1093/jac/dkx028.
504 [13] Taccone F, Van den Abeele A-M, Bulpa P, Misset B, Meersseman W, Cardoso T, et al.
505 Epidemiology of invasive aspergillosis in critically ill patients: clinical presentation, underlying
506 conditions, and outcomes. Crit Care 2015;19:7. doi:10.1186/s13054-014-0722-7.
507 [14] Garcia-Rubio R, Cuenca-Estrella M, Mellado E. Triazole Resistance in Aspergillus Species: An
508 Emerging Problem. Drugs 2017;77:599–613. doi:10.1007/s40265-017-0714-4.
509 [15] Gonçalves SS, Souza ACR, Chowdhary A, Meis JF, Colombo AL. Epidemiology and molecular
510 mechanisms of antifungal resistance in *Candida* and *Aspergillus*. Mycoses 2016;59:198–219.
511 doi:10.1111/myc.12469.
512 [16] Brown GD, Denning DW, Gow NAR, Levitz SM, Netea MG, White TC. Hidden killers: Human
513 fungal infections. Sci Transl Med 2012;4. doi:10.1126/scitranslmed.3004404.
514 [17] Parker JE, Warrilow AGS, Price CL, Mullins JGL, Kelly DE, Kelly SL. Resistance to antifungals
515 that target CYP51. J Chem Biol 2014;7:143–61. doi:10.1007/s12154-014-0121-1.
516 [18] Hargrove TY, Wawrzak Z, Lamb DC, Guengerich FP, Lepesheva GI. Structure-Functional
517 Characterization of Cytochrome P450 Sterol 14α-Demethylase (CYP51B) from Aspergillus
518 fumigatus and Molecular Basis for the Development of Antifungal Drugs. J Biol Chem
519 2015;290:23916–34. doi:10.1074/jbc.M115.677310.
520 [19] Conner KP, Vennam P, Woods CM, Krzyaniak MD, Bowman MK, Atkins WM. 1,2,3-Triazole-
521 heme interactions in cytochrome P450: functionally competent triazole-water-heme complexes.
522 Biochemistry 2012;51:6441–57. doi:10.1021/bi300744z.
523 [20] Mellado E, Diaz-Guerra TM, Cuenca-Estrella M, Rodriguez-Tudela JL. Identification of two
524 different 14-alpha sterol demethylase-related genes (cyp51A and cyp51B) in Aspergillus
525 fumigatus and other Aspergillus species. J Clin Microbiol 2001;39:2431–8.
526 doi:10.1128/JCM.39.7.2431-2438.2001.
527 [21] Georgopapadakou NH, Walsh TJ. Antifungal agents: chemotherapeutic targets and
528 immunologic strategies. Antimicrob Agents Chemother 1996;40:279–91.
529 [22] Vermeulen E, Maertens J, De Bel A, Nulens E, Boelens J, Surmont I, et al. Nationwide

530 Surveillance of Azole Resistance in Aspergillus Diseases. Antimicrob Agents Chemother
531 2015;59:4569–76. doi:10.1128/AAC.00233-15.
532 [23] Snelders E, Huis In 't Veld RAG, Rijs AJMM, Kema GHJ, Melchers WJG, Verweij PE. Possible
533 environmental origin of resistance of Aspergillus fumigatus to medical triazoles. Appl Environ
534 Microbiol 2009;75:4053–7. doi:10.1128/AEM.00231-09.
535 [24] Faria-Ramos I, Farinha S, Neves-Maia J, Tavares P, Miranda IM, Estevinho LM, et al.
536 Development of cross-resistance by Aspergillus fumigatus to clinical azoles following exposure
537 to prochloraz, an agricultural azole. BMC Microbiol 2014;14:155. doi:10.1186/1471-2180-14-
538 155.
539 [25] Hagiwara D, Watanabe A, Kamei K, Goldman GH. Epidemiological and Genomic Landscape of
540 Azole Resistance Mechanisms in Aspergillus Fungi. Front Microbiol 2016;7:1382.
541 doi:10.3389/fmicb.2016.01382.
542 [26] Chowdhary A, Sharma C, Meis JF. Azole-Resistant Aspergillosis: Epidemiology, Molecular
543 Mechanisms, and Treatment. J Infect Dis 2017;216:S436–44. doi:10.1093/infdis/jix210.
544 [27] Arendrup MC, Cuenca-Estrella M, Lass-Flörl C, Hope WW, European Committee on
545 Antimicrobial Susceptibility Testing Subcommittee on Antifungal Susceptibility Testing
546 (EUCAST-AFST). EUCAST technical note on Aspergillus and amphotericin B, itraconazole,
547 and posaconazole. Clin Microbiol Infect 2012;18:E248-50. doi:10.1111/j.1469-
548 0691.2012.03890.x.
549 [28] Hope WW, Cuenca-Estrella M, Lass-Flörl C, Arendrup MC, European Committee on
550 Antimicrobial Susceptibility Testing-Subcommittee on Antifungal Susceptibility Testing
551 (EUCAST-AFST). EUCAST technical note on voriconazole and Aspergillus spp. Clin Microbiol
552 Infect 2013;19:E278-80. doi:10.1111/1469-0691.12148.
553 [29] Arendrup MC, Meletiadis J, Mouton JW, Guinea J, Cuenca-Estrella M, Lagrou K, et al.
554 EUCAST technical note on isavuconazole breakpoints for Aspergillus, itraconazole breakpoints
555 for Candida and updates for the antifungal susceptibility testing method documents. Clin
556 Microbiol Infect 2016;22:571.e1-571.e4. doi:10.1016/j.cmi.2016.01.017.
557 [30] CLSI. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous
558 Fungi. 3rd Ed. CLSI standard M38. 2017.

559 [31] Content Antifungal Agents European Committee on Antimicrobial Susceptibility Testing
560 Breakpoint tables for interpretation of MICs. n.d.
561 [32] Sanguinetti M, Posteraro B. Susceptibility Testing of Fungi to Antifungal Drugs. J Fungi 2018;4.
562 doi:10.3390/JOF4030110.
563 [33] CLSI. Epidemiological Cutoff Values for Antifungal Susceptibility Testing 2nd Ed. CLSI
564 standard M59. 2018.
565 [34] Espinel-Ingroff A, Turnidge J. The role of epidemiological cutoff values (ECVs/ECOFFs) in
566 antifungal susceptibility testing and interpretation for uncommon yeasts and moulds. Rev
567 Iberoam Micol 2016;33:63–75. doi:10.1016/j.riam.2016.04.001.
568 [35] Geißel B, Loiko V, Klugherz I, Zhu Z, Wagener N, Kurzai O, et al. Azole-induced cell wall
569 carbohydrate patches kill Aspergillus fumigatus. Nat Commun 2018;9:3098.
570 doi:10.1038/s41467-018-05497-7.
571 [36] Sun X, Wang K, Yu X, Liu J, Zhang H, Zhou F, et al. Transcription Factor CCG-8 as a New
572 Regulator in the Adaptation to Antifungal Azole Stress. Antimicrob Agents Chemother
573 2014;58:1434–42. doi:10.1128/AAC.02244-13.
574 [37] Wei X, Zhang Y, Lu L. The molecular mechanism of azole resistance in Aspergillus fumigatus:
575 from bedside to bench and back. J Microbiol 2015;53:91–9. doi:10.1007/s12275-015-5014-7.
576 [38] Hawkins NJ, Cools HJ, Sierotzki H, Shaw MW, Knogge W, Kelly SL, et al. Paralog Re-
577 Emergence: A Novel, Historically Contingent Mechanism in the Evolution of Antimicrobial
578 Resistance. Mol Biol Evol 2014;31:1793–802. doi:10.1093/molbev/msu134.
579 [39] Dudakova A, Spiess B, Tangwattanachuleeporn M, Sasse C, Buchheidt D, Weig M, et al.
580 Molecular Tools for the Detection and Deduction of Azole Antifungal Drug Resistance
581 Phenotypes in Aspergillus Species. Clin Microbiol Rev 2017;30:1065–91.
582 doi:10.1128/CMR.00095-16.
583 [40] Martel CM, Parker JE, Warrilow AGS, Rolley NJ, Kelly SL, Kelly DE. Complementation of a
584 Saccharomyces cerevisiae ERG11/CYP51 (Sterol 14 -Demethylase) Doxycycline-Regulated
585 Mutant and Screening of the Azole Sensitivity of Aspergillus fumigatus Isoenzymes CYP51A
586 and CYP51B. Antimicrob Agents Chemother 2010;54:4920–3. doi:10.1128/AAC.00349-10.
587 [41] Mousavi B, Hedayati MT, Teimoori-Toolabi L, Guillot J, Alizadeh A, Badali H. cyp51A gene

588 silencing using RNA interference in azole-resistant *Aspergillus fumigatus*. Mycoses
589 2015;58:699–706. doi:10.1111/myc.12417.
590 [42] Hu W, Sillaots S, Lemieux S, Davison J, Kauffman S, Breton A, et al. Essential Gene
591 Identification and Drug Target Prioritization in Aspergillus fumigatus. PLoS Pathog 2007;3:e24.
592 doi:10.1371/journal.ppat.0030024.
593 [43] Mellado E, Garcia-Effron G, Buitrago MJ, Alcazar-Fuoli L, Cuenca-Estrella M, Rodriguez-
594 Tudela JL. Targeted Gene Disruption of the 14- Sterol Demethylase (cyp51A) in Aspergillus
595 fumigatus and Its Role in Azole Drug Susceptibility. Antimicrob Agents Chemother
596 2005;49:2536–8. doi:10.1128/AAC.49.6.2536-2538.2005.
597 [44] Paul RA, Rudramurthy SM, Dhaliwal M, Singh P, Ghosh AK, Kaur H, et al. Magnitude of
598 Voriconazole Resistance in Clinical and Environmental Isolates of *Aspergillus flavus* and
599 Investigation into the Role of Multidrug Efflux Pumps. Antimicrob Agents Chemother 2018;62.
600 doi:10.1128/AAC.01022-18.
601 [45] Warrilow AGS, Parker JE, Price CL, Nes WD, Kelly SL, Kelly DE. In Vitro biochemical study of
602 CYP51-mediated azole resistance in Aspergillus fumigatus. Antimicrob Agents Chemother
603 2015;59:7771–8. doi:10.1128/AAC.01806-15.Address.
604 [46] Lepesheva GI, Waterman MR. Sterol 14alpha-demethylase cytochrome P450 (CYP51), a P450
605 in all biological kingdoms. Biochim Biophys Acta 2007;1770:467–77.
606 doi:10.1016/j.bbagen.2006.07.018.
607 [47] Parker JE, Warrilow AGS, Price CL, Mullins JGL, Kelly DE, Kelly SL. Resistance to antifungals
608 that target CYP51. J Chem Biol 2014;7:143–61. doi:10.1007/s12154-014-0121-1.
609 [48] Liu M, Zheng N, Li D, Zheng H, Zhang L, Ge H, et al. *cyp51A* -based mechanism of azole
610 resistance in *Aspergillus fumigatus* : Illustration by a new 3D Structural Model of *Aspergillus*
611 *fumigatus* CYP51A protein. Med Mycol 2016;54:400–8. doi:10.1093/mmy/myv102.
612 [49] Lockhart SR, Frade JP, Etienne KA, Pfaller MA, Diekema DJ, Balajee SA. Azole resistance in
613 Aspergillus fumigatus isolates from the ARTEMIS global surveillance study is primarily due to
614 the TR/L98H mutation in the cyp51A gene. Antimicrob Agents Chemother 2011;55:4465–8.
615 doi:10.1128/AAC.00185-11.
616 [50] Garcia-Rubio R, Alcazar-Fuoli L, Monteiro MC, Monzon S, Cuesta I, Pelaez T, et al. Insight into

the Significance of Aspergillus fumigatus cyp51A Polymorphisms. Antimicrob Agents Chemother 2018;62. doi:10.1128/AAC.00241-18.

[51] Mann PA, Parmegiani RM, Wei S-Q, Mendrick CA, Li X, Loebenberg D, et al. Mutations in Aspergillus fumigatus resulting in reduced susceptibility to posaconazole appear to be restricted to a single amino acid in the cytochrome P450 14alpha-demethylase. Antimicrob Agents Chemother 2003;47:577–81. doi:10.1128/AAC.47.2.577-581.2003.

[52] Nascimento AM, Goldman GH, Park S, Marras SAE, Delmas G, Oza U, et al. Multiple resistance mechanisms among Aspergillus fumigatus mutants with high-level resistance to itraconazole. Antimicrob Agents Chemother 2003;47:1719–26.

[53] Krishnan Natesan S, Wu W, Cutright JL, Chandrasekar PH. In vitro-in vivo correlation of voriconazole resistance due to G448S mutation (cyp51A gene) in Aspergillus fumigatus. Diagn Microbiol Infect Dis 2012;74:272–7. doi:10.1016/j.diagmicrobio.2012.06.030.

[54] Bader O, Weig M, Reichard U, Lugert R, Kuhns M, Christner M, et al. cyp51A-Based mechanisms of Aspergillus fumigatus azole drug resistance present in clinical samples from Germany. Antimicrob Agents Chemother 2013;57:3513–7. doi:10.1128/AAC.00167-13.

[55] Camps SMT, van der Linden JWM, Li Y, Kuijper EJ, van Dissel JT, Verweij PE, et al. Rapid induction of multiple resistance mechanisms in Aspergillus fumigatus during azole therapy: a case study and review of the literature. Antimicrob Agents Chemother 2012;56:10–6. doi:10.1128/AAC.05088-11.

[56] Kidd SE, Goeman E, Meis JF, Slavin MA, Verweij PE. Multi-triazole-resistant Aspergillus fumigatus infections in Australia 2015. doi:10.1111/myc.12324.

[57] Alanio A, Sitterle E, Liance M, Farrugia C, Foulet F, Botterel F, et al. Low prevalence of resistance to azoles in Aspergillus fumigatus in a French cohort of patients treated for haematological malignancies. J Antimicrob Chemother 2011;66:371–4. doi:10.1093/jac/dkq450.

[58] Lescar J, Meyer I, Akshita K, Srinivasaraghavan K, Verma C, Palous M, et al. Aspergillus fumigatus harbouring the sole Y121F mutation shows decreased susceptibility to voriconazole but maintained susceptibility to itraconazole and posaconazole. J Antimicrob Chemother 2014;69:3244–7. doi:10.1093/jac/dku316.

646 [59] Wiederhold NP, Gil VG, Gutierrez F, Lindner JR, Albataineh MT, McCarthy DI, et al. First
647 Detection of TR34 L98H and TR46 Y121F T289A Cyp51 Mutations in Aspergillus fumigatus
648 Isolates in the United States. J Clin Microbiol 2016;54:168–71. doi:10.1128/JCM.02478-15.
649 [60] Mellado E, Garcia-Effron G, Alcazar-Fuoli L, Cuenca-Estrella M, Rodriguez-Tudela JL.
650 Substitutions at methionine 220 in the 14alpha-sterol demethylase (Cyp51A) of Aspergillus
651 fumigatus are responsible for resistance in vitro to azole antifungal drugs. Antimicrob Agents
652 Chemother 2004;48:2747–50. doi:10.1128/AAC.48.7.2747-2750.2004.
653 [61] Howard SJ, Cerar D, Anderson MJ, Albarrag A, Fisher MC, Pasqualotto AC, et al. Frequency
654 and evolution of Azole resistance in Aspergillus fumigatus associated with treatment failure.
655 Emerg Infect Dis 2009;15:1068–76. doi:10.3201/eid1507.090043.
656 [62] Bellete B, Raberin H, Morel J, Flori P, Hafid J, Manhsung RT. Acquired resistance to
657 voriconazole and itraconazole in a patient with pulmonary aspergilloma. Med Mycol
658 2010;48:197–200. doi:10.3109/13693780902717018.
659 [63] Bueid A, Howard SJ, Moore CB, Richardson MD, Harrison E, Bowyer P, et al. Azole antifungal
660 resistance in Aspergillus fumigatus: 2008 and 2009. J Antimicrob Chemother 2010;65:2116–8.
661 doi:10.1093/jac/dkq279.
662 [64] Snelders E, Karawajczyk A, Schaftenaar G, Verweij PE, Melchers WJG. Azole Resistance
663 Profile of Amino Acid Changes in Aspergillus fumigatus CYP51A Based on Protein Homology
664 Modeling. Antimicrob Agents Chemother 2010;54:2425–30. doi:10.1128/AAC.01599-09.
665 [65] Albarrag AM, Anderson MJ, Howard SJ, Robson GD, Warn PA, Sanglard D, et al. Interrogation
666 of Related Clinical Pan-Azole-Resistant Aspergillus fumigatus Strains: G138C, Y431C, and
667 G434C Single Nucleotide Polymorphisms in *cyp51A* , Upregulation of *cyp51A* , and Integration
668 and Activation of Transposon *Atf1* in the *cyp51A* Promoter. Antimicrob Agents Chemother
669 2011;55:5113–21. doi:10.1128/AAC.00517-11.
670 [66] Krishnan-Natesan S, Chandrasekar PH, Alangaden GJ, Manavathu EK. Molecular
671 characterisation of cyp51A and cyp51B genes coding for P450 14α-lanosterol demethylases A
672 (CYP51Ap) and B (CYP51Bp) from voriconazole-resistant laboratory isolates of Aspergillus
673 flavus. Int J Antimicrob Agents 2008;32:519–24. doi:10.1016/j.ijantimicag.2008.06.018.
674 [67] Denning DW, Park S, Lass-Florl C, Fraczek MG, Kirwan M, Gore R, et al. High-frequency

675 triazole resistance found In nonculturable Aspergillus fumigatus from lungs of patients with
676 chronic fungal disease. Clin Infect Dis 2011;52:1123–9. doi:10.1093/cid/cir179.
677 [68] Fraczek MG, Bromley M, Buied A, Moore CB, Rajendran R, Rautemaa R, et al. The cdr1B
678 efflux transporter is associated with non-cyp51a-mediated itraconazole resistance in
679 Aspergillus fumigatus. J Antimicrob Chemother 2013;68:1486–96. doi:10.1093/jac/dkt075.
680 [69] Liu W, Sun Y, Chen W, Liu W, Wan Z, Bu D, et al. The T788G mutation in the cyp51C gene
681 confers voriconazole resistance in Aspergillus flavus causing aspergillosis. Antimicrob Agents
682 Chemother 2012;56:2598–603. doi:10.1128/AAC.05477-11.
683 [70] Paul RA, Rudramurthy SM, Meis JF, Mouton JW, Chakrabarti A. A Novel Y319H Substitution in
684 CYP51C Associated with Azole Resistance in Aspergillus flavus. Antimicrob Agents Chemother
685 2015;59:6615–9. doi:10.1128/AAC.00637-15.
686 [71] Sharma C, Kumar R, Kumar N, Masih A, Gupta D, Chowdhary A. Investigation of Multiple
687 Resistance Mechanisms in Voriconazole-Resistant *Aspergillus flavus* Clinical Isolates from a
688 Chest Hospital Surveillance in Delhi, India. Antimicrob Agents Chemother 2018;62.
689 doi:10.1128/AAC.01928-17.
690 [72] Howard SJ, Harrison E, Bowyer P, Varga J, Denning DW. Cryptic Species and Azole
691 Resistance in the Aspergillus niger Complex. Antimicrob Agents Chemother 2011;55:4802–9.
692 doi:10.1128/AAC.00304-11.
693 [73] Iatta R, Nuccio F, Immediato D, Mosca A, De Carlo C, Miragliotta G, et al. Species distribution
694 and *in vitro* azole susceptibility of *Aspergillus* section *Nigri* from clinical and environmental
695 settings. J Clin Microbiol 2016;54:JCM.01075-16. doi:10.1128/JCM.01075-16.
696 [74] Hashimoto A, Hagiwara D, Watanabe A, Yahiro M. Drug Sensitivity and Resistance
697 Mechanism in Aspergillus Section Nigri Strains from Japan. Antimicrob Agents Chemother
698 2017;61:1–10.
699 [75] Arendrup MC, Jensen RH, Grif K, Skov M, Pressler T, Johansen HK, et al. In Vivo Emergence
700 of Aspergillus terreus with Reduced Azole Susceptibility and a Cyp51a M217I Alteration. J
701 Infect Dis 2012;206:981–5. doi:10.1093/infdis/jis442.
702 [76] Zoran T, Sartori B, Sappl L, Aigner M, Sánchez-Reus F, Rezusta A, et al. Azole-Resistance in
703 Aspergillus terreus and Related Species: An Emerging Problem or a Rare Phenomenon? Front

Microbiol 2018;9:516. doi:10.3389/fmicb.2018.00516.

[77] Osherov N, Kontoyiannis DP, Romans A, May GS. Resistance to itraconazole in Aspergillus nidulans and Aspergillus fumigatus is conferred by extra copies of the A. nidulans P-450 14alpha-demethylase gene, pdmA. J Antimicrob Chemother 2001;48:75–81. doi:10.1093/jac/48.1.75.

[78] Buied A, Moore CB, Denning DW, Bowyer P. High-level expression of cyp51B in azole-resistant clinical aspergillus fumigatus isolates. J Antimicrob Chemother 2013;68:512–4. doi:10.1093/jac/dks451.

[79] Pérez-Cantero A, López-Fernández L, Guarro J, Capilla J. New insights into the Cyp51 contribution to azole resistance in Aspergillus section Nigri. Antimicrob Agents Chemother 2019;63. doi:10.1128/AAC.00543-19.

[80] Fattahi A, Zaini F, Kordbacheh P, Rezaie S, Safara M, Fateh R, et al. Evaluation of mRNA Expression Levels of cyp51A and mdr1, Candidate Genes for Voriconazole Resistance in Aspergillus flavus. Jundishapur J Microbiol 2015;8:e26990. doi:10.5812/jjm.26990.

[81] Mellado E, Garcia-Effron G, Alcázar-Fuoli L, Melchers WJG, Verweij PE, Cuenca-Estrella M, et al. A New Aspergillus fumigatus Resistance Mechanism Conferring In Vitro Cross-Resistance to Azole Antifungals Involves a Combination of cyp51A Alterations. Antimicrob Agents Chemother 2007;51:1897–904. doi:10.1128/AAC.01092-06.

[82] Snelders E, Karawajczyk A, Verhoeven RJA, Venselaar H, Schaftenaar G, Verweij PE, et al. The structure–function relationship of the Aspergillus fumigatus cyp51A L98H conversion by site-directed mutagenesis: The mechanism of L98H azole resistance. Fungal Genet Biol 2011;48:1062–70. doi:10.1016/j.fgb.2011.08.002.

[83] Snelders E, Camps SMT, Karawajczyk A, Rijs AJMM, Zoll J, Verweij PE, et al. Genotype–phenotype complexity of the TR46/Y121F/T289A cyp51A azole resistance mechanism in Aspergillus fumigatus. Fungal Genet Biol 2015;82:129–35. doi:10.1016/j.fgb.2015.06.001.

[84] Hodiamont CJ, Dolman KM, Ten berge IJM, Melchers WJG, Verweij PE, Pajkrt D. Multiple-azole-resistant *Aspergillus fumigatus* osteomyelitis in a patient with chronic granulomatous disease successfully treated with long-term oral posaconazole and surgery. Med Mycol 2009;47:217–20. doi:10.1080/13693780802545600.

[85] Rodriguez-Tudela JL, Alcazar-Fuoli L, Mellado E, Alastruey-Izquierdo A, Monzon A, Cuenca-Estrella M. Epidemiological Cutoffs and Cross-Resistance to Azole Drugs in Aspergillus fumigatus. Antimicrob Agents Chemother 2008;52:2468–72. doi:10.1128/AAC.00156-08.

[86] van der Linden JWM, Camps SMT, Kampinga GA, Arends JPA, Debets-Ossenkopp YJ, Haas PJA, et al. Aspergillosis due to Voriconazole Highly Resistant Aspergillus fumigatus and Recovery of Genetically Related Resistant Isolates From Domiciles. Clin Infect Dis 2013;57:513–20. doi:10.1093/cid/cit320.

[87] Garcia-Rubio R, Escribano P, Gomez A, Guinea J, Mellado E. Comparison of Two Highly Discriminatory Typing Methods to Analyze Aspergillus fumigatus Azole Resistance. Front Microbiol 2018;9:1626. doi:10.3389/fmicb.2018.01626.

[88] Dhingra S, Cramer RA. Regulation of Sterol Biosynthesis in the Human Fungal Pathogen Aspergillus fumigatus: Opportunities for Therapeutic Development. Front Microbiol 2017;8:92. doi:10.3389/fmicb.2017.00092.

[89] Blosser SJ, Cramer RA. SREBP-dependent triazole susceptibility in Aspergillus fumigatus is mediated through direct transcriptional regulation of erg11A (cyp51A). Antimicrob Agents Chemother 2012;56:248–57. doi:10.1128/AAC.05027-11.

[90] Hagiwara D, Watanabe A, Kamei K. Sensitisation of an Azole-Resistant Aspergillus fumigatus Strain containing the Cyp51A-Related Mutation by Deleting the SrbA Gene. Sci Rep 2016;6:38833. doi:10.1038/srep38833.

[91] Camps SMT, Dutilh BE, Arendrup MC, Rijs AJMM, Snelders E, Huynen MA, et al. Discovery of a hapE Mutation That Causes Azole Resistance in Aspergillus fumigatus through Whole Genome Sequencing and Sexual Crossing. PLoS One 2012;7:e50034. doi:10.1371/journal.pone.0050034.

[92] Paul S, Stamnes M, Thomas GH, Liu H, Hagiwara D, Gomi K, et al. AtrR Is an Essential Determinant of Azole Resistance in *Aspergillus fumigatus*. MBio 2019;10. doi:10.1128/mBio.02563-18.

[93] Hagiwara D, Miura D, Shimizu K, Paul S, Ohba A, Gonoi T, et al. A Novel Zn2-Cys6 Transcription Factor AtrR Plays a Key Role in an Azole Resistance Mechanism of Aspergillus fumigatus by Co-regulating cyp51A and cdr1B Expressions. PLOS Pathog 2017;13:e1006096.

762 doi:10.1371/journal.ppat.1006096.
763 [94] Misslinger M, Gsaller F, Hortschansky P, Mü C, Bracher F, Bromley MJ, et al. The cytochrome
764 b 5 CybE is regulated by iron availability and is crucial for azole resistance in A. fumigatus.
765 Metallomics 2017;9:1655. doi:10.1039/c7mt00110j.
766 [95] Rajendran R, Mowat E, McCulloch E, Lappin DF, Jones B, Lang S, et al. Azole resistance of
767 Aspergillus fumigatus biofilms is partly associated with efflux pump activity. Antimicrob Agents
768 Chemother 2011;55:2092–7. doi:10.1128/AAC.01189-10.
769 [96] Coleman JJ, Mylonakis E. Efflux in fungi: la pièce de résistance. PLoS Pathog
770 2009;5:e1000486. doi:10.1371/journal.ppat.1000486.
771 [97] Perlin DS, Shor E, Zhao Y. Update on Antifungal Drug Resistance. Curr Clin Microbiol Reports
772 2015;2:84–95. doi:10.1007/s40588-015-0015-1.
773 [98] Law CJ, Maloney PC, Wang D-N. Ins and outs of major facilitator superfamily antiporters. Annu
774 Rev Microbiol 2008;62:289–305. doi:10.1146/annurev.micro.61.080706.093329.
775 [99] Meneau I, Coste AT, Sanglard D. Identification of *Aspergillus fumigatus* multidrug transporter
776 genes and their potential involvement in antifungal resistance. Med Mycol 2016;54:616–27.
777 doi:10.1093/mmy/myw005.
778 [100] Sipos G, Kuchler K. Fungal ATP-binding cassette (ABC) transporters in drug resistance &
779 detoxification. Curr Drug Targets 2006;7:471–81.
780 [101] Paul S, Diekema D, Moye-Rowley WS. Contributions of Aspergillus fumigatus ATP-binding
781 cassette transporter proteins to drug resistance and virulence. Eukaryot Cell 2013;12:1619–28.
782 doi:10.1128/EC.00171-13.
783 [102] Paul S, Diekema D, Moye-Rowley WS. Contributions of both ATP-Binding Cassette
784 Transporter and Cyp51A Proteins Are Essential for Azole Resistance in Aspergillus fumigatus.
785 Antimicrob Agents Chemother 2017;61:e02748-16. doi:10.1128/AAC.02748-16.
786 [103] Ferreira ME da S, Malavazi I, Savoldi M, Brakhage AA, Goldman MHS, Kim HS, et al.
787 Transcriptome analysis of Aspergillus fumigatus exposed to voriconazole. Curr Genet
788 2006;50:32–44. doi:10.1007/s00294-006-0073-2.
789 [104] da Silva Ferreira ME, Capellaro JL, dos Reis Marques E, Malavazi I, Perlin D, Park S, et al. In
790 Vitro Evolution of Itraconazole Resistance in Aspergillus fumigatus Involves Multiple

Mechanisms of Resistance. Antimicrob Agents Chemother 2004;48:4405–13. doi:10.1128/AAC.48.11.4405-4413.2004.

[105] Natesan SK, Lamichchane AK, Swaminathan S, Wu W. Differential expression of ATP-binding cassette and/or major facilitator superfamily class efflux pumps contributes to voriconazole resistance in Aspergillus flavus. Diagn Microbiol Infect Dis 2013;76:458–63. doi:10.1016/J.DIAGMICROBIO.2013.04.022.

[106] Slaven JW, Anderson MJ, Sanglard D, Dixon GK, Bille J, Roberts IS, et al. Increased expression of a novel Aspergillus fumigatus ABC transporter gene, atrF, in the presence of itraconazole in an itraconazole resistant clinical isolate. Fungal Genet Biol 2002;36:199–206. doi:10.1016/S1087-1845(02)00016-6.

[107] Alcazar-Fuoli L, Mellado E. Ergosterol biosynthesis in Aspergillus fumigatus: its relevance as an antifungal target and role in antifungal drug resistance. Front Microbiol 2012;3:439. doi:10.3389/fmicb.2012.00439.

[108] Rybak JM, Ge W, Wiederhold NP, Parker JE, Kelly SL, Rogers PD, et al. Mutations in *hmg1* , Challenging the Paradigm of Clinical Triazole Resistance in *Aspergillus fumigatus*. MBio 2019;10. doi:10.1128/mBio.00437-19.

[109] Hagiwara D, Arai T, Takahashi H, Kusuya Y, Watanabe A, Kamei K. Non- *cyp51A* Azole-Resistant *Aspergillus fumigatus* Isolates with Mutation in HMG-CoA Reductase. Emerg Infect Dis 2018;24:1889–97. doi:10.3201/eid2410.180730.

[110] Shekhova E, Kniemeyer O, Brakhage AA. Induction of Mitochondrial Reactive Oxygen Species Production by Itraconazole, Terbinafine, and Amphotericin B as a Mode of Action against Aspergillus fumigatus. Antimicrob Agents Chemother 2017;61. doi:10.1128/AAC.00978-17.

[111] Bromley M, Johns A, Davies E, Fraczek M, Mabey Gilsenan J, Kurbatova N, et al. Mitochondrial Complex I Is a Global Regulator of Secondary Metabolism, Virulence and Azole Sensitivity in Fungi. PLoS One 2016;11:e0158724. doi:10.1371/journal.pone.0158724.

[112] Qiao J, Liu W, Li R. Truncated Afyap1 Attenuates Antifungal Susceptibility of Aspergillus fumigatus to Voriconazole and Confers Adaptation of the Fungus to Oxidative Stress. Mycopathologia 2010;170:155–60. doi:10.1007/s11046-010-9309-2.

[113] Lessing F, Kniemeyer O, Wozniok I, Loeffler J, Kurzai O, Haertl A, et al. The *Aspergillus*

fumigatus Transcriptional Regulator AfYap1 Represents the Major Regulator for Defense against Reactive Oxygen Intermediates but Is Dispensable for Pathogenicity in an Intranasal Mouse Infection Model. Eukaryot Cell 2007;6:2290–302. doi:10.1128/EC.00267-07.

[114] Ukai Y, Kuroiwa M, Kurihara N, Naruse H, Homma T, Maki H, et al. Contributions of yap1 Mutation and Subsequent atrF Upregulation to Voriconazole Resistance in Aspergillus flavus. Antimicrob Agents Chemother 2018;62:AAC.01216-18. doi:10.1128/AAC.01216-18.

[115] Cowen LE. Hsp90 orchestrates stress response signaling governing fungal drug resistance. PLoS Pathog 2009;5:e1000471. doi:10.1371/journal.ppat.1000471.

[116] Lamoth F, Juvvadi PR, Soderblom EJ, Moseley MA, Asfaw YG, Steinbach WJ. Identification of a key lysine residue in heat shock protein 90 required for azole and echinocandin resistance in Aspergillus fumigatus. Antimicrob Agents Chemother 2014;58:1889–96. doi:10.1128/AAC.02286-13.

[117] Song J, Zhai P, Lu L. Damage resistance protein (Dap) contributes to azole resistance in a sterol-regulatory-element-binding protein SrbA-dependent way. Appl Microbiol Biotechnol 2017;101:3729–41. doi:10.1007/s00253-016-8072-9.

[118] Song J, Zhai P, Zhang Y, Zhang C, Sang H, Han G, et al. The Aspergillus fumigatus Damage Resistance Protein Family Coordinately Regulates Ergosterol Biosynthesis and Azole Susceptibility. MBio 2016;7:e01919-15. doi:10.1128/MBIO.01919-15.

[119] Juvvadi PR, Lee SC, Heitman J, Steinbach WJ. Calcineurin in fungal virulence and drug resistance: Prospects for harnessing targeted inhibition of calcineurin for an antifungal therapeutic approach. Virulence 2017;8:186. doi:10.1080/21505594.2016.1201250.

[120] Liu F, Pu L, Zheng Q, Zhang Y, Gao R, Xu X, et al. Calcium signaling mediates antifungal activity of triazole drugs in the Aspergilli. Fungal Genet Biol 2015;81:182–90. doi:10.1016/j.fgb.2014.12.005.

[121] Dirr F, Echtenacher B, Heesemann J, Hoffmann P, Ebel F, Wagener J. AfMkk2 is required for cell wall integrity signaling, adhesion, and full virulence of the human pathogen Aspergillus fumigatus. Int J Med Microbiol 2010;300:496–502. doi:10.1016/J.IJMM.2010.03.001.

[122] Chung D, Thammahong A, Shepardson KM, Blosser SJ, Cramer RA. Endoplasmic reticulum localized PerA is required for cell wall integrity, azole drug resistance, and virulence in

Aspergillus fumigatus. Mol Microbiol 2014;92:1279–98. doi:10.1111/mmi.12626.

[123] Delattin N, Cammue BP, Thevissen K. Reactive oxygen species-inducing antifungal agents and their activity against fungal biofilms. Future Med Chem 2014;6:77–90. doi:10.4155/fmc.13.189.

[124] Fanning S, Mitchell AP. Fungal Biofilms. PLoS Pathog 2012;8:e1002585. doi:10.1371/journal.ppat.1002585.

[125] Ramage G, Rajendran R, Sherry L, Williams C. Fungal Biofilm Resistance. Int J Microbiol 2012;2012. doi:10.1155/2012/528521.

[126] Chowdhary A, Sharma C, Hagen F, Meis JF. Exploring azole antifungal drug resistance in *Aspergillus fumigatus* with special reference to resistance mechanisms. Future Microbiol 2014;9:697–711. doi:10.2217/fmb.14.27.

[127] Xiong Q, Hassan SA, Wilson WK, Han XY, May GS, Tarrand JJ, et al. Cholesterol import by Aspergillus fumigatus and its influence on antifungal potency of sterol biosynthesis inhibitors. Antimicrob Agents Chemother 2005;49:518–24. doi:10.1128/AAC.49.2.518-524.2005.

[128] Sanglard D. Finding the needle in a haystack: Mapping antifungal drug resistance in fungal pathogen by genomic approaches. PLOS Pathog 2019;15:e1007478. doi:10.1371/journal.ppat.1007478.

FIGURE LEGENDS

Figure 1. Summary on azole resistance mechanisms described in *Aspergillus.*

Figure 2. Cyp51 phylogenetic tree of *Aspergillus*. Sequences were retrieved from the JGI and AspGD databases and the phylogenetic tree was constructed by MEGA7 software. Genome localization of the *cyp51* genes is indicated as SC (Supercontig), S (Scaffold) and C (Chromosome).

Figure 3. Cyp51 amino acids involved in azole resistance in the Cyp51A protein in *A. fumigatus* (a), *A. flavus* (b), *A. niger* (c), *A. tubingensis* (d) and *A. terreus* (e); Cyp51B (f) and Cyp51C (g) from *A. flavus.* Conserved domains are highlighted: transmembrane domains associated to the endoplasmic reticulum (black) and cytoplasmic regions (grey)

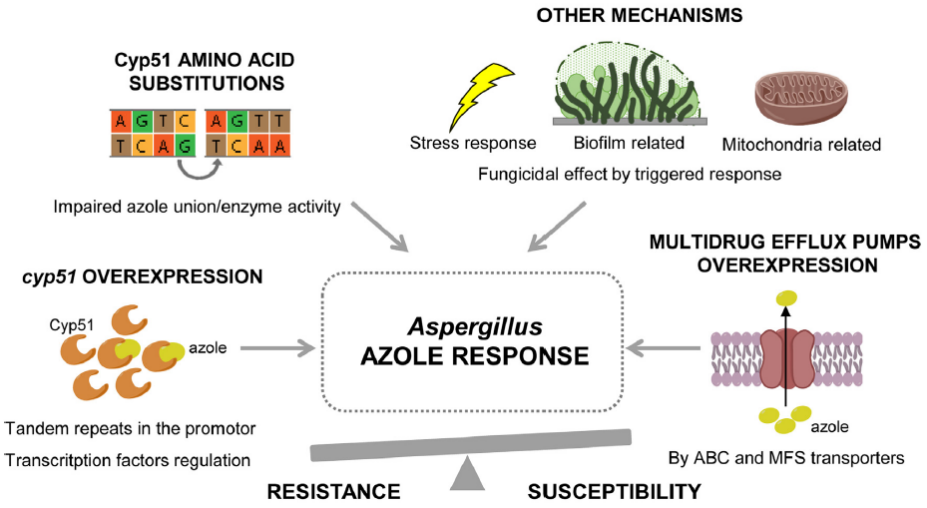

Figure 1.

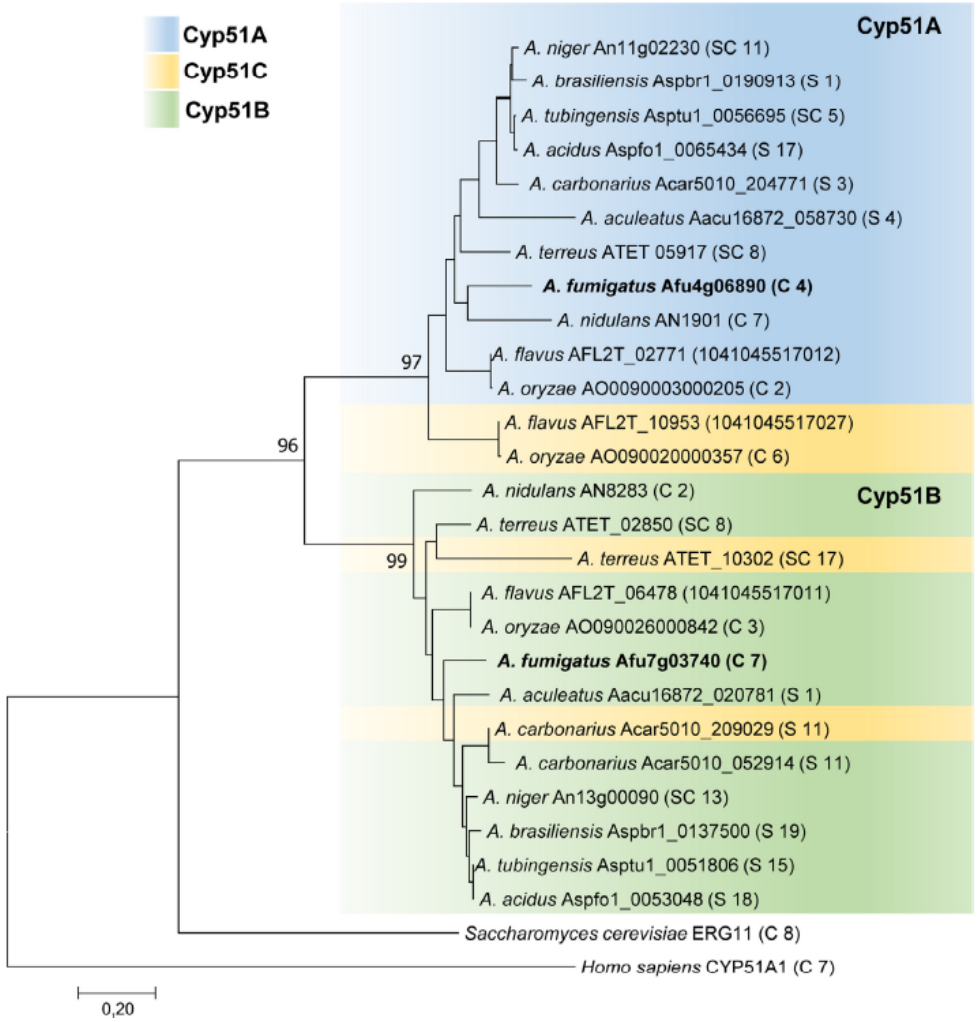

Figure 2.

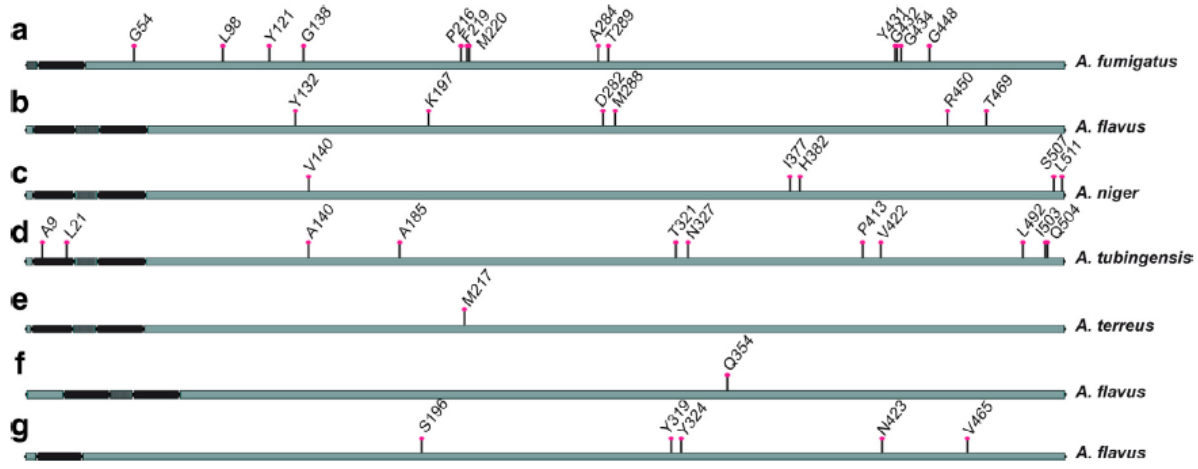

Figure 3.

907 **Table 1.** Reported *A. fumigatus* genes involved in azole resistance.

Gene	Accession nº	Mutant phenotypes	Expression studies
Ergosterol synthesis			
cyp51A	Afu4g06890	Increased azole susceptibility without *cyp51B* expression alteration [43]	Induced by azoles [18] Overexpression reduces azole susceptibility [77] Silencing by siRNA increases azole susceptibility [41]
cyp51B	Afu7g03740	No effect on azole susceptibility [42]	Not induced by azoles [18]
ABC transporters			
cdr1B (abcB, abcC, atrG)	Afu1g14330	Large increase in azole susceptibility [68,101,102]	Upregulated by VRC [103]
mdr1 (abcA')	Afu5g06070	No effect on *in vitro* azole susceptibility [68] *mdr1* overproduction results increases i*n vivo* azole tolerance [101]	Highly induced by VRC and ITC and occasionally overexpressed in non-wt strains [101,103,104]
mdr2	Afu4g10000	No effect on azole susceptibility [68]	Overexpressed in non-wt strains [104]
mdr3	Afu3g03500	No effect on azole susceptibility [68]	Overexpressed in non-wt strains [52,104]
mdr4	Afu1g12690	No effect on azole susceptibility [68]	Occasionally upregulated by ITC [52,104]
abcD	Afu6g03470	-	Upregulated by VRC [103]
abcE	Afu7g00480	-	Upregulated by VRC [103]
atrI	Afu3g07300	-	Higher expression in non-wt strains [99]
atrF	Afu6g04360	No effect on azole susceptibility [68]	Overexpressed and upregulated by ITC in non-wt strains [99,106]
MFS transporters			
mdrA	Afu1g13800	Increased susceptibility towards ITC and VRC [99]	-
mfs56	Afu1g05010	No effect in azole susceptibility [68]	Overexpressed in some non-wt strains but no general correlation to MICs [68]
mfsA	Afu8g05710	-	Upregulated by VRC [103]
mfsB	Afu1g15490	-	Upregulated by VRC [103]
mfsC	Afu1g03200	-	Upregulated by VRC [103]

908

909

910 **Table 2.** Reported amino acid changes in Cyp51 with effects in azole susceptibility.

Species	Substitution	Reduced susceptibility
A. fumigatus	G54R, -W, -E, -K [a]	ITC and/or POS [51,52]
	Y121F [a]	VRC [58]
	G138C, -S [a]	ITC, PSC, VRC [59,65]
	P216L [a]	ITC, PSC [55,61,63]
	F219I, -C, -S [a]	ITC, PSC [54,55,59]
	M220V, -K, -T, -I [a]	ITC and/or POS and/or VRC [59,60,64]
	A284T [a]	ITC, PSC, VRC [63]
	Y431C, -S [a]	PSC, VRC and/or ITC [56,61,65]
	G432S [a]	ITC [57]
	G434C [a]	ITC, PSC, VRC [61,65]
	G448S [a]	ITC, VRC [53,62]
	TR34/L98H [a]	ITC, PSC, VRC [81,85]
	TR46/Y121F/T289A [a]	VRC [86]
	TR53 [a]	ITC, VRC [84]
A. flavus	Y132N/T469S [a]	VRC [66]
	K197N [a]	VRC [66]
	K197N/D282E/M288L [a]	VRC [66]
	R450S [a]	VRC [71]
	Q354K [b]	VRC [71]
	S196F/A324P/N423D/V465M [c]	VRC [71]
	Y319H [c]	VRC [70]
A. niger	V104I [a]	ITC [74]
	H382R [a]	ITC [74]
	I377V/S507I/L511M [a]	ITC, VRC [74]
A. tubingensis	L21F [a]	ITC and / or PSC [73,74]
	A9V/L21F/A140V/P413S/D505E [a]	ITC [74]
	T321A [a]	VRC [74]
	A185G/T321A/N327S/V422I/L492M/I503F/Q504P [a]	ITC, VRC [74]
A. terreus	M217T, -V, -I [a]	PSC and/or ITC [75,76]

911

912 **Notes:** [a] substitution present in Cyp51A; [b] substitution present in Cyp51B; [c] substitution present in Cyp51C

913 **Abbreviations:** ITC, itraconazole; PSC, posaconazole; VRC, voriconazol

914

915

916