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Chapter 1

Biofilm Formation as a Pathogenicity Factor of Medically Important Fungi

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Additional information is available at the end of the chapter

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Abstract

To cause disease, the infectious agent makes use of both invasiveness factors—the pathogen virulence factors—and the ability to resist and evade the host immune system. The success of the infection process is the result of a complex equation involving pathogen interaction with the host, wherein the expression of several virulence factors (and not just one or the other) will favor the establishment of the pathogen in the host. Fungal pathogens are frequently associated with biofilm formation.

Biofilms are communities of microbial cells adhered to a biotic or abiotic surface and surrounded by an extracellular matrix secreted by the biofilm cells. Pathogenic fungi are capable of forming biofilms inside patients, which is often related to invasive and drug-resistant fungal infections. The most dangerous characteristic associated with biofilm development is the increased resistance of biofilm cells to antifungal drugs, which results from a multifactorial association of mechanisms. General properties of fungal biofilms, resistance behavior and its correlation with pathogenicity are discussed in this chapter.

Keywords: Fungal biofilms, *Candida*, *Aspergillus*, Extracellular matrix, Resistance, Fungal Infection

1. Introduction

Since the seventeenth century, biofilms have been described in multiple systems. Most bacteria preferentially grow as biofilms, in all self-sustaining aquatic ecosystems, and these sessile bacterial cells differ deeply from their planktonic counterparts (cells in suspension) [1]. The definitions of biofilm have evolved over the years, in parallel to the advances of the biology area and research studies on the subject. The definition used today was proposed by Donlan and Costerton in 2002, and it describes a biofilm as a microbial community in which the cells

are connected to a substrate, or to each other, embedded in a extracellular matrix of polymeric substances (produced by themselves) and exhibit an altered phenotype regarding the rate of growth and transcription of genes [2].

In fungi, the ability to colonize surfaces and to form biofilms was initially demonstrated for *Candida albicans* and *Saccharomyces cerevisiae*, in the 1990s and early 2000s [3, 4]. However, the growing awareness on the importance of fungal biofilms can be confirmed by the increased number of publications upon biofilm formation by other *Candida* species [5–7], as well as other yeasts that cause opportunistic infections and pneumonia in humans, such as *Malassezia pachydermatis* [8], *Rhodotorula* sp. [9], *Trichosporon asahii* [10], *Blastoschizomyces* [11], *Pneumocystis* spp. [12] and *Cryptococcus neoformans* [13]. Moreover, the ability to form biofilms has also been demonstrated in several filamentous fungi, including *Aspergillus fumigatus* [14] and *Fusarium* spp. [15], in fungi that cause endemic mycoses such as *Histoplasma capsulatum* [16], *Paracoccidioides brasiliensis* [17] and *Coccidioides immitis* [18] and in zygomycetes such as *Mucorales* [19].

Until now, several superficial reports about the ability of a wide range of fungal species to form biofilms *in vitro* and *in vivo* have popped up, demonstrating that this is possibly due to a favorable lifestyle organization used by most medically important fungi. Deeper knowledge about those biofilms is still a challenge. As they account for the first and second leading fungal infections on hospitals, *Candida* and *Aspergillus* biofilms are the most studied examples. Therefore, the next sections of this chapter highlight the most important features of *Candida* and *Aspergillus* biofilms, as they are known up to this date. An additional section will summarize recently published features of biofilms of other species of fungi.

2. *Candida* spp. biofilms

Candida spp. are often identified as the causative agent of candidemia, hospital pneumonia and urinary tract infections and, almost invariably, these infections are associated with the use of a medical device and biofilm formation on its surface [20]. The most commonly colonized medical device is the central venous catheter (CVC), used for administration of fluids, nutrients and medicines [21]. The infusion fluid or the catheter may be contaminated, but, more often, yeasts are introduced from the skin of the patient or the hands of health professionals [21]. Alternatively, these yeasts can migrate into the catheter from a pre-existing lesion. However, if *Candida* spp. that colonize the gastrointestinal tract as a commensal start to develop a pathogenic behavior, they are able to penetrate the intestinal mucosa, spread through the bloodstream and, then, circulating yeast may colonize the catheter endogenously. This could be a common dissemination mechanism in cancer patients because cancer chemotherapy leads to damage to the intestinal mucosa [22]. In non-neoplastic patients, infected catheters are the most important source of bloodstream infections followed by widespread invasive candidiasis. The catheter removal is recommended in patients with disseminated *Candida* spp. infection to facilitate disinfection of the blood and to improve prognosis [23–25].

Candidemia and other forms of invasive candidiasis (i.e., infection involving normally sterile sites) are the most prevalent invasive mycoses worldwide [20, 26] with mortality rates close to 40% [27, 28]. *Candida albicans* is the most commonly isolated species; however, in the past few decades, several surveillance studies reported an increased incidence of infections caused by *Candida* non-*albicans* species (CNA), like *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, *C. guilliermondi* and *C. lusitaniae* [29, 30]. This epidemiological swift is of utmost importance because resistance to fluconazole and echinocandins (two of the three antifungal options in the clinical practice) has been shown to be more common in CNA species compared with *C. albicans* [31], especially due to some CNA species that are inherently resistant to antifungals, such as *C. krusei* to fluconazole [31], or have a greater propensity to develop antifungal resistance, such as *C. glabrata* [32, 33]. *Candida* spp., including *C. albicans* and the main CNA species related to candidemia, can colonize surfaces and develop biofilms, as demonstrated by several *in vitro* and *in vivo* studies [6, 34–38]. *C. albicans* is the third leading cause of catheter-related infections, the second main cause of colonization-followed-by-infection [39, 40] and the mortality rate in patients with candidemia associated to catheter use is as high as 41% [41, 42].

Candida biofilm development can be didactically described in four sequential steps (Figure 1): (a) adherence—initial phase, in which the yeast in suspension and those circulating (planktonic cells) adhere to the surface, first 1–3 h; (b) intermediate phase, concerning the development of biofilm, 11–14 h; (c) maturation phase, in which the polymeric matrix completely soaks all layers of cells adhered to the surface in a three-dimensional structure, 20–48 h; (d) dispersion, in which the most superficial cells leave the biofilm and colonize areas surrounding the surface, after 24 h [43].

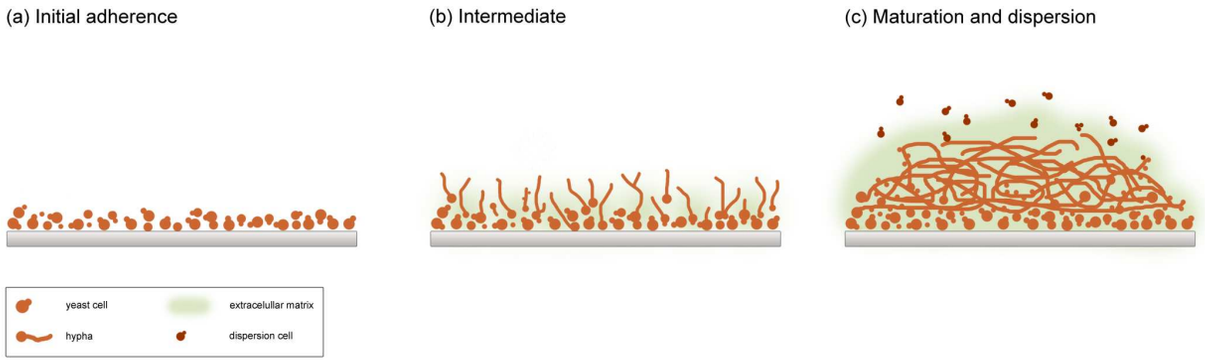

Figure 1. *Candida* biofilm development model: (a) initial phase of adherence, in which the yeast in suspension (planktonic cells) adhere to the surface; (2) intermediate phase, accounts for growth of colonies and initial extracellular matrix secretion; (3) maturation phase, in which the ECM completely soaks all layers of cells adhered to the surface in a three-dimensional structure. After maturation, dispersion events, when the most superficial cells leave the biofilm and colonize areas surrounding the surface, may occur.

The mature biofilm consists of a dense network of cells in the form of yeasts, hyphae and pseudohyphae (Figure 2A) soaked by polymeric extracellular matrix and with water channels between the cells, which facilitate the diffusion of nutrients from the environment through the biomass to the lower layers and which also allow the elimination of waste [43–45].

Biofilms of CNA species are less complex in structure because true-hyphae is not present, culminating in a biofilm formed predominantly by yeasts (*C. parapsilosis* and *C. glabrata* biofilms, Figure 2B and 2C, respectively) or, as observed for *C. tropicalis*, a mix of yeasts and some pseudo-hyphae [35].

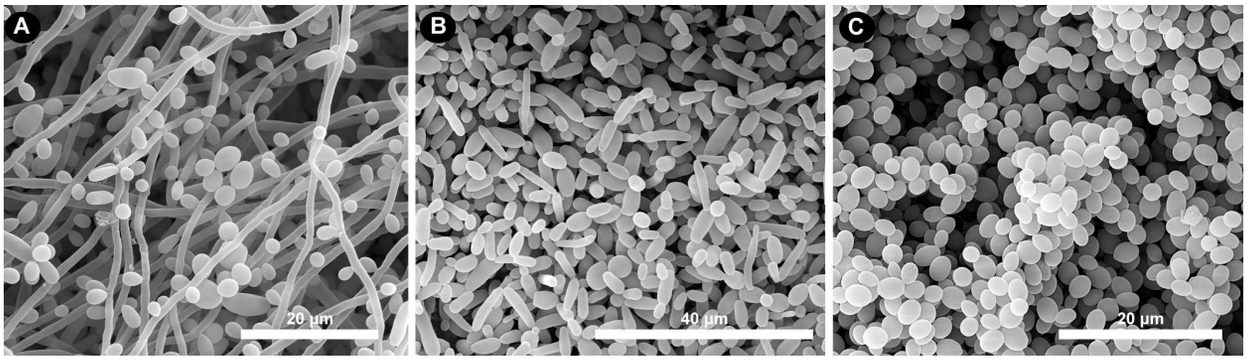

Figure 2. Scanning electron micrograph of *in vitroCandida* spp. biofilms. (A) *Candida albicans* biofilms, formed by a dense network of cells in the form of yeasts, hyphae and pseudohyphae. (B) *Candida parapsilosis* biofilm, formed mainly by yeast cells, either round or elongated in shape; (C) *Candida glabrata* biofilm, formed exclusively by smaller, spherical yeast cells.

Biofilms of *Candida* spp., formed using *in vivo* models, seem to follow the same sequence of *in vitro* formation [36]; however, maturation occurs more rapidly and the final thickness is increased. Mostly, *C. albicans* forms bi-layered biofilms, with a bottom layer formed of yeasts tightly attached to the surface and upper layers formed by hyphae; however, the final architecture of the biofilm is variable and depends, in part, on the substrate on which it is formed and on the growing conditions [43].

3. *Aspergillus fumigatus* biofilms

Aspergillus spp. are filamentous fungi and their spores are commonly found in soil, water and decaying organic matter. Many species have been identified in nature, but a small portion is recognized as causative agents of aspergillosis, and associated with human infections, being *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus* and *Aspergillus nidulans* the most clinically relevant species.

Hundreds of *A. fumigatus* conidia are inhaled daily and reach the alveoli of the human host. In immunocompetent individuals, conidia are efficiently eliminated by pulmonary macrophages. However, depending on the immune status of the host or predisposing conditions, *A. fumigatus* can lead to the development of disease in immunocompetent patients as in aspergiloma patients with pre-existing pulmonary cavities or chronic obstructed sinuses (generated by tuberculosis, bronchiectasis or cystic fibrosis), in allergic rhinitis mediated by immunoglobulin E, in pneumonia and in allergic bronchopulmonary aspergillosis (ABA; clinical condition developed by patients with cystic fibrosis and asthma caused by *A. fumigatus* antigens). In immunocompromised patients, the pulmonary infection can spread into the bloodstream

(invasive pulmonary aspergillosis; IPA) leading to the involvement of multiple organs. Invasive aspergillosis (IA) is the major infectious cause of morbidity in deeply immunocompromised patients, especially post-transplant and/or with prolonged neutropenia; and mortality rates range from 40–90% [46]. *A. fumigatus* is responsible for approximately 90% of cases of IA [17, 47] and is, therefore, the most studied species. The initial establishment of chronic *A. fumigatus* infection involves the germination of conidia into mycelia and then subsequent invasion of the mycelial structure into pulmonary epithelial and endothelial cells [48]. In 2007, Beauvais et al. used scanning electron microscopy to show that the aerial hyphae of the mycelial colony formed over pulmonary cells were bounded together by a dense hydrophobic ECM, and that those colonies were more resistant to amphotericin B than liquid-submerged colonies, raising the hypothesis of biofilm formation during pulmonary *Aspergillus* colonization [49]. Confirmation was published by the same group, in 2010, using an *in vivo* model to demonstrate and characterize the presence of mature *A. fumigatus* biofilms (composed of hyphae covered with extracellular matrix) in aspergilomas and during the development of disseminated aspergilosis [46].

Following the first report in 2007, several studies demonstrated that *A. fumigatus* is able to grow as biofilms under *in vitro* conditions on polystyrene microtitre plates seeded with both human bronchial epithelial cells and cystic fibrosis (CF) human bronchial epithelial cells [50–52]. Later on, *A. fumigatus* adherence and colonization of medical devices such as catheters, prostheses, cardiac pacemakers, heart valves and even breast implants have been extensively described [17, 53–55].

Compared to *C. albicans*, biofilm development is slower for *Aspergillus*, as a lag phase of approximately 10 h (conidial adhesion and germination) stands between the initial conidial seeding and the formation of an initial monolayer (early phase, 10–16 h). Then within the next few hours, intense hyphae grow and ECM secretion leads to increased structural complexity (intermediate, 48 h), culminating with a dense and mature biofilm after 72h (maturation phase) [50, 51].

Despite the lack of clinical studies substantiating *A. fumigatus* biofilm development *in vivo*, evidence such as high mortality in neutropenic cancer patients suffering from IA (40–90%) [46] and resistance of chronic infections to potent antifungal drugs *in vitro* [51, 56, 57] clearly indicates the formation of *A. fumigatus* biofilms *in vivo*. Additionally, histological and microscopic examination of bronchopulmonary lavage samples from the lungs have revealed the presence of numerous *A. fumigatus* hyphae in the form of dense intertwined mycelial balls or grains, referred to as mycetoma, which is similar to the biofilms formed by *Candida* species *in vivo* [36]. In fact, in 2009, Mowat et al. raised the discussion whether mycetomas should be considered biofilms [14].

4. Biofilms of other medically important fungi

Cryptococcosis, caused by yeasts of the genus *Cryptococcus* sp., is the third most prevalent disease in HIV-positive individuals. It is estimated that one million cases per year are associ-

ated with cryptococcosis in HIV-positive patients worldwide [58]. Infection by *Cryptococcus* occurs through inhalation of yeast spores in the environment and is considered a primary pulmonary infection that may progress to disseminated infection. Disseminated infection can affect the central nervous system (CNS), causing more severe forms of the disease like meningitis, encephalitis or meningoencephalitis [58]. More than 600,000 deaths are attributed to the 1 million new cases of cryptococcal meningitis that occur every year [59].

The main pathogenic species to humans are *C. neoformans* and *C. gatti*, with *C. neoformans* being the agent of opportunistic infections while *C. gatti* may also affect immunocompetent hosts. *Cryptococcus* sp. yeasts are able to colonize and form biofilms over various prosthetic devices such as peritoneal dialysis fistulas, hip prostheses and heart valves [55]. These biofilms include yeast cells with a vast amount of polysaccharide composing the extracellular matrix responsible for preventing its eradication by environmental agents and antimicrobials. Because *Cryptococcus* sp. in essentially an environmental fungus that adapted to the human host, biofilm formation is an expected survival strategy in harsh environmental conditions (e.g., ultraviolet light, dryness and natural antimicrobial substances). There are only a few studies on biofilms of *Cryptococcus* sp.; however, it is known that their formation is dependent on the presence of their polysaccharide capsule, mainly composed of glucuronoxylomanana (GXM), since anti-GXM antibodies specifically inhibit biofilm formation [60].

Invasive infections caused by *Candida* spp., *Cryptococcus* spp. and *Aspergillus* spp. are more frequently observed; however, other rare opportunistic fungi such as filamentous hyaline fungi (*Fusarium* spp., *Acremonium* spp. and species from the *Pseudallescheria*/*Scedosporium* complex) may also cause diseases that may vary from superficial to life-threatening invasive infections that may be fatal for immunocompromised individuals.

Fusarium species are common soil saprophytes and also important pathogens of plants and humans, causing superficial, invasive or disseminated infections. Twelve species are associated with human infections, and *Fusarium solani*, *Fusarium oxysporum*, *Fusarium verticillioidis* and *Fusarium moniliforme* are the most important species in the human infection context [61]. As with aspergillosis, the clinical form of *Fusarium* depends on the immune status of the host. Among immunocompetent hosts, keratitis and onychomycosis are the most common infections; therefore, in immunocompromised hosts, disseminated fusariosis is the second most common infection with filamentous fungus and affects especially patients undergoing therapy with high-dose corticosteroids with severe and prolonged neutropenia, in which a mortality rate up to 100% can be observed [61]. *Fusarium* spp. is also a major cause of microbial keratitis, and the formation of biofilms has been suggested as a contributing factor in recent outbreaks, especially associated with the use of contact lenses [62]. In addition to eye infections, *Fusarium* sp. is also commonly isolated as the causative agent of onychomycosis. In nails, fungal cells generally form thick biomasses, containing embedded elements in a fungal extracellular matrix [63]. Several factors, including the firm adhesion to the nail plate, the presence of "persister cells" and the difficulty of eradicating the infection, suggest that biofilms are an important factor in the pathogenesis of onychomycosis [64]. *Fusarium* biofilm formation on polyestyrene surfaces, contact lenses and over human fingernails has been demonstrated *in vitro* and *in vivo* [15, 65–67] and may possibly occur on other medical devices, contributing to the high virulence and mortality observed in invasive infections.

5. Biofilm resistance behavior

Currently, antifungal therapy is based on four major classes of antifungal drugs: the polyene agents, azoles, allylamines and echinocandins. However, the therapeutic arsenal is limited by several problems, including selectivity, toxicity and development of resistance. Considering invasive mycoses, options are even more restricting, comprising amphotericin B, fluconazole (with several restrictions), itraconazole and voriconazole being the most suitable drugs. Although amphotericin B is considered to be the gold standard drug for these infections, its high degree of hepatotoxicity and nephrotoxicity [68] may turn it unacceptable for most patients predisposed to invasive fungal infections. Furthermore, some *Candida* species such as *C. krusei* and *C. glabrata* show less susceptibility to azole agents, which can lead to a therapeutic failure and often to death of the patient [32, 33]. From a clinical standpoint, resistance is the persistence or progression of an infection despite adequate medical therapy [69].

Fungal infections associated with biofilm formation are often poorly susceptible or even refractory to conventional antifungal therapies, which implies the need for higher dosages—not always possible, as discussed above—or antifungal combination therapy for better penetration of drugs in biofilms. The ineffectiveness of the azole antifungals and classical formulations of amphotericin B (deoxycholate) against biofilms of *Candida* spp. was demonstrated by several groups over the past few years [70–72], whereas only the echinocandins and lipid formulations of amphotericin B showed good activity against biofilms of *C. albicans* and *C. parapsilosis* [71]. Similarly, *Cryptococcus neoformans* and *C. laurenttii* biofilms were resistant to all tested azoles (itraconazole, fluconazole and voriconazole) [73, 74], but were susceptible to amphotericin B [74]. Importantly, biofilms of *A. fumigatus* were resistant to both voriconazole and echinocandins (anidulafungin and caspofungin) in two published studies [57, 75], being amphotericin B the only available antifungal drug with demonstrated activity against *A. fumigatus* biofilms available for clinical use [75]. Finally, *F. solani* and *F. oxysporum in vitro* biofilms also showed reduced susceptibility to all tested antifungal agents, including amphotericin B, voriconazole, itraconazole and fluconazole [66, 76]. Thus, the current scenario shows the scarcity of drugs available for the treatment of invasive fungal infections derived from biofilms, which are increasingly frequent in the hospital environment and frequently associated with severe clinical conditions.

6. Biofilm mechanisms of resistance

According to the definition of a biofilm, the cells that compose this structure have an altered phenotype and differ from the planktonic cells (free-floating cells) in the expression of genes, rate of growth and also in its susceptibility to antifungal agents. The increased resistance to antifungals in *Candida* spp. grown as biofilms, in comparison to its planktonic forms, is the most medically relevant behavioral change associated to biofilms in the clinical setting [77]. Multiple mechanisms have been suggested to explain the increased antifungal resistance of the biofilm, including cell density, alteration of drug targets, expression of drug efflux pumps,

the extracellular matrix and presence of persistent cells [55, 77–81]. Each of these mechanisms will be addressed separately in the next paragraphs, in the context of our chosen biofilm model (*C. albicans*), and recent finds concerning other fungi will be inserted when appropriate.

7. Cell density

The biofilm architecture is highly ordered to allow the infusion of nutrients and waste expulsion. Mature biofilms, even having high cell density, exhibit spatial heterogeneity with microcolonies and water channels, common feature of both biofilm bacteria and fungi [55]. It has been shown that both planktonic cells and cells resuspended from biofilms exhibit sensitivity to azoles when the cell density is low (10^3 cells/ml) and became more resistant when cell density is increased ten-fold [78]. It is believed, therefore, that the cell density is an important resistance factor within complex biofilms, particularly to azoles.

8. Drug target alteration

The antifungal agents of the azole class, including fluconazole, itraconazole, voriconazole and posaconazole, act by inhibiting sterol 14-α-demethylase enzyme encoded by ERG11 gene. The main target of azoles, Erg11p protein, can develop point mutations or be overexpressed, reducing the drug activity and culminating in an ineffective treatment. Treatment of *C. albicans* biofilms with fluconazole induces upregulation of genes encoding enzymes involved in the ergosterol biosynthesis (CaERG1, CaERG3, CaERG11 and CaERG25), this feature being even more pronounced in biofilms exposed for longer periods (22 h). Yet, treatment of *C. albicans* biofilms with amphotericin B results in increased expression of CaSKN1 predominantly and a modest upregulation of CaKRE1 (both related to the cell wall) [82]. Upregulation of genes from the ergosterol biosynthetic pathway were also reported in biofilms of *C. dubliniensis* [83] and *C. parapsilosis* [84] and in a *in vivo* model of *C. albicans* biofilms using central venous catheters [85]. Additionally, the analysis of sterol composition of the biofilm cells of *C. albicans* has shown that the levels of ergosterol (the main sterol of fungal cell membrane) were significantly lower in the intermediate stages (12 h) and maturation (48 h) compared with the initial phase (6 h) of biofilm development [81]. Changing ergosterol exposition in the membranes of biofilm cells could explain their resistance both to azole agents as to polyenic, targeting the ergosterol molecule.

9. Drug efflux pumps expression

The primary molecular mechanism leading to resistance to the azoles, in *C. albicans*, is the increased efflux of the drug, mainly mediated by transporters from the ABC family and the MFS facilitators superfamily. The ABC transporters (ATP Linked), in *C. albicans*, constitute a

multigene family, which includes multiple genes CDR (CDR1-4). Among the MFS family members, whom are secondary carriers and use the proton motive force, the MDR1 gene encodes an important mediator, which has been implicated in the resistance of *C. albicans* exclusively against fluconazole [55]. Various antifungal agents may be substrates for these pumps, and, therefore, its overexpression can lead to cross-resistance between different drugs, particularly azoles.

The increased expression of genes encoding drug efflux pumps has been reported in *C. albicans* [77, 79, 81], *C. glabrata* [86] and *C. tropicalis* [5] biofilms. Interestingly, the expression of CaCDR1, CaCDR2 and CaMDR1 is differentially regulated during development of the biofilm and after its exposure to antimicrobial drugs [77, 81, 87, 88]. Using *C. albicans* single, double and triple mutants for the main efflux pump genes (Δcdr1, Δcdr2, Δmdr, Δcdr1/Δcdr2 and Δmdr/Δcdr1/Δcdr2), Mukherjee and colleagues (2004) demonstrated that 6 h after formation, biofilms of double and triple mutants were 4–16 times more sensitive to fluconazole than biofilms of the wild type, while the biofilm from all strains become highly resistant to this azole after 12 and 48 h of development [81]. The lack of involvement of efflux pumps in mature biofilm resistance has been previously demonstrated by Ramage et al., also using *C. albicans* strains [77]. Collectively, the available literature supports the hypothesis that efflux pump overexpression is an important, but not exclusive, determinant of fungal resistance to azoles biofilms and may play an important role in the initial phases of biofilm development. Their primary function may be to allow the first cells to establish within complex environments and to protect them from acute toxicity, thus ensuring the permanence of these cells and allowing the biofilm to start to grow [81]. In the clinical setting, early exposure to azoles can, then, increase the expression of efflux pumps in early-established cells and contribute to induce clinical resistance.

10. Role of the extracellular matrix of the biofilm in resistance

In most biofilms, the population of microorganisms corresponds to 10% of the total mass and the extracellular matrix (ECM) corresponds to 90%. The ECM is a key biofilm component, which exerts a physical barrier function, protecting the cells from environmental factors such as host immunity and antifungal agents [21]. In 2004, Al-Fattani and Douglas demonstrated that, although the diffusion of small molecules can be hampered by the presence of a dense ECM, reducing the penetration of antifungal drugs does not play a key role in biofilm resistance [89]. Recent studies have provided new insights suggesting that the chemical composition of the ECM and its regulation may play the central role in resistance.

The overall composition of the ECM of *C. albicans* biofilms was first characterized by Baillie and Douglas [90] and confirmed later by Al-fattani et al. [89]. Recently, an extensive analysis of the ECM composition of *C. albicans* biofilms was published, where proteins appear as the major component (55%), followed by carbohydrates (25%), lipids (15%) and nucleic acids, mostly e-DNA (5%) [91]. Nuclear magnetic resonance (NMR) of exopolysaccharide fractions detected three major polysaccharides, similar to those found in the cell wall, but in quite

different relative abundance. While β-1,3-glucan is the most abundant polysaccharide in the cell wall of *C. albicans* planktonic cells, the amount of β-1,3-glucan present in the ECM of its biofilms was surprisingly low. The most abundant polysaccharides are, actually, mannans and α-1,6-1,2-branching mannans, which appears to be associated with β-1,6-glucans, forming a glucan-mannan complex [91]. Much less is known about the ECM composition of biofilms of other *Candida* species and or fungi. Therefore, ECM may also resemble cell wall components in other species, as demonstrated for *A. fumigatus*, in which ECM is composed of galactomannan, α-1,3-glucans, galactosaminogalactan, monosaccharides and polyols, melanin and proteins [49].

The contribution of the β-1,3-glucan for the biofilm resistance in *C. albicans* was confirmed by a series of studies, which demonstrated that (*i*) the digestion of β-1,3-glucan residues by the addition of β-1,3-glucanase significantly improved the *in vitro* anti-biofilm activity of both fluconazole and amphotericin B drugs; (*ii*) the addition of exogenous ECM and/or β-1,3-glucan residues reduced the *in vitro* antifungal activity of fluconazole against *C. albicans* planktonic cells [92], resembling a biofilm-like behavior by the presence of the ECM. Also, β-1,3-glucan is responsible for sequestering all major drugs from the ECM environment, including azoles, echinocandins, polyenes and pyrimidines [93–95], behaving like a "drug sponge" and contributing to the increased resistance of the biofilm. A recent study published by Dr. Andes group (2014) upon this subject suggests that the most abundant polysaccharide in the ECM is not β-1,3-glucan (as previously thought), but a polysaccharide complex comprising an association of glucan-mannan residues, which is also capable of binding to fluconazole molecules and contributes to the resistance [91]. The work emphasizes that, most possibly, a large proportion of polysaccharides in the ECM may act as drug-sequestering molecules and contribute to biofilm resistance to antifungal agents.

In addition to polysaccharides, the extracellular DNA (eDNA) present in ECM of *C. albicans* biofilms also appears to have a role in resistance to non-azole agents. This feature was confirmed by Martins and colleagues, in 2010 and 2012, using DNase enzymes in association with antifungal drugs and confirming that destroying the eDNA with the enzyme led to an increased *in vitro* anti-biofilm activity of polyenes and echinocandins, but not azoles [96, 97]. Studies by Rajendran et al. have now also demonstrated that eDNA is also an important structural constituent of *A. fumigatus* ECM and plays an important functional role in maintaining the structural and architectural integrity of its biofilms. Furthermore, in this species, the release of eDNA by autolysis in biofilms is significantly associated with the levels of antifungal resistance, suggesting that eDNA plays an important role in *A. fumigatus* biofilm resistance to antifungals [98].

Other than physical components, transcription factors that regulate glucan synthesis and hydrolases are also associated with biofilm resistance. The CaZAP1 transcription factor is a negative regulator of the release of soluble β-1,3-glucan for the ECM in *C. albicans* biofilms. Yet, a group of alcohol dehydrogenases (CaADH5, CaCSH1 and CaLFD6) is associated with the production of ECM as they act as "quorum sensing" molecules, coordinating the maturation of biofilm [99]. In general, ECM production in *C. albicans* biofilms is highly regulated and is a key factor for resistance.

The ability to form *in vitro* biofilms containing ECM and its participation in the resistance has been described in other *Candida* species, including *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. dubliniensis* [35] and, also, in other fungi, such as: *Cryptococcus neoformans*, *C. gattii*, *Pneumocystis* spp., *Blastoschizomyces capitatus*, *Malassezia pachydermatis*, *Saccharomyces cerevisiae*, *Rhizopus oryzae*, *Lichtheimia corymbifera*, *Rhizomucor pusillus* and *Apophysomyces elegant*, *Rhodotorula* spp., *Aspergillus fumigatus*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Coccidioides immitis*, *Fusarium*, *Trichosporon asahii* and *Mucorales* (Revised in [17, 55]), corroborating the hypothesis that the ECM plays a critical role in fungal resistance and is one of the most significant mechanisms and regulated in the resistant phenotype of biofilm.

11. Persister cells

Persister cells are an important mechanism of tolerance in chronic infections and recently have received special attention in fungi biofilms [55]. By definition, these cells are "dormant variants of regular cells inside a microbial population that are highly tolerant to antibiotics" [100]. The main disruptive effect of antifungal agents in the cells relates to its interference with metabolic processes (synthesis of cell membrane, cell wall or DNA). The main characteristic of a "dormant" or "persister" cell is the reduction of its metabolism and cell division. So, because they are not metabolizing substrates and not dividing, these cells are no longer a target for the antifungal and become tolerant to its presence [100]. The presence of persister cells has been demonstrated in biofilms of *C. albicans*, *C. krusei* and *C. parapsilosis* treated with amphotericin B [101]. In an evidentiary study, re-inoculation of biofilm cells that survived the treatment with amphotericin B produced a new biofilm with a new subpopulation of persistent cells, suggesting that they were not mutants, but phenotypic variants of the wild type and adhesion on the substrate has triggered the formation of a persister subpopulation. Thus, in this clinically relevant scenario, inefficient and prolonged antifungal therapy may be beneficial for this subpopulation of the biofilm, which may be responsible for the ineffectiveness of the treatment and relapses [55].

In summary, the major studies published to date that attempt to elucidate the main factors involved in antifungal resistance of biofilms were performed with *C. albicans* biofilms and therefore, little is known about the specific resistance mechanisms for biofilms of other *Candida* species, or other biofilm-former fungi. It is likely that the ECM also acts as a barrier to the penetration of antifungal in those biofilms; however, as the ECM composition is different for each species, the role of the resistance to antifungal agents probably will not be the same. Likewise, the patterns of gene expression and sterol metabolism membrane will also be specific for each species.

12. Biofilm and pathogenesis

Pathogenesis involves the interaction between the pathogen and the host. To cause disease, the infectious agent makes use of both invasiveness factors—the pathogen virulence factors—

and the ability to resist and evade the host immune system. Often these two topics communicate, mainly because the molecules and metabolic adaptations produced by the pathogen to escape the immune response are considered as virulence factors.

The ability to grow as a biofilm cannot be considered a *classic* virulence factor, as the definition of virulence factor states that lack of the featured characteristic leads to non-virulent strains. Several fungi that do not form biofilm are still able to cause infection; however, those who do grow as biofilms are constantly linked to severe disease. Interestingly enough, a new molecule that impairs *C. albicans* biofilm formation does so by inhibiting the filamentation, an important virulence factor of this species. *In vivo* inhibition of filamentation and consequently biofilm formation depletes oral infection of immunocompromised mice [102]. This corroborates to the hypothesis that biofilm formation might be an important pathogenic factor and, thus, an important drug target.

The relationship between biofilm and pathogenicity relies mainly on two unique features of this community life-style: its increased resistance and the dispersion of infectious cells. Biofilms are a natural survival strategy of microorganisms to resist environmental threats [2]. In the clinical setting, the encased highly dense colony of fungal cells is protected not only from antifungal penetration, as discussed above, but also from the immune system. A single yeast or hyphae cell can be recognized and eliminated by the innate immunological response, either via phagocytosis by macrophages or induction of apoptosis by degranulation of mast cells. However, biofilms are too big to be phagocytosed and, yet, ECM may impair recognition of fungal surface epitopes. Thus, biofilm formation may also contribute to the escape from the host immunological response, favoring the establishment of the infection.

Candida mature biofilms (and possibly all fungi biofilms), after reaching a critical biomass, find a dynamic equilibrium in which the increase in cell density is offset by the release of superficial yeasts from the top, in a phenomenon called dispersion.

Cells that are released from mature biofilms are called "dispersion cells" and may colonize adjacent surfaces, expanding the biofilm or, in a clinically relevant scenario, use the bloodstream to disseminate the infection and allow the colonization of deep organs [103]. Additionally, *C. albicans* dispersion cells exhibit significant phenotypic changes and are more virulent than those grown as planktonic (non-biofilm) cells. Alterations include: increased adherence to polystyrene, significantly higher germ tube formation, which is important because filamentation is essential for *C. albicans* virulence, more robust biofilm formation and increased virulence in a murine model of disseminated candidiasis [103]. Therefore, when a catheter is infected with fungal biofilm, "dispersion cells" with increased virulence potential may gain access to the bloodstream and disseminate the infection.

Recently, a prospective analysis of patients with *Candida* bloodstream infection (BSI) performed in Scotland confirmed that biofilm formation is a risk factor for mortality in patients with disseminated *C. albicans* infection [42]. Several previous works also showed that removal of a catheter within the first 24 h of candidemia diagnosis improves the clinical outcome and results in a shorter duration of candidemia with decreased mortality [42, 104, 105], confirming that biofilms function as reservoirs and are directly correlated to the dissemination of the

infection. In fact, the current *guideline for the management of catheter-associated infections and their clinical management* states that, where possible, the catheter should be removed in non-neutropenic patients [23, 24, 106, 107].

13. Final considerations

The ability to form biofilms is widespread among pathogenic fungi, but understanding of the mechanisms that govern their formation, physiology and drug resistance is still limited. The continuous development of knowledge of the molecular mechanisms underlying biofilm formation, maintenance and molecular basis of metabolic dormancy of subpopulations of cells, such as persister cells, could lead to a drug-based strategy that could help us solve clinical diseases associated with fungal biofilms.

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