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The Quiet and Underappreciated Rise of Drug-Resistant Invasive Fungal Pathogens

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Abstract: Human fungal pathogens are attributable to a significant economic burden and mortality worldwide. Antifungal treatments, although limited in number, play a pivotal role in decreasing mortality and morbidities posed by invasive fungal infections (IFIs). However, the recent emergence of multidrug-resistant Candida auris and Candida glabrata and acquiring invasive infections due to azole-resistant C. parapsilosis, C. tropicalis, and Aspergillus spp. in azole-naïve patients pose a serious health threat considering the limited number of systemic antifungals available to treat IFIs. Although advancing for major fungal pathogens, the understanding of fungal attributes contributing to antifungal resistance is just emerging for several clinically important MDR fungal pathogens. Further complicating the matter are the distinct differences in antifungal resistance mechanisms among various fungal species in which one or more mechanisms may contribute to the resistance phenotype. In this review, we attempt to summarize the burden of antifungal resistance for selected non-albicans Candida and clinically important Aspergillus species together with their phylogenetic placement on the tree of life. Moreover, we highlight the different molecular mechanisms between antifungal tolerance and resistance, and comprehensively discuss the molecular mechanisms of antifungal resistance in a species level.

Keywords: Candida glabrata; Candida parapsilosis; Candida tropicalis; Candida auris; Aspergillus terreus; Aspergillus fumigatus; antifungal resistance mechanisms

1. Introduction

Numerous fungal species, from yeasts and yeast-like fungi to molds, constitute human mycobiome and inhabit the gastrointestinal tract of healthy individuals [1]. However, the gut-resident fungi can translocate from the gut to the bloodstream and cause lethal invasive fungal infection (IFI) when the immune system is impaired [2]. Fungi profoundly affect human health. Based on global estimations,
they cause 1.7 billion benign superficial infections, and IFI that is responsible for 1.5 million patient deaths annually [3]. The main causative species belong to the *Candida*, *Cryptococcus*, and *Aspergillus* genera. The incidence of fatal invasive fungal diseases is rising because of an increasing population at risk in developed countries, e.g., individuals with immunological deficiency, hematological malignancy, solid organ transplant recipients, and those with chronic obstructive pulmonary disease or exposed to continued corticosteroid therapy [4].

Although limited in number and chemical classes, antifungal treatments and/or prophylaxis are central to reducing comorbidities and mortalities caused by fungal infections. Yet it is considered as a driving force that replaces sensitive fungal species with other species exhibiting intrinsic and/or acquired resistance [5]. These emerging species are associated with longer hospitalizations, increased therapeutic failure, and increased costs, when compared to *C. albicans*, the most predominant fungal species causing bloodstream infection in humans [6,7]. Currently, clinical guidelines endorse treating IFIs caused by *Candida* and *Aspergillus* species by echinocandins and mold-active triazoles, respectively [8,9]. Underlying host conditions, antifungal pharmacokinetics and pharmacodynamics, and fungal attributes may alone or collectively contribute to therapeutic failure. Fungal factors resulting in antifungal resistance involve various subcellular mechanisms, including alteration of the drug target, overexpression of efflux pumps and drug target, and gross chromosomal changes [10].

Antifungals have different modes of action and belonged to three major classes, namely azoles (fluconazole, voriconazole, itraconazole, isavuconazole, and posaconazole, etc.), polyenes [amphotericin B (AMB)], and echinocandins (caspofungin, micafungin, and anidulafungin). Azoles disrupt fungal ergosterol production by binding to one of the critical enzymes (Erg1p) in the ergosterol biosynthesis pathway, which results in the accumulation of toxic sterols; polyenes bind to ergosterol and cause fungal cell death by forming pores on the cell membrane and disturbance of osmotic pressure; and echinocandins inhibit the biosynthesis of a key cell wall polymer, β-1,3-n-glucan, by blocking the catalytic subunit of glucan synthase enzyme, encoded by the *FKS* gene [10]. The modes of action and fungal cell fate depend on the cellular target, fungal species, and antifungal used. For instance, azoles are fungistatic against *Candida*, meaning that they do not kill the *Candida* cells but prevent cell division, while echinocandins exert fungicidal activity against *Candida* causing cell death. It is important to highlight the difference between tolerance and resistance. The former encompasses rapid cellular changes that lead to transient (phenotypic) tolerance to the antifungal drugs, which is visible after 48 h, while the latter involves heritable genomic changes, ranging from point mutations to gross chromosomal changes resulting in permanent antifungal resistance, which is visible after 24 h (reviewed in [11]).

In the current review, we provide an overview of the epidemiology and molecular mechanisms of tolerance and resistance to antifungals of three most prevalent non-albicans *Candida* (NAC) species, namely *Candida glabrata*, *C. parapsilosis*, and *C. tropicalis*, the multidrug-resistant (MDR) *C. auris*; and most prevalent molds, namely *Aspergillus fumigatus* and *Aspergillus terreus*. Although, *Candida krusei* (*Pichia kudriavzevii*) shows intrinsic resistance to fluconazole, this species is not included in the scope of this paper, which is extensively described in a recent study published in 2020 [12]. Rather we focus on other NAC species ranked as the first to fourth most common cause of candidemia (except for *C. auris*). Additionally, biofilms exert intrinsic resistance against antifungals, but this topic has been extensively reviewed elsewhere [13–17] and will not be significantly addressed in the current paper.

2. **Taxonomic Placement of Target Non-albicans Candida and Aspergillus Species**

2.1. *Candida*

Despite their shared name, *Candida* species do not constitute a genus in the phylogenetic sense. Indeed, when molecular data are used to place them in the Saccharomycotina phylogenetic tree, they are spread at different positions, intermingled with other non-*Candida* species [18]. The list of *Candida* species that causes candidiasis is long, with over 30 different species, although most of them are only
rarely isolated from patients. Among the NAC pathogens covered in the current review, _C. parapsilosis_ and _C. tropicalis_ are relatively close to _C. albicans_, and belong to the _Lodderomyces_ clade. Nevertheless, within this clade they belong to clearly different lineages that are separated by non-pathogenic species. Of note, _C. parapsilosis_ belongs to a species complex that comprises other, less-prevalent pathogenic species that are hybrids such as _C. orthopsilosis_ and _C. metapsilosis_, and for which hybridization has been proposed as a virulence emergence mechanism [19]. _Candida glabrata_ is distantly related to _C. albicans_, being more closely related to the model yeast _Saccharomyces cerevisiae_, and belonging to a clade of yeasts that underwent whole-genome duplication (WGD) via hybridization approximately 100 million years ago [18]. In this post-WGD clade, only _C. glabrata_ and some of its closest relatives (in the _Nakaseomyces_ clade) can be considered regular opportunistic pathogens. Finally, _C. auris_ is more distant from _C. albicans_ than _C. glabrata_ and belongs to the so-called _Metschnikowia_ clade, which diverged earlier within the Saccharomycotina tree (Figure 1) [20]. The taxonomic classification and naming of Saccharomycotina yeasts is currently being revisited and will ultimately entered into the classification, delimitation and phylogeny of this group of closely related species. Nevertheless, the ecological niche occupied by _C. auris_ is distantly related to _C. orthopsilosis_ and _C. metapsilosis_. The fact that opportunistic _Candida_ pathogens belong to such diverged clades indicates that their ability to infect human has emerged independently multiple times during evolution, which is further highlighted by the variable molecular mechanisms of virulence and differential antifungal susceptibility patterns [18,21,22].

**Figure 1.** Phylogenetic tree of _Candida_ species studied in the current review, i.e., _C. glabrata_, _C. parapsilosis_, _C. tropicalis_, and _C. auris_ (highlighted in blue). This tree was constructed using maximum likelihood of 11,570 core genes based on 1000 replicates. Asterisks does not serve any specific definitions. This figure was adopted permission from Munoz et al., 2018 [20].

### 2.2. Aspergillus

By contrast, the _Aspergillus_ genus comprises more than 340 species [23], which are fungal saprophytes that are found in diverse ecological niches around the world. More predominantly, species within the _Fumigati_ and _Terrei_ sections are associated with clinical complications in humans, such as chronic and allergic pulmonary aspergillosis, saprophytic colonization, asthma with fungal sensitization and invasive aspergillosis (IA) [3].
The Aspergillus section Funigati contains up to 63 species, although it is doubtful if some of them should be considered as species and possibly are synonymous with other species [24]. Species delimitation within this complex relies on several features that define five clades proposed based on them [25]: (I) A. fumigatus, (II) A. lentulus and A. funisynnematus, (III) A. funigatiifinis and A. novofunigatus, (IV) A. viridinutans, A. udagawaee, and other atypical strains; and (V) A. hiratsukae, A. brevipes, A. duricaulis, and A. unilateralis.

The Aspergillus terreus species complex is found in a wide variety of habitats, such as the soil, compost, and dust, but a specific niche is not known. The spectrum of diseases caused by these fungi covers allergic, chronic, invasive and disseminated forms of aspergillosis [26]. The section Terrei comprises 16 accepted species, namely A. terreus sensu stricto (s.s.), A. alabamensis, A. allahabadii, A. ambiguus, A. aureoterreus, A. bicephalus, A. carneus, A. citrinoterreus, A. floccosus, A. iranicus, A. hortai, A. microcysticus, A. neoaficanus, A. neoindicus, A. niveus, and A. pseudoterreus [27]. The production of aleurioconidia by A. terreus s.s., A. carneus, A. flavipes, and A. niveus seems to be a unique feature among the Aspergillus species. These morphologically distinct lateral conidia (aleurioconidia) are attached directly to hyphae and their function is as yet unknown [28].

3. Antifungal Tolerance Molecular Mechanisms and Its Implications as a Potential Therapeutic Option

Antifungal tolerance involves acute cellular responses to stressors, such as antifungals that threaten the integrity of the fungal cells. The fungal cells are constantly challenged by extrinsic stressors; hence, the cell wall and cell membrane are the two most important physical barriers responsible for cellular homeostasis. The viability and fate of fungal cell are largely determined by the abilities to sense the stress, integrate intracellular responses, and subsequently orchestrate a proper response. Stressors may lead to lethal consequences by destabilizing and degrading cellular proteins. However, molecular chaperones, such as HSP90, counteract the stressor effects by stabilizing critical and essential downstream client proteins, such as Mkp and calcineurin, leading to stressor withstanding and tolerance [29,30]. Notably, master components involved in antifungal tolerance also play a role in virulence and biofilm formation [30]. These compensatory mechanisms orchestrate a rapid and appropriate response to stress, allowing the cell to “buy time” to acquire mutations in critical genes and/or undertake gross chromosomal changes, consequently leading to permanent resistance [31,32]. The acquisition of such mutations in genes associated with resistance may occur in the presence of specific mutations/absence of DNA repair mechanisms, such as mismatch repair (MSH2), resulting in increased antifungal tolerance and virulence [32,33]. Of note, the link between MSH2, in vitro tolerance, and clinical tolerance is uncertain [34,35].

Echinocandins and azoles disrupt the cell wall and cell membrane integrity, respectively. Upon fungal cell exposure to echinocandins, cellular sensors detect the presence of drug molecules. This is followed by the engagement of main signal transduction pathways involved in stress adaptation and cell wall integrity, including protein kinase C (PKC), high-osmolarity glycerol (HOG), and calcineurin pathways, activation of appropriate transcription factors, and, finally, expression of response element genes, such as FKS, and CHS2, and CHS8 (Figure 2) [36,37]. Activation of these pathways results in increased chitin levels in the cell wall, as a substitute for the reduced quantity or loss of β-1,3-d-glucan [37]. Interestingly, A. fumigatus displays paradoxical growth effect (PGE) when exposed to caspofungin, i.e., inhibition of growth at minimum inhibitory concentration (MIC) (0.5 μg/mL), but not above MIC (4 μg/mL). The inhibition of growth at MIC involves a relocalization of β-1,3-d-glucan synthase complex (Fks1p and Rho) from cell wall to vacuole, while continuous exposure to high MIC after 48 h results in returning of this complex to cell wall, which results in normal growth [38]. New lines of studies have found that a transcription factor, FhdA, plays an important role in PGE, which is involved in iron metabolism and mitochondrial respiratory function [39]. Importantly, addition of farnesol can block PGE in A. fumigatus when exposed to caspofungin [38]. Further, generally, azole tolerance results from an independent contribution of PKC and calcineurin pathways, leading to
the activation of efflux pumps (Figure 1) [29,31]. Antifungal tolerance exerted by biofilm, however, does not prominently engage calcineurin and PKC pathways, in which the β-1,3-β-glucan matrix is significantly increased [40].

![Mechanisms of antifungal tolerance](image)

**Figure 2.** Mechanisms of antifungal tolerance. The mechanisms include rapid coordination of numerous signal transduction pathways that depend on the antifungal drug used. Echinocandin tolerance mechanism, known as the cell wall integrity pathway, involves protein kinase C (PKC), high-osmolarity glycerol (HOG), and calcineurin pathways, followed by the overexpression of chitin synthase, and FKS1 and FKS2 to compensate for the reduction of β-1,3-β-glucan level in the cell wall. Membrane integrity pathway orchestrates the azole tolerance pathways, which includes PKC and calcineurin pathways. As it is shown, HSP90 plays a critical role in antifungal tolerance by stabilizing the key regulatory proteins.

Tolerant cells exhibit the same level of minimum inhibitory concentration (MIC) as susceptible ones after 24 h incubation, therefore could be misidentified as susceptible isolates. If incubated for 48 h, however, tolerant cells can grow at a drug level higher than MIC, which is due to slow growth of tolerant cells in presence of antifungal drugs relative to drug-resistant isolates [11]. Of note, the level of tolerance is driven by the number of tolerant cells, which varies among isolates and could be measured quantitatively [11]. Some studies have shown the clinical implication of tolerant cells and observed that isolates with a high level of tolerant cells poorly respond to fluconazole when compared to isolates having medium- and low-tolerance cells [11,41]. Genes involved in tolerance, including the ones encoding HSP90 and calcineurin, are highly evolutionarily conserved such that a human ortholog of HSP90 is still functional in yeast [42]. Therefore, HSP90 and calcineurin inhibitors designed for immunosuppression in human are highly active against *C. albicans* and *A. fumigatus*. It is known that caspofungin and fluconazole are fungistatic in *A. fumigatus* and *C. albicans*, respectively [43]. However, in vivo and in vitro studies revealed that genetic impairment of HSP90 and/or calcineurin inhibitors potentiate the efficacy of fluconazole and caspofungin, rendering them fungicidal [43]. In addition, HSP90 inhibitors elicit aberrant biofilm morphology and restrict the dispersal and viability of yeast cells [40]. Even though acquisition of mutation in an antifungal drug target results in antifungal resistance independent of HSP90 and calcineurin, the effect of echinocandin against *C. glabrata* isolates harboring mutations in FKS1 and/or FKS2 can be potentiated when used in combination with HSP90 inhibitors [36]. Similarly, the combination of caspofungin and fluphenazine, a calmodulin inhibitor, potentiated the effect of caspofungin in *C. glabrata* isolates harboring prominent known FKS mutations and increased the survival of the infected *Galleria mellonella* relative to those treated with caspofungin alone [44]. These observations may suggest the fact that both tolerance mechanisms and FKS point
mutations synergistically may contribute to echinocandin resistance in vitro and therapeutic failure in vivo.

Although HSP90 and calcineurin inhibitors may hold promise for future use in combination with antifungal drugs, mutations in HSP90 and CNA1, the catalytic subunit of calcineurin, leading to resistance to the respective inhibitors, have been identified [45,46]. Further, human HSP90 inhibitors are pumped out of the fungal cell by efflux pumps [45] and their profound immunosuppressive effect is lethal in a murine model of invasive candidiasis [43] and places the host at high risk of secondary infection. This highlights the importance of developing of HSP90 inhibitors specific to fungi.

4. Antifungal Resistance Overview

Antifungal resistance is either acquired or innate (inherent). The former involves permanent resistance evolved during the course of antifungal therapy, while the latter is defined when a species intrinsically exhibits show elevated MIC values toward an antifungal. An example of innate resistance fungi is most C. auris isolates, which has been recently recognized as a globally emerging multidrug-resistant species [47]. An important consideration regarding the issue of susceptibility to antifungal drugs of different pathogens is that resistance can have different origins. On the one hand, different species have different intrinsic susceptibility to different antifungal drugs [21,22], which defines a shared trait of a species, but is more or less variable among strains. Here again, different species might have different potentials to adapt to different drugs, and hence, the acquired resistance is not entirely independent of the phylogenetic background. Below, we focus on the acquired antifungal resistance mechanisms, from single-nucleotide polymorphisms to gross chromosomal changes.

4.1. Point Mutations Leading to Antifungal Resistance

Molecular mechanisms of antifungal resistance vary depending on the class of antifungals. Azole resistance is primarily orchestrated by the upregulation of the gene encoding the drug target (ERG11) and those encoding efflux pumps belonging to the major facilitator superfamily (MFS), such as MDR1, and ATP-binding cassette (ABC) transporters, such as CDR1 and CDR2 [48]. MFS efflux pumps are composed of 12–14 transmembrane proteins transferring azoles out of the fungal cell using a proton motive force [48]. ABC transporters are composed of two transmembrane and two cytoplasmic nucleotide-binding domains, and use ATP to pump azoles and/or toxic metabolites out of the cell [49]. Upregulation of ERG11, MDR1, and ABC transporter genes occurs mainly as a result of gain-of-function (GOF) mutations in genes encoding zinc finger transcription factors (Zn2-Cys6), such as UPC2, MRR1, and TAC1 (PDR1 in C. glabrata). Although the transactivators are promising druggable targets, high-resolution structures are not available, except for Upc2p [50]. Modification of the azole drug target, Erg11p, is another prominent mechanism of azole resistance. Acquisition of mutations resulting in amino acid substitutions at specific positions near the heme-binding site, including Y132F, K143R, and G464S, lowers the affinity of Erg11p to azoles, with a subsequent azole resistance [51].

Echinocandins are not efflux pump targets and resistance to this class of antifungals mainly develops through acquisition of mutation in short specific regions of the FKS1 and FKS2 genes, within hotspot (HS) regions [52]. Echinocandin-resistant yeasts from the Lodderomyces and Metschnikowia clades carry accountable mutations in HS1 and HS2 of FKS1, while mutations in HS1 of FKS1 and FKS2 are the most prevalent causes of echinocandin resistance in C. glabrata [52]. Elevated MIC values vary depending on the position and the nature of the amino acid substitution [52,53]. Although sequencing of HS regions is the most convenient way of determining echinocandin resistance mechanisms, accountable mutations outside of the HS regions were recently identified, reinforcing the importance of sequencing the whole FKS gene [54].

The rare occurrence of AMB resistance lead to a limited number of studies dedicated to deciphering AMB resistance mechanisms. The limited studies available implicated a role of ERG3, ERG2, and ERG6 as the possible mechanisms involved in AMB resistance [55–57]. Future studies are warranted to comprehensively examine AMB resistance mechanisms.
4.2. Gross Chromosomal Changes Leading to Antifungal Resistance

Beyond point mutations, gross genomic rearrangements have been reported to confer resistance to different antifungal drugs. Indeed, it has been suggested that such genomic rearrangements may commonly predate the appearance of point mutations [21]. This would explain why resistance rapidly appears in cell populations exposed to low densities of an antifungal. Some gross genomic re-arrangements, such as aneuploidy, occur at higher rates than a specific point mutation, particularly under stress conditions, and they seem to be well tolerated by the yeast cell [58,59]. Aneuploidy results in a combined down- or upregulation of several genes. This may allow survival in the presence of a drug and selection for the aneuploidy until a more favorable point mutation occurs. In this regard, azole resistance in *C. albicans* has been linked with a specific segmental aneuploidy leading to the duplication of *ERG11* and *TAC1* genes involved in ergosterol synthesis and drug efflux, respectively [59]. Genomic rearrangements have also been suggested to play a role in the adaptation of *C. glabrata* to stressful conditions, including exposure to antifungals [60]. However, similar chromosomal aneuploidies appear spontaneously in the *C. glabrata* cultivated under non-stressful conditions [61]. Further, recent whole-genome sequencing analysis failed to identify consistent links between aneuploidy involving genes associated with drug resistance and increased-resistance profiles [62,63]. *Candida parapsilosis* and *C. tropicalis* are even less studied in this regard. Hence, although it is established that aneuploidy plays a role in mediating drug resistance in *C. albicans*, the impact of this resistance mechanism in other species remains to be clarified. Finally, other gross genomic changes such as copy-number variation and loss of heterozygosity (LOH) (in heterozygous species such as *C. albicans* and hybrids of the *C. parapsilosis* clade), have also been proposed as possible mechanisms mediating rapid cell adaptation to antifungals [21,64].

4.3. Antifungal Resistance and Fitness Cost

Although resistance is a favorable trait for fungus in the presence of antifungals, considering the associated alteration of major cellular components, resistant isolates are typically less fit than their susceptible counterparts when examined in the absence of antifungal agent [45,65]. Small genomic changes, such as those leading to amino acid substitution, might be associated with a trivial fitness cost, while some gross chromosomal changes render resistant isolates more susceptible to killing by macrophage [45]. The exception is *C. glabrata* where GOF resistance mutations increase fitness by both protecting from drug through induction of the key drug efflux transporter but also by decreasing immune surveillance by macrophages (more details provided in Section 6.1) [66]. Although replacement of resistant isolates by susceptible ones in the absence of antifungal drugs is a rational assumption compatible with the evolutionary concept of natural selection [45], it is not always the case.

5. Fungal-, Host-, and Drug-Related Factors Facilitating the Emergence of Antifungal Resistance

The gastrointestinal (GI) tract is considered to be a major source of invasive candidiasis, as well as a barrier for the penetration of antifungal drugs, especially echinocandins [67]. Furthermore, fungal cells robustly produce biofilms inside the GI tract, further impeding the penetration of target cells at an infection site by antifungal drugs. On the other hand, the low permeability of echinocandins across the intestinal barrier necessitates the use of a high dose of echinocandins to attain a sufficiently high concentration in the GI tract so that the drugs would exert fungicidal activity [68]. At four times the humanized dosage of caspofungin (20 mg/kg), the fungal burden in the GI tract of mice dropped, which reduced dissemination to other organs. However, rebound was associated with the emergence of echinocandin-resistant strains harboring mutation in *FKS1/FKS2*, [69]. Some species, such as *C. glabrata*, which can cause intraabdominal candidiasis, show a pronounced immune evasion and reduced neutrophil influx, resulting in progression from peritonitis to abscesses [67]. Consequently, as discussed above, various factors associated with the host, pathogen, and antifungal drugs together facilitate the emergence of antifungal resistance [70]. Delivering drugs at an appropriate level at the site...
of infection is critical to achieve pharmacodynamic targets attainment to maximize clinical outcome. For intraabdominal infections, the recommended standard of care drugs, the echinocandin micafungin, fails to achieve sufficient levels during therapy, while newer related drugs in clinical development show superior penetration properties [71].

6. Epidemiology and Mechanisms of Antifungal Resistance in NAC and Aspergillus Species

6.1. Candida glabrata

*Candida glabrata* is a prominent cause of bloodstream infection (candidemia) worldwide and the second leading cause in some countries, including USA [72], Canada [73], Australia [74], and Scandinavian countries [75–80]. According to numerous epidemiological studies, the number of candidemia cases caused by *C. glabrata* exhibits a temporal increasing trend [80–84]. The elderly, patients undergoing abdominal surgeries, and those previously exposed to echinocandins and azoles are susceptible to acquiring candidemia caused by *C. glabrata* [85,86]. *Candida glabrata* is known for its significant tolerance of antifungal drugs [32] and it can rapidly develop resistance during the course of antifungal therapy, which ultimately leads to therapeutic failure [85,87–95]. Based on a recent worldwide study evaluating the burden of candidemia and antifungal resistance, the SENTRY Antifungal Surveillance Program, the incidence of fluconazole-resistant (FLZR) *C. glabrata* isolates has increased from 8.6% to 10.1% during 1997–2014, and Latin American and Asian Pacific countries noted the highest rate of fluconazole resistance (10.6% and 6.8%) [79]. Although echinocandin resistance is not common among the other *Candida* species (except for *C. auris*), this phenomenon is apparent in *C. glabrata*, with the worldwide prevalence ranging from 1.7–3.5% depending on the echinocandin drug tested [79]. More importantly, 5.5–7.6% of FLZR *C. glabrata* isolates reported by the SENTRY study are co-resistant to echinocandins and considered MDR [79]. At the institutional level, the prevalence of echinocandin resistance can vary significantly, reaching up to >13% at some centers [96].

Except for a single study associating ERG11<sup>C315D</sup> withazole resistance [97], GOF mutations in *PDR1* appear to be a prominent factor driving azole resistance in *C. glabrata* in vitro and in vivo [98]. As already discussed, the expression of efflux pump genes, including *CDR1*, *CDR2* (*PDH1*), and *SNQ2*, is regulated by Pdr1p [99]. The protein is comprised of four domains, namely, DNA-binding domain (DBD), inhibitory domain (ID) (equivalent of a xenobiotic-binding domain in *Saccharomyces cerevisiae*), middle-homology domain (MHD), and activator domain (AD) (Figure 3) [100]. Once an azole reaches a cell, it binds to the MHD and activates *PDR1*, followed by interaction of *PDR1* and Gal11A on PDRE, RNA polymerase II recruitment, and the overexpression of downstream genes, such as efflux pumps [101]. GOF mutations in the regions encoding ID and AD disrupt the inhibition and induce the activation of *PDR1*, respectively, while those in a region encoding MHD obviate the need for xenobiotic activation (here, azoles) [98]. As discussed earlier, the GOF mutations in *PDR1* play role in virulence and immune evasion [102]. Further, overexpression of the transcription factor gene *CgSTB5* abrogates azole resistance by downregulating the expression of efflux pump genes (but not *SNQ2*) [103]. Interestingly, a small molecule, iKIX1, inhibits the interaction between CgGal11A and CgPdr1, and not only significantly reduces the fungal tissue burden in mice systemically infected with WT-<i>CgPDR1</i> and <i>CgPDR1</i>_L280F isolates when used in combination with fluconazole, but also reduces the adhesion of *C. glabrata* in a mouse model of urinary tract infection when used alone [104]. In addition, according to a recent study, chemical and/or genetic inhibition of histone acetyltransferase, Cgn5p, is lethal in *C. glabrata* isolates harboring GOF mutations in *PDR1*; it showed a significantly reduced frequency of GOF mutations in CGN5-inhibited *C. glabrata* isolates compared with non-inhibited *C. glabrata* isolates in an evolutionary model of FLZR [100].

It was recently shown that the mechanism of azole resistance in *C. glabrata* involves a complicated circuitry of zinc cluster transcription factors other than Pdr1, such as Upc2A (Figure 3) [105,106]. Interestingly, double deletion of Upc2A (*upc2AΔ*<sup>Δ</sup>) in both fluconazole-susceptible and FLZR *C. glabrata* isolates results in a 16-fold decrease of FLZ MIC, and downregulation of *CDR1*, *PDH1*, and *PDR1* upon
induction by FLZ [106]. Indeed, Upc2A directly binds to the CDR1 and PDR1 promoters, leading to the overexpression of these genes and FLZR (Figure 4) [105]. However, PDR1 and CDR1 upregulation in the upc2AΔ isolate [105] may suggest that FLZR in C. glabrata is more complicated than currently thought, and may involve other zinc-cluster transcription factors.

![Figure 3](image-url)  
Figure 3. Pdr1p contains four domains. Numerous GOF mutations (black bars) can cause azole resistance. Adopted the permission from Ferrari et al., 2009 [98].

![Fluconazole resistance diagram](image-url)  
Figure 4. Fluconazole resistance is mediated by both Upc2p and Pdr1p in C. glabrata.

Resistance to echinocandins appears to be more straightforward, and mainly associated with non-synonymous mutations in HS1 of FKS1 and FKS2. S629P in Fks1, and S663P and F659Deletion in Fks2p are the most prominent substitutions involved in both in vitro and in vivo resistance (Table 1). Of note, mutations occurring outside of these HS regions can also lead to echinocandin therapeutic failure [54]. Therefore, isolates displaying echinocandin resistance without known mutations in the HS regions may harbor non-synonymous mutations located anywhere in the FKS genes. Importantly, it has been documented that occasionally C. glabrata blood isolates carrying mutation in HS1-Fks1 (S629T) are fully susceptible to echinocandins, while the patient infected with such isolate showed therapeutic failure [107]. Therefore, combination of both antifungal susceptibility testing (AFST) and FKS sequencing can more precisely predict therapeutic failure when treating candidemia patients with echinocandins. Of note, this finding warrants further confirmation by larger studies and not all routine laboratories have direct access to both methods.
Table 1. Mutations involved in echinocandin resistance and their in vitro and in vivo impact.

| Species          | FKS1-HS1 MIC (µg/mL) | FKS2-HS1 MIC (µg/mL) | Therapeutic Failure | Reference                  |
|------------------|----------------------|----------------------|---------------------|----------------------------|
|                  | Mutation             | CSP (µg/mL) | MCF (µg/mL) | AND (µg/mL) | Mutation             | CSP (µg/mL) | MCF (µg/mL) | AND (µg/mL) | CSP (µg/mL) | MCF (µg/mL) | AND (µg/mL) | Reference                  |
|                  | fks1∆ + E655K        | 8                  | 16              | 8             | Y657del + F659Y         | 0.25–0.5 | 0.25          | 1             | fks1∆ + E655K                  |
|                  | Fe625S               | 2                  | 0.25            | 1             | F659L                  | 1-2      | 0.03–0.06     | 0.06–0.12     | Fe659L                                      |
|                  | F625C                | 0.12               | NA              | NA            | F659uml                | 0.06–0.32 | 0.12–4        | 0.12–4        | F659uml                                      |
|                  | Fe625I + P667T        | 4                  | 2               | 0.25          | F659L                  | 0.5–2    | 0.012–0.25    | 0.25–1         | F659L                                      |
|                  | S626P + S663F         | 1–32               | 4               | 0.5–2         | F659L                  | 0.25–1   | 0.03–0.25     | 0.06–1         | F659L                                      |
|                  | H627V                | 0.06               | 0.25            | 0.03          | F659L                  | 2        | 0.25          | 0.5            | F659L                                      |
|                  | S629P + D666V         | 0.12–4             | 0.5–1           | 0.12–2        | F659L + L664V           | 4        | 0.5           | 1             | S629P + D666V                                 |
|                  | S629P                | 0.06–16            | 0.06–16         | 0.5–8         | F659L + D666N           | 16       | 2             | >4                         | S629P + S629P + S629P                         |
|                  | R631G                | 0.12–0.5           | 0.25–0.5        | 0.06–0.5      | F659L                  | 1        | 0.25          | 1             | S629P + D666V                                 |
|                  | R631G + D666V         | 0.25               | 0.5             | 0.5           | F659L + S663A + D666E   | 4        | 0.25          | 1             | S629P + D666V                                 |
|                  | D632H                | 2                  | 0.5             | 0.5           | S663F                  | 0.25–4   | 0.125–4       | 0.5–4          | S629P + D666V                                 |
|                  | D632Y                | 0.12–2             | 0.06–0.5        | 0.25–0.5      | L664R                  | 1        | 0.06–0.12     | 0.12          | S629P + D666V                                 |
|                  | D632V                | 0.12–2             | 0.25–2          | 1–2           | R665G                  | 0.12–0.5 | 0.25–1        | 0.06–1         | S629P + D666V                                 |
|                  | D666H                | 0.5                | 0.06            | 0.12          | D666E                  | 1        | 0.06          | 0.25          | S629P + D666V                                 |
|                  | D666N                | 0.5–2              | 0.06            | 0.12–0.25     | P667H                  | 0.25–2   | 0.12–0.25     | 0.25–2         | S629P + D666V                                 |
|                  | P667T                | 2                  | 0.015           | 0.25          | P667T                  | 2        | 0.015         | 0.25          | S629P + D666V                                 |

| Species          | FKS1-HS1 MIC (µg/mL) | FKS1-HS2 MIC (µg/mL) | Therapeutic Failure | Reference                  |
|------------------|----------------------|----------------------|---------------------|----------------------------|
|                  | Mutation (Frequency) | CSP (µg/mL) | MCF (µg/mL) | AND (µg/mL) | Mutation (Frequency) | CSP (µg/mL) | MCF (µg/mL) | AND (µg/mL) | CSP (µg/mL) | MCF (µg/mL) | AND (µg/mL) | Reference                  |
|                  | F641L                | 1                  | 0.5             | 0.5           | NF                     | NA/WT     | NA/WT         | NA/WT         | F641L                                      |
|                  | F641S                | 4–2                | 1               | 1             | NF                     | NA/WT     | NA/WT         | NA/WT         | F641S                                      |
|                  | L644W                | 2                  | NA              | NA            | NF                     | NA/WT     | NA/WT         | NA/WT         | F641S                                      |
|                  | S645P                | 4–32               | 0.5–2           | 0.25–4        | NF                     | NA/WT     | NA/WT         | NA/WT         | F641S                                      |
|                  | R647G                | NA                 | 0.25            | 0.06          | NF                     | NA/WT     | NA/WT         | NA/WT         | R647G                                      |
|                  | R658G                | >8                 | 2–4             | NF            | NA/WT                 | NA/WT     | NA/WT         | NA/WT         | R658G                                      |
|                  | S639F                | 4–16               | 16              | 8             | NF                     | NA/WT     | NA/WT         | NA/WT         | S639F                                      |
|                  | S639Y                | 8                  | 8               | 8             | NF                     | NA/WT     | NA/WT         | NA/WT         | S639F                                      |
|                  | S639P                | >16                | 8               | 8             | NF                     | NA/WT     | NA/WT         | NA/WT         | S639F                                      |
|                  | A. fumigatus         | F675S              | 2                | 2             | ND                     | NA/WT     | NA/WT         | NA/WT         | F675S                                      |

**C. glabrata**

**C. tropicalis**

**C. parapsilosis**

**C. auris**

**A. fumigatus**
Recently, it was proposed that the presence of mutation(s) in a gene of DNA repair pathway, \textit{MSH2}, increases the propensity of clinical \textit{C. glabrata} isolates to acquire in vitro resistance to antifungal drug(s) \cite{60}. This notion has been evaluated in French \cite{136}, Chinese \cite{124}, Spanish \cite{35}, and Indian \cite{137} clinical isolates of \textit{C. glabrata}, and it appears that the presence of a mutation in \textit{MSH2} is associated with the genotype but not with the acquisition of antifungal resistance. Although, these clinical studies reported little or no resistance, which makes biological associations seems suspicious. Nevertheless, some \textit{MSH2} mutations, but not all, are associated with antifungal multidrug resistance and future studies are warranted to identify the accountable mutations \cite{32}.

6.2. Candida tropicalis

\textit{Candida tropicalis} is the primary cause of candidemia in India \cite{138}, Tunisia \cite{139}, and Algeria \cite{140}, the second cause of candidemia in Asian Pacific countries \cite{141}, and fourth cause worldwide \cite{79}. It is considered to be the most virulent species after \textit{C. albicans} \cite{142} and like \textit{C. glabrata}, this yeast can develop antifungal resistance during the course of antifungal therapy \cite{125,126,143}. Candidemia patients infected with \textit{C. tropicalis} show the poorest prognosis and highest mortality rate compared with patients infected with other NAC species \cite{144}. Patients suffering from leukemia and neutropenia are considered to be highly susceptible to developing \textit{C. tropicalis} candidemia \cite{7}. The SENTRY study has noted a two-fold increase in the number of FLZR \textit{C. tropicalis} isolates in the years 1997–2014, with the highest rate detected in Asian Pacific countries \cite{79}. Indeed, significant increase of the incidence of FLZR \textit{C. tropicalis} isolates has been noted by numerous institutional and nationwide studies, and ranges from 6.7\% to 42.7\% \cite{95,145–148}. This could be associated with disproportionate azole use in the clinic. Surprisingly, according to studies conducted in Taiwan \cite{146}, Japan \cite{147}, Iran \cite{149}, and Turkey \cite{150}, almost 50\% of candidemia patients infected with FLZR \textit{C. tropicalis} isolates are azole naïve. Unfortunately, as is the case with \textit{C. parapsilosis}, the vast majority of FLZR \textit{C. tropicalis} isolates are identified in developing countries, hampering the efficacy of FLZ in these countries. Although, the global rate of echinocandin resistance remains low (0.5–0.7\%), an increasing trend for echinocandin resistance has been noted for \textit{C. tropicalis} in the years 2015–2016 \cite{79}.

As for other NAC species, MDR was reported in some studies and, strikingly, almost 1\% of the Indian \textit{C. tropicalis} blood isolates are resistant to the three major classes of antifungals \cite{138}. Unlike \textit{C. parapsilosis}, it appears that \textit{ERG11} overexpression is a more prominent FLZR mechanism than efflux pump activity in \textit{C. tropicalis} \cite{151–154}, and \textit{MDR1} overexpression \cite{152,155,156} is more prevalent than \textit{CDR1} overexpression \cite{152,156}. Surprisingly, FLZR \textit{C. tropicalis} isolates lacking any accountable mutation in \textit{ERG11} and not overexpressing efflux pumps were also identified, suggesting the involvement of other, unknown, mechanisms \cite{156}. In one study, mutations in the promoter region of \textit{UPC2} were identified but the authors never tested whether they could cause \textit{UPC2} and \textit{ERG11} overexpression and, in turn, azole resistance \cite{157}. The presence of non-synonymous mutations in regulator genes, and their effect on azole resistance and target genes, were assessed by a limited number of studies. Considering the increasing number of FLZR \textit{C. tropicalis} isolates, especially in developing countries, better understanding of the mechanism of azole resistance in this species is of paramount importance. Y132F is the most prevalent accountable amino acid change in Erg11p (Table 2) and is most frequently observed together with S154F; the latter does not confer azole resistance \cite{152}. Various mutations linked to echinocandin resistance have been identified; S645P in HS1 of Fks1 is the most prominent amino acid change (Table 1). The mode of transmission of this species remains inconclusive, with some studies suggesting horizontal transfer from a contaminated hospital environment \cite{158–160}, while others assuming colonization of individuals outside of the clinic, via agricultural and crop sources (see Section 7), followed by subsequent dissemination in the hospital \cite{146}.
Table 2. Mutations involved in azole resistance and their in vitro and in vivo impacts.

| Species | Protein | Mutation | MIC (µg/mL) | Therapeutic Failure | References |
|---------|---------|----------|-------------|---------------------|------------|
|         |         |          | FLZ | VRZ | ITZ | PSZ | FLZ | VRZ |
| C. tropicalis | Erg11p | P56S | 8–64 | ≥0.125–16 | ≥16 | ND |
|         |         | P56S + V234F | 8 | 1 | 0.5 | ND |
|         |         | Y132F | 8–256 | 0.5–32 | 0.25 | Y132F | Y132F |
|         |         | Y132F + S154F | 2–256 | 0.125–8 | 0.25–2 | 0.12–1 | Y132F + S154F |
|         |         | Y132F + F145L + S154F | ≥256 | ≥8 | NA | 1 |
|         |         | V125F | 8 |
|         |         | Y257H | 8–32 |
|         |         | V234F | 8 | 1 | 0.5 |
|         |         | G464S | 2–64 | 0.12–2 | 0.12–2 | 0.25–1 | G464S |
|         |         | K143R | 64–84 | 4–8 | 1–8 | 1–4 |
|         |         | L333I | 4 | 0.5 | 1 | 0.25 |
|         |         | G464D | >64 | >8 | ND | ND | G464D | G464D |
|         |         | Δ44aa + D275V + P511A | >64 | >8 | ND | ND |
|         | Upc2p  | Δ301–304 (AQSP) + Q320PPQ (Proline insertion) | 16 | 1 | 2 | 0.5 | Δ301–304 (AQSP) + Q320PPQ |
|         |         | T241A | 8 | 0.06 | 0.25 | ND |
|         |         | Q340H+T381S | 64 | 4 | 16 | ND |
|         | Mrr1p  | T255P | 8 | 1 | 0.5 | ND |
|         |         | A647S | 8 | 1 | 0.5 | ND |
|         | Tac1p  | R47Q + N164I | 8 | 1 | 0.5 | ND |
| Species       | Protein | Mutation | MIC (µg/mL) | Therapeutic Failure | References |
|---------------|---------|----------|-------------|---------------------|------------|
|               |         |          | FLZ | VRZ | ITZ | PSZ | FLZ | VRZ |
| C. parapsilosis | Erg11p  | Y132F    | 2–>256 | ≤0.03–2 | ≤0.03–0.25 | <0.015–0.125 | Y132F |
|               |         | K143R    | 4   | ≤0.03–0.5 | ≤0.03–1 | <0.015–0.25 |
|               |         | Y132F + K143R | 32–>32 | 0.06–4 | ND | ND | Y132F + K143R |
|               |         | G458S    | 16–>32 | 0.5–1 | ND | ND |
|               |         | G307A + Y132F | 16–>32 | 0.5–2 | ND | ND |
|               |         | Q250K + G458S | 16   | 0.25 | ND | ND |
|               |         | G458S + T519A | 16   | 0.5 | ND | ND |
|               |         | G_53A | 4–16 | NI | ND | ND |
|               |         | -102_101-insT | 8   | NI | ND | ND |
|               |         | P250S | 8   | 0.12 | ND | ND |
|               |         | I283R | 64  | 0.5 | 25 | ND |
|               |         | P295R | 32  | 0.5 | ND | ND |
|               |         | P295L + Q1074Stop | 16 | 0.25 | ND | ND | [151,164–171] |
|               | Mrr1p   | R479K | 128 | 2 | 1 | ND |
|               |         | G583R | >64 | 2 | ND | ND |
|               |         | A619V | 4–8 | NI | ND | ND |
|               |         | L779F | 32 | NI | ND | ND |
|               |         | A854V | 64 | 1 | 0.5 | ND |
|               |         | A859T | 8 | NI | ND | ND |
|               |         | W872C | 32 | 0.12 | ND | ND |
|               |         | K873N | 64 | 2 | ND | ND |
|               |         | L926Stop | 32 | 0.5 | ND | ND |
|               |         | G927D | 16 | 0.25 | ND | ND |
|               |         | L986P | 32 | 0.5 | Yes | ITZ |
|               |         | S1081P | 8 | 0.25 | ND | ND |
| Species | Protein | Mutation          | MIC (µg/mL) | Therapeutic Failure | References            |
|---------|---------|-------------------|-------------|---------------------|-----------------------|
|         |         |                   | FLZ       | VRZ     | ITZ     | PSZ     | FLZ       | VRZ                 |
| C. parapsilosis | Tac1p    | A21V               | 8–32       | 0.06–1  | ND      | ND      | [151,164–171]          |
|         |         | G490R + S760R + A761G | 8         | 0.12    | ND      | ND      |
|         |         | D603V + P803L      | 8         | 0.12    | ND      | ND      |
|         |         | G650E              | 1278–256  | 4       | 0.5     | ND      |
|         |         | N900D              | 8         | 0.12    | ND      | ND      |
|         |         | Q656K + M966V      | 32        | 0.5     | ND      | ND      |
|         | Upc2p   | L978W              | 128       | 8       | 0.5     | ND      |
|         |         | P45H               | 8–32       | 0.12–1  | ND      | ND      |
|         |         | Q371H              | 16        | 0.5     | ND      | ND      |
| C. auris | Erg11p  | Y132F              | 1–256     | 0.06–16 | 0.03–0.8 | 0.015–8 | [130,131,133,172,173] |
|         |         | K143R              | 4–256     | 0.03–16 | 0.03–0.5 | 0.015–0.25 |
|         |         | K143F              | 64        | 0.5     | 0.5     | 0.25    |
6.3. *Candida parapsilosis*

*Candida parapsilosis* is the second leading cause of candidemia in Latin American countries [174], and in some Asian [83,175–178], European [179–181], and African countries [140,182], and the third cause of candidemia worldwide [79]. Neonates, patients receiving total parenteral nutrition, and those with central venous catheters (CVC) are most prone to developing candidemia caused by *C. parapsilosis* [183]. Recently, the SENTRY study revealed that Latin American (4.6%) and Asian Pacific countries (0.6%) have the highest and lowest percentage of FLZR *C. parapsilosis* isolates, respectively [79]. However, this is beyond the scale reported from a South African multicenter nationwide study, according to which more than 50% of *C. parapsilosis* isolates are FLZR, with 44% among these cross-resistant to VRZ (VRZR) [182]. Furthermore, a Korean single-center study reported a significant increase in the prevalence of FLZR among 2015–2016 isolates when compared to 2011–2015 isolates (14.3% vs. 0.9%, respectively) [184]. Unfortunately, this wave of FLZR *C. parapsilosis* isolates has also been observed in Kuwait [166], USA [165,185], Brazil [167,168], South Korea [164], India [169], South Africa [182] and Turkey [170]. The high rate of FLZR for a species that used to be susceptible to azoles may have arisen from the disproportionate use of azoles in the hospital [170,182]. The notable increase in the prevalence of FLZR *C. parapsilosis* isolates could be a major threat in developing countries, in which the vast majority of candidemia cases are treated with FLZ [138,169,182,186]. By contrast, according to the SENTRY study, echinocandin resistance is a rare phenomenon among *C. parapsilosis* isolates (up to 0.1%) [79].

Evaluation of FLZR *C. parapsilosis* isolates revealed that FLZR mechanisms involve ERG11 mutations, and upregulation of CDR1 and MDR1, and in few cases ERG11, which might arise from GOF mutations in the respective zinc cluster regulators, TAC1, MRR1, and UPC2, accordingly (Table 2). Although some studies link the substitutions R478K, G583R, L779F, and K873N in Mrr1p to azole resistance [129], the role of other mutations in the aforementioned regulators is largely unknown. Surprisingly, the overexpression of CDR1, MDR1, and ERG11 in the absence of any mutations in the corresponding regulators [165,185], and 2-fold dilution decrease in FLZ MIC values when either CDR1 or MDR1 are deleted [185], are indications for the involvement of other mechanisms. Therefore, a comprehensive transcriptomic and proteomic analysis of FLZR *C. parapsilosis* isolates may allow a better understanding of the possible mechanisms of azole resistance in *C. parapsilosis*. Y132F and K143R are presumably the most frequently identified amino acid changes causing FLZR and/or VRZR, and we noticed that isolates harboring Y132F in Erg11p are significantly associated with a mortality rate that is higher than that associated with Y132F+K143R isolates [170]. Beyond the common paradigm of ERG11 and efflux pump involvement in azole resistance, whole genome sequence analysis showed that a laboratory-driven, posaconazole-resistant *C. parapsilosis* isolate harbored an amino acid substitution in Erg3, R135I, which is believed to confer azole resistance and prevent the formation of toxic sterol intermediates [188].

A naturally occurring polymorphism in HS1 of FKS1 (P660A) in *C. parapsilosis* results in elevated echinocandin MIC values in this species [189], yet an effect on the drug–target interaction seems weak and the infected candidemia patients treated with echinocandins show a favorable clinical outcome compared to those treated with FLZ [190,191]. Interestingly, despite the identification of clinical echinocandin-resistant *C. parapsilosis* isolates, no other mutations in HS1 and HS2 of FKS1 were identified to date. Most recently, we evaluated a large collection of Turkish *C. parapsilosis* blood isolates (2007–2019) [129]. For the first time, we identified four isolates that were resistant to micafungin and carried R658G in HS1 of FKS1 (Table 1). Interestingly, three of those isolates represented the same genotype, were also further resistant to FLZ, and carried Y132F+K143R in Erg11p [129]. This represented an unprecedented clonal expansion of MDR *C. parapsilosis* isolates [129]. *Candida parapsilosis* is well-known for biofilm production on biotic and abiotic surfaces, and the hands of the healthcare workers are considered as one of the major sources of bloodstream infection [183]. Consequently, antifungal-naïve patients might acquire antifungal-resistant *C. parapsilosis* isolates from the hospital.
environment, which may in turn result in therapeutic failure. Therefore, strict adherence to hygiene and CVC removal could greatly reduce C. parapsilosis-associated candidemia.

6.4. Candida auris

Candida auris was described for the first time in 2009, causing an ear infection in a Japanese patient [192]. However, it soon became one of the most worrisome MDR pathogenic fungal species known, causing infection in over 35 countries on six continents [193]. This species is the third to fifth common cause of candidemia in South Africa [194] and India [138], respectively. Of note, in some hospitals in India, C. auris is the second most common cause of candidemia [195]. One of the most dramatic examples of the dominance of this species was documented in a course of a nationwide South African candidemia study, with C. auris identified as a rare cause of candidemia in 2009 but becoming the third most common cause of candidemia 7 years later [194] and on its way to become the leading candidemia cause. Its ability to persistently colonize the skin, hospital equipment, and environment [196], and survival on plastic surfaces for 4 weeks [197], combined with the inefficiency of the currently used disinfectants [198] necessitates intense infection control measures, such as those recommended for challenging bacterial species that cause nosocomial outbreaks, such as methicillin-resistant Staphylococcus aureus and Clostridium difficile [196]. Several hypotheses have tried to elucidate the simultaneous worldwide emergence of C. auris [199,200], yet its recent bizarre dominance remains enigmatic. Whole-genome sequencing revealed that the identified clinical and environmental isolates belong to four clades (clusters) representing geographical origin of the isolates, namely, the South Asian clade (clade I), East Asian Clade (clade II), South African Clade (clade III), and South American clade (clade IV) (Figure 5) [172]. Of note, according to an updated whole-genome study, a single isolate of Iranian C. auris potentially represents a fifth clade (not shown in Figure 4), consolidated by hundreds of thousands of base–pair differences with the closest clade (II) and the notion that the infected individual did not travel outside of Iran [193]. The concept that isolates from those clades have been detected in the healthcare setting hundreds to thousands of kilometers apart [130,172,201] suggests clonal expansion of C. auris, possibly via travel [193]. Catheter insertion, recent surgery, and previous exposure to antifungals are among the potential risk factors for the development of infection caused by C. auris [172,194].

Figure 5. Whole-genome sequence analysis of the US C. auris (up to 2018) isolates reveals the existence of four major clades. Isolates representing all identified clades have been recovered from the U.S. The Iranian clade is not shown in this figure. Adopted the permission from Chow et al., 2018 [201].

Antifungal susceptibility profiles vary depending on the clade [131,172,173,195], with the drug resistance reaching up to 7%, 35%, and >90% against echinocandins [172], AMB [172], and fluconazole [131], respectively. Similar to C. glabrata, resistance against two or three major classes of antifungals is frequently observed [131,172]. Amino acid changes in Erg11p are closely associated
with resistance and seems to be clade-specific, where clade III is prone to harbor F126T; Y132F is most prevalent in clade IV [172]; and Y132F and K143R are prominent in clade I (Table 2) [131]. Of note, according to a recent study examining the South Korean isolates, only a small proportion of FLZR isolates (3/38, 7.8%) harbor K143R and the remaining isolates lack accountable mutations [173]. This underscores the involvement of other, yet to be identified pathways, such as efflux pumps, with the emphasis on CDR1. CDR1 plays a prominent role in azole resistance, as CDR1 disruption significantly decreases the MIC value to azoles containing target site amino acid substitutions [202]. Furthermore, CDR1 expression in C. auris 5–25 min post-exposure to fluconazole is 14.4–6.7 times higher than that in C. glabrata [203]. Since HSP90 inhibitors do not abrogate the azole resistance conferred by CDR1 overexpression [204], it is not unreasonable to assume an involvement of TAC1 overexpression, achieved via GOF mutations, in the resistance. Therefore, profiling and evaluation of GOF mutations in a transcriptional regulator of efflux pumps may further elucidate the azole resistance mechanisms. Moreover, CDR1 plays an important role in the intermediate and late stages of biofilm growth, and inhibiting CDR1 causes a 4–16-fold decrease in the FLZ MIC values [205]. Indeed, consistent with this hypothesis, the most recent study found that GOF mutations in TAC1 are other players implicated in azole resistance in C. auris [206].

Although constitutive overexpression of ERG11 has been observed in some isolates [131], this phenomenon is an unlikely player in azole resistance. Further, sectional genomic duplication (12–153 kb), with the largest occurring in clade III, ERG11 is associated with azole resistance and/or elevated MIC values [20,207]. Echinocandin resistance is mainly associated with a substitution of serine 639 to proline (S639P), tyrosine, or phenylalanine (HS1 of FKS1). Among these, S639P is the most prominent amino acid change (Table 1) [132].

Finally, the AMB resistance can be a combination of overexpression [20] and/or non-synonymous mutations in a number of genes, and more studies are warranted to identify the role of these mutations in resistance [20,172].

6.5. Aspergillus fumigatus

Aspergillus fumigatus is the principal causative agent of human aspergillosis, accounting for more than half of all isolates in most studies [208,209]. Azole drugs are the main antifungal compounds used both in agriculture and the clinical setting, and the emergence of azole resistance is rising and spreading worldwide [210,211]. Based on a prospective multicenter international study involving 19 countries, the prevalence of azole-resistant A. fumigatus is 3.2% [212].

Generally, azole-resistant isolates are acquired via two routes. In the clinical setting, azole resistance may develop during long periods of azole treatment. It is associated with single-point mutations in a lanosterol-14-α sterol-demethylase gene (Cyp51A), encoding a key protein in the ergosterol biosynthesis pathway, that lead to amino acid changes (G54, G138, P216, M220, and G448) [213]. Alternatively, extended use of demethylation inhibitors (DMIs) in agriculture is associated with tandem repeat (TR) integrations of different sizes in the Cyp51A promoter, followed, or not, by point mutations in the gene (TR34/L98H, TR46/Y121F/T289A, and TR53) [4]. Hence, azole selective pressure elicits the development of different azole resistance mechanisms and also different azole susceptibility patterns. Apart from azole drug target, some studies have indicated the emergence of notable number of azole-resistant A. fumigatus isolates displaying WT CYP51A [214,215] for which the azole resistance phenotype was corroborated by the overexpression of efflux pumps, especially Cdr1B [214]. Moreover, acquisition of various mutations in Hmg1 [216] HapE [217] also confer azole resistance in A. fumigatus. Other species of the Aspergillus section Fumigati that are also human pathogens show azole resistance, such as A. lentulus, A. viridinutans, A. fumigatiaffinis, and A. fischeri [218,219]. Although rarely used as a main treatment among patients suffering invasive aspergillosis, recent studies have revealed that mutation in HS1-FKS1 [135] and also changes posed to the microenvironment of β-1,3-d-glucan synthase in A. fumigatus can result in echinocandin resistance [220].
6.6. Aspergillus terreus

In the past, infections caused by *A. terreus* species complex were classified as rare, but their clinical incidence has recently increased. According to a prospective international multicenter surveillance study, the prevalence of *A. terreus* species complex among patients with mold-positive cultures is 5.2%, attributed *A. terreus* s.s. (86.8%), followed by *A. citrinoterreus* (8.4%), *A. hortai* (2.6%), *A. alabamensis* (1.6%), *A. neoaficanus* (0.2%), and *A. floccosus* (0.2%) (221). Frequent occurrences are noted at certain geographic locations, such as Innsbruck (Austria) and Houston (TX, USA). Of special concern is the high mortality of disseminated disease caused by this species [221]. The at-risk population for infections caused by *Aspergillus* section *Terrei* is the same as that for individuals suffering from *A. fumigatus* diseases, and comprises mainly immunocompromised individuals. However, non-immunocompromised individuals may also be affected [221].

*Aspergillus terreus* species complex holds an exceptional position within the aspergilli, as it displays polyene resistance in vitro and in vivo [222]. Generally, AMB MIC values range from 0.125 to 32 mg/L. The underlying AMB resistance mechanisms of *A. terreus* are only partly understood and are multifaceted [222,223]. AMB resistance seems to be related to basal superoxide dismutase activity and an enhanced oxidative stress response in *A. terreus*.

According to a recent study, approximately 5% of *A. terreus* s.s. isolates are resistant to posaconazole in vitro [224]. The prevalence of resistance differs geographically, and ranges from 0% in the Czechia, Greece, and Turkey, to 13.7% in Germany. The highest rates of resistance are observed in Austria, Germany, and the UK. Azole resistance in *A. terreus* s.s. is associated with mutations in the Cyp51A gene. In the Cyp51A protein, M217 position correlates with the posaconazole-resistant phenotype, with substitutions M217T and M217V reported [224] (Table 3). By contrast, azole resistance among cryptic species is rare, and observed only in *A. citrinoterreus* and *A. alabamensis*.

### Table 3. Mutations involved in azole resistance in *A. terreus* and *A. fumigatus* and their in vitro and in vivo impacts. The frequencies mentioned do not necessarily consider all studies published and might be a rough approximation of the actual frequencies.

| Species | Protein | Mutation | MIC (µg/mL) | Therapeutic Failure | References |
|---------|---------|----------|-------------|---------------------|------------|
| *A. fumigatus* | Cyp51A | G54R | 0.125–0.5 | 2–8 | G54 | [209,211, 225–236] |
| | | G54E | 0.125–0.25 | 2–16 | 0.25–2 |
| | | G54W | 0.125–0.25 | 1–16 | >16 |
| | | G54V | 1 | >8 |
| | | G138C | 8 | >16 | >16 |
| | | P216L | 1 | 8–16 | 1–16 |
| | | M220R | 2 | >16 | 2 |
| | | M220I | 1 | >16 | 0.5 |
| | | M220V | 1–4 | 8–32 | 0.5–2 |
| | | M220K | 2 | 16 | >16 |
| | | M220L | 0.5 | >8 | >8 |
| | | M220T | 0.5–2 | 32 | 0.06–0.25 |
| | | G448S | 4–8 | 0.5–8 | 0.125–1 |
| | | TR34/L98H | 4–8 | >32 | 0.5–1 |
| | | TR46/Y121F/T289A | >16 | >16 | 2 |
| | | TR53 | 16 | >16 | 0.25 |
| | HapE | P88L | 1 | >16 | 0.125 | [217] |
| *A. terreus* | Cyp51A | M220L | <1 | <1 | >0.25 | Not evaluated |
| | | M217T | <1 | <1 | >0.25 | Not evaluated |

References:

[217], [218], [224], [225–236]
7. Role of Agriculture in the Development of Resistance

Azole drugs are the only class of compounds that are used both in agriculture and in the clinical setting [211]. In the context of agriculture, they are extensively used for crop protection, preservation of the yield and quality of crops against plant fungal diseases, and prevention of contamination by yeasts (Candida spp., Trichosporon penicillatium, and Cryptococcus spp.) and filamentous fungi (Aspergillus spp., Fusarium spp., and Alternaria spp.) during the pre- and post-harvest periods. The global pesticide use increased significantly during the years 2012–2016, with the highest use in Asia, 2 M tons collectively (52%); followed by America, with 1 M tons (32.7%); Europe, with 477 K tons (11.6%); Africa, with 95 K tons (2.3%); and Oceania, with approximately 55 K tons (1.4%) [237]. The increased use of antifungals in agriculture in recent years has paralleled the detection rate of fungicide-resistant pathogenic fungi. As A. fumigatus spores are ubiquitous in the environment, and environmentally acquired azole-resistant isolates, especially those in compost heaps [238], may exhibit in vivo and/or in vitro resistance to medically important azole drugs, such agricultural pesticides may threaten human health by exposure through contact, inhalation, or ingestion of contaminated food or water.

The hypothesis that an environmental source of resistant A. fumigatus isolates could underpin the emergence of azole resistance is supported by the identification of primary IA cases caused by azole-resistant A. fumigatus in patients who have never been treated with azoles [239]. Since environmental isolates harboring TR resistance mechanisms are identified in azole-naïve patients on five continents, and the same resistance mechanism was identified in environmental isolates treated with MDIs that share a higher genetic similarity with wild-type isolates, this strongly suggests that clinically acquired azole-resistant isolates are primarily acquired from the environment [4]. A recent study, however, through the use of whole-genome sequencing identified that azole-resistant A. fumigatus isolates harboring TR can also develop during the course of antifungal treatment, which shows the same genotype as the initial azole-susceptible A. fumigatus isolates [240]. It seems that the emergence of azole-resistant fungus in the clinic with the environmental source is not exclusive to molds. Recently, the same phenomenon was observed for C. tropicalis and 55.2% of azole-resistant isolates were recovered from patients never treated with azoles [146]. Interestingly, multi-locus sequence typing (MLST) revealed that the clinical azole-resistant isolates share a high degree of similarity with an azole-resistant isolate recovered from fruit, indicating an increasing danger of acquisition of environmental azole-resistant fungi that represent a wide spectrum of species, ranging from molds to yeasts [146].

8. Genomic Tools for Early Diagnosis of Resistance

AFST is a popular culture-based method of analysis. It is a phenotypic approach for the visual determination of the susceptibility of a fungal species to a specific antifungal, by reporting the MIC value. MIC is the lowest concentration of an antifungal that results in growth inhibition of a fungal species when compared to a positive control. AFST broth microdilution protocols are standardized by the Clinical Laboratory Standard Institute (CLSI; http://www.clsi.org) and European Committee for Antimicrobial Susceptibility Testing (EUCAST; http://www.eucast.org}). Although AFST plays a central role in patient management by aiding the prescription of an appropriate antifungal, it is time-consuming and data interpretation varies depending on the protocol used and between laboratories.

In some cases, sequencing of drug target genes is a better predictor of patient outcome than AFST [86]. Consequently, various polymerase chain reaction (PCR)-based techniques have been developed for the identification of mutations in drug target genes, facilitating timely administration of an appropriate antifungal [241]. Any PCR-based or hybridization-based method that is able to detect the presence of single-point-mutations or genetic variations can be used to test for the existence of a set of known resistance-conferring mutations [242]. Such approaches are highly sensitive, and require a low amount of input DNA that does not need to be of the highest quality. However, these methods are designed to target a set of known resistance-conferring mutations. Hence, a negative result does not always imply the absence of resistance, they are best used to confirm known prominent mutations.
conferring antifungal resistance. Whole-genome sequencing or targeted sequencing of genomic regions known to confer resistance can be used for the detection of genomic alterations that potentially confer resistance in an isolate of interest. Although the obtained data should be crosschecked against a catalogue of known resistance-conferring mutations, these approaches can potentially uncover new variations (e.g., by assessing the potential impact on proteins encoded by the mutated genes), and they do not need to be re-designed every time the catalogue is expanded [242]. Nevertheless, although these approaches are promising, they have different limitations that delay their introduction in the clinic. The associated costs, required expertise, need for a high amount of template DNA, difficulty of direct probing clinical samples, and requirement for on-site technology are some of the challenges that need to be addressed [242,243].

9. Future Perspectives

The increasing number of fungal species resistant to antifungals and the emergence of MDR fungal species parallels with the global increase in the use of DMIs pose a serious threat to patient outcome, especially those in developing countries where azoles are the main antifungal used to treat invasive fungal infections. Therefore, epidemiological studies and constant monitoring of the burden of antifungal resistance on worldwide scale should be coupled to revisiting the antifungal stewardship protocols in both environment and clinics. Moreover, broadening our understanding about the mechanisms of antifungal resistance not only allows designing more rapid molecular techniques to rapidly diagnose antifungal resistance but also may lead to designing more efficient new fungal-specific antifungal drugs given the high genetic similarity of fungi with human. Therefore, comprehensive and species-specific multimics studies and high-resolution structural approaches play an integral role in this context. Introduction of antifungal drugs showing optimal clinical profiles into the clinic and extending research to identify naturally occurring secondary metabolites showing promising antifungal drugs are more than ever needed. New online platforms, such as www.theyeasts.org, encourages researchers throughout the world to deposit the microbiological and therapeutic failure data of mutations occurring in drug target and efflux pump regulators allowing clinicians to promptly predict the possible MIC values of any mutation and if they can cause therapeutic failure. We hope that the rise of technical advances will accompany the extensive international collaboration that is needed to tackle the pressing challenge of antifungal resistance.

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