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REVIEW ARTICLE

Our current clinical understanding of *Candida* biofilms: where are we two decades on?

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Clinically we have been aware of the concept of *Candida* biofilms for many decades, though perhaps without the formal designation. Just over 20 years ago the subject emerged on the back of progress made from the bacterial biofilms, and academic progress pace has continued to mirror the bacterial biofilm community, albeit at a decreased volume. It is apparent that *Candida* species have a considerable capacity to colonize surfaces and interfaces and form tenacious biofilm structures, either alone or in mixed species communities. From the oral cavity, to the respiratory and genitourinary tracts, wounds, or in and around a plethora of biomedical devices, the scope of these infections is vast. These are highly tolerant to antifungal therapies that has a measurable impact on clinical management. This review aims to provide a comprehensive overight of our current clinical understanding of where these biofilms cause infections, and we discuss existing and emerging antifungal therapies and strategies.

Key words: *Candida*; biofilm; fungi; antifungals; yeast; Clinical microbiology.

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Directly or indirectly, biofilms are responsible for over 80% of all microbial infections (1–3), which can vary from superficial to more serious and deep infections, with high mortality rates associated (1,2). *Candida* species biofilms are among the most common microorganisms in clinical settings, being commonly found in patients' skin or on the hands of nursing staff (4–7), adhered to biomedical devices, growing as biofilms, capable of withstanding extraordinarily high antifungal concentrations (5,8). The first description of a *Candida albicans* biofilms from oral and urinary sources was made in 1981, and since then our overall appreciation and undertesting of them has improved (9).

The importance of fungi in human health cannot be understated, so much so that their impact has now been fully recognized by the world health organization (WHO) within their recent publication of priority fungal pathogens (10). Despite the lack of definitive data to demonstrate the burden of disease, some have estimated that over 1 billion people are affected by fungal disease, which in turns kills 1.5 million annually (11). Among these pathogens is *Candida albicans* that has been identified within the critical priority group, alongside *Aspergillus fumigatus*, *Cryptococcus neoformans* and *Candida*

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auris. Tens of millions are affected by mucosal candidiasis, and an estimated further 750 000 people with systemic candidiasis, of which the latter has mortality rates of around 50% (11). These statistics highlight the critical importance that candidiasis has in human disease. One of the critical factors in managing these infections is our ability, or lack thereof, to successfully diagnose these infections (12).

Notably, one of the key contributing factors to this burden of health is the ability of *Candida* species to form an aggregative biofilm phenotype upon mucosal surfaces, intimately attached to indwelling biomedical implants or as aggregates surrounding adjacent tissue to biomaterials (13). Biofilms may be present as mono-species consortia of yeast and hyphal cells embedded within polymeric matrix (14), but also as aggregates (or floccules) of cells (15). Fig. 1 illustrates morphological appearance of *C. albicans* biofilms grown *in vitro* upon polystyrene and polymethylmethacrylate, and *C. auris* grown on a cellulose matrix. More frequently they are co-associated with bacteria as interkingdom populations. Irrespective of their constituent parts, they are surprisingly recalcitrant to antifungal agents, and this tolerance makes them a significant clinical issue (16). This review aims to provide a detailed insight into the strides made in increasing our understanding of *Candida* biofilms over the past two decades ever since its mainstream acceptance as a clinical entity.

WHO ARE THE RISK GROUPS FROM FUNGAL BIOFILM INFECTIONS?

Those at greatest risk from these infections are those with weakened immunity or those with underlying health issues (17). This includes chronic lung disease, HIV, cancer, diabetes, and many other serious diseases. Those critically ill patients in the ICU, those undergoing invasive procedures and those receiving immunosuppressants or broad-spectrum antibiotics are all high-risk groups. Patients within these groups will inevitably continue to expand, especially as the world population grows past 8 billion inhabitants in 2022. Patients undergoing treatment for cancer, including immunotherapy and chemotherapy, a patient population that continues to advance at pace and will undoubtedly lead to more within these risk groups. We also observed the consequence of this during the COVID-19 pandemic laid bare, and the necessity to use immunotherapies and a range of supportive measures that result in co-morbid invasive fungal disease in this patient group (18). The critical care environment coupled with severely ill patients provided the perfect storm for biofilm-related disease.

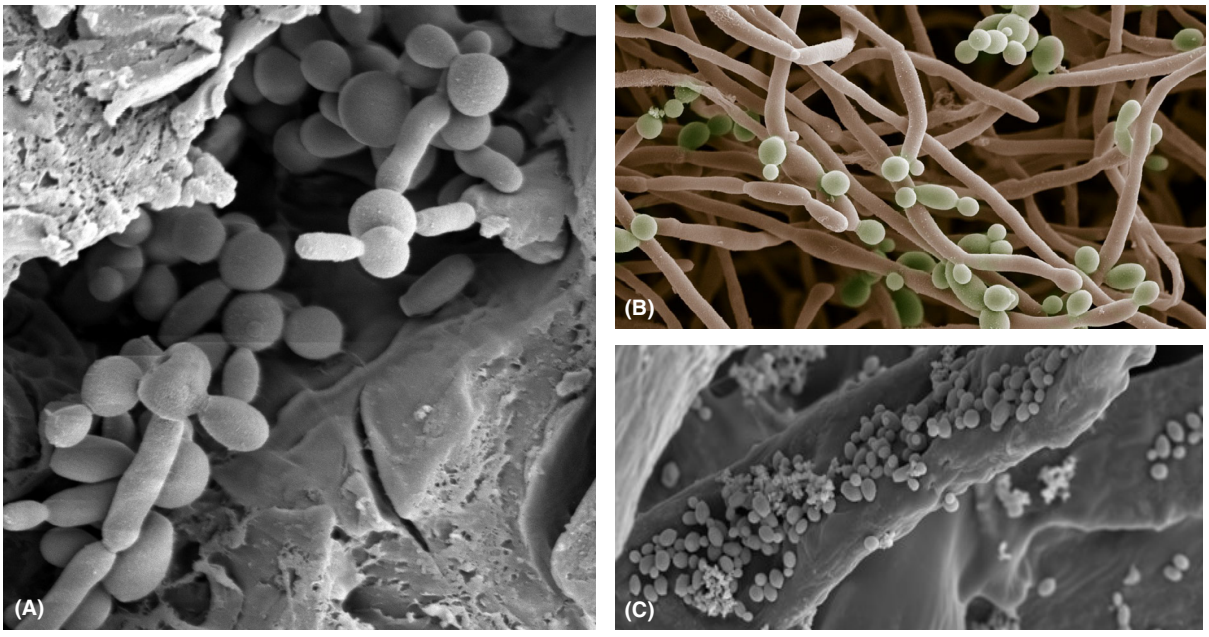

Fig. 1. *Candida* spp. biofilms on different surfaces. (A) *Candida albicans* grown on an irregular polymethylmethacrylate (denture prosthesis) substrate. (B) *Candida albicans* grown on polystyreme and pseudocoloured green blastospores and orange hyphae. (C) *Candida auris* grown on a cellulose matrix – note the dominant yeast morphology.

Biofilm-related infection plays an additional roles in patients with any form of biomaterial, for example prosthetic heart valve, total hip arthroplasty, knee joint, presence of an indwelling venous or urinary catheter, artificial lens, cochlear implants, etc (13). Moreover, the risk of biofilm-related infection is increased in patients with wound-related trauma, which may be disease related (*e.g.* diabetic ulcers), or in the form of burns or trauma (19). Biofilms can also exist out with the patient, adhering to fomites and medical equipment around the clinical environment (20). For example, *C. auris* was shown to persist as a resilient yeast and spread rapidly throughout a critical care ward in the UK (21).

Collectively, this paints a particularly gloomy outlook for an ageing population who will increasingly rely on these medical interventions and be exposed to challenges brought about by innovative immunotherapies. Whilst the relative risks of biofilm-related infection remain stable, the increasing population profile means more and more patients will be exposed to these hard-to-treat infections. With a limited arsenal of antifungal agents available for clinical use, the successful management of these patients is challenging. Table 1 illustrates the breath of risk factors posed by an increasing population.

CANDIDA BIOFILMS ARE IMPORTANT IN SUPERFICIAL AND DEEP INFECTIONS

The mucosal barriers of the oral cavity, oropharynx, respiratory, gastrointestinal, and genitourinary tracts are all potential sites for the genus *Candida* to reside, colonize and potentially initiate pathogenesis. Alongside an exhaustive list of 'who's who' among the human microbiome (22), *Candida* species have the capacity to either co-aggregate, co-exist or be antagonized by bacteria in both yeast and hyphal forms. Notably, *Candida* spp. appear to preferentially interact as innocent bystanders in these relationships (23). Irrespective of these inter-kingdom relationships, *Candida* spp. have been shown within the most accessible of these clinical sites (*i.e.* the oral cavity and vagina) to have the capacity to form biofilms that have the clinical appearance of white patches, or pseudomembranes (24,25). Beyond this they have the capacity to hijack wounds, catheter lines and indwelling devices to gain systemic access, and to cause debilitating and life-threatening infections (13,26), some of which are now discussed. Fig. 2 provides a schematic overview of the breadth of possible *Candida* spp. biofilm infections.

Table 1. Risk factors influencing candidal infections

Local	Systemic
Indwelling prosthetic devices	Broad spectrum antibiotic treatment
Xerostomia	Immunosuppressive therapy or condition *e.g.* Organ transplant
Dentures	Genetic susceptibility
Burns	Cytotoxic chemotherapy
Trauma	Radiotherapy
Wounds	Human Immunodeficiency Virus (HIV)
Vaginal douching	Hyperglycaemia
Contraceptive pills	Pregnancy
	Infections such as tuberculosis
	Chronic renal failure
	Nutrition *e.g.* Iron, folate and vitamin C, B, A
	Impaired liver function
	Steroid use

Oropharynx

Candidal infections of the oral cavity are mainly opportunistic in nature, and frequently co-aggregate with microbial species in the form of biofilms on biological and inert substrates, or as aggregates within saliva. Oral candidiasis (candidosis) are generally superficial infections (27), a result of the overgrowth of mainly *C. albicans*, though other non-*albicans* species, *Candida dubliniensis*, *Candida krusei*, *Candida parapsilosis*, *Candida stellatoidea*, *Candida glabrata*, *Candida tropicalis*, and *Candida guilliermondii* contribute to oral candidiasis, but to a lesser extent. Within the oral environment these yeasts coalesce upon mucosal surfaces and give the clinical appearance of thick white plaques. Microscopically, these appear as mixtures of yeasts and hyphae intertwined and covered thoroughly by a glucans matrix, a substance shared by the genus (28). Moreover, this glue-like material supports architecture and tolerance within an interkingdom biofilm (29).

Diagnosis of oral candidiasis is usually first based on a clinical presentation, followed up if necessary with histopathological examinations of the infected tissue (24). Routinely, oral swabs and rinses are used for microbiological analysis, with microscopy being particularly useful for detecting the presence of *C. albicans* hyphae, a useful biomarker for differentiating against azole-insensitive yeast such as *C. krusei* and *C. glabrata*. This is an important factor in empirical treatment of these diseases (24). These procedures can diagnose *Candida* species in pseudomembranous candidosis, angular chelitis and denture-induced stomatitis (DIS). DIS differs from these other infections as the biofilm tends to reside on the denture-fitting surface (30), and its intimate

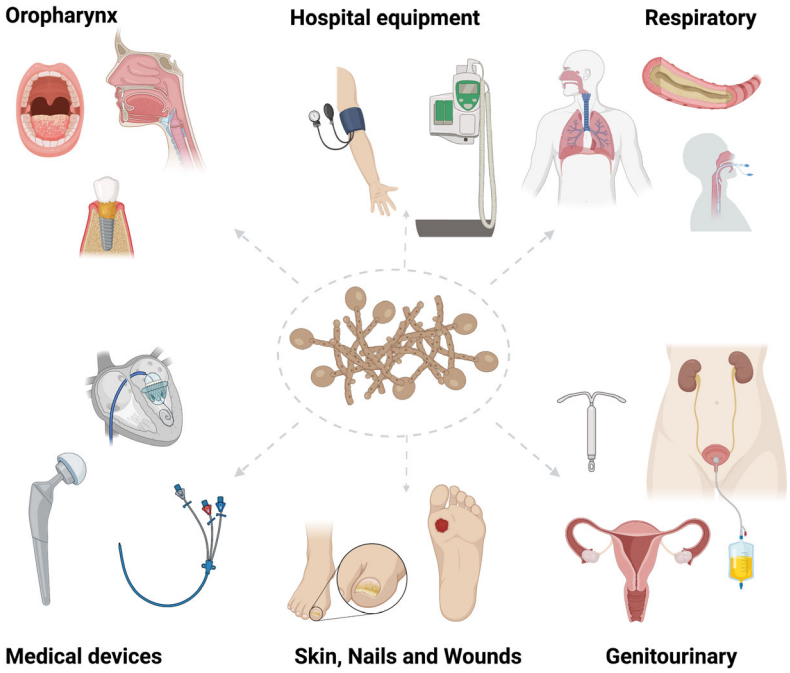

Fig. 2. Clinically important sites where *Candida* biofilms are known to be problematic.

association with the palatal surface results in inflammation. Here swabs of the tissue and denture are important, but also consideration of the sonication of the denture to maximize quantifiable bioburden (31), a technique first optimized in prosthetic joint biofilms (32). Our studies have shown that *Candida* species play an important role in these biofilms as resilient cells within interkingdom biofilms, but that bacteria occupy the biofilms by up to 2 logs greater than yeasts (33). This has an impact for the consideration for therapeutic control, though it is clear that frequent daily denture cleansing extra-orally is the most effective preventative strategy (34).

Other prevalent oral chronic biofilm diseases in humans are dental caries and periodontal diseases, both of which are considered primarily bacterial driven diseases (35,36). However, the role of yeasts within these diseases is often overlooked and widely disregarded despite the presence of yeasts in saliva. Elevated levels of *Candida* species have been detected in children with caries (37), though whether they are directly associated with dental caries remains unconfirmed (38). Their presence may be indicative of disease rather than directly causality (39). Similar detection rates have been reported in patients with periodontal diseases, much higher than in healthy patients, and shown to correlate with disease severity (40–42). This is somewhat confirmed in what limited data exist within a recent systematic review from 21 available studies (43). Taken together, and until proved otherwise, it would appear that we observe elevated of *Candida* levels in these biofilm diseases as a consequence of microbial dysbiosis and host derived factors, though we cannot exclude their indirect effects contributing to pathological processes (23). Indeed, we know that that key periodontal pathogens are pathogenically primed on encountering *C. albicans* (44).

The oropharynx is another important site for *Candida* biofilms, especially in those with a voice prosthesis (45). This is commonly associated in patients with a laryngopharyngeal malignancy that need to undergo a laryngectomy, which can result impact air control, swallowing, phonation, and coughing. The rapid colonization of silicone voice prostheses by resident yeasts leads to device failure and the need for removal, as these have a lifespan of 4–6 months (46). This is important as these microbes have the capacity to deteriorate silicone materials if unmanaged, though there are possibilities to coat with antimicrobials and prevent this biodegradation (47).

Respiratory tract

The proximity of the lungs to the oropharynx makes microbial spread to the respiratory tract possible, often facilitated by endotracheal tubes (48).

The trachea can be colonized by *C. albicans* in critical care environments, which has significant biofilm-related implications for patients intubated with endotracheal tubes that require respiratory assistance (49). The lungs, an organ commonly associated with biofilms, are therefore an important site for *Candida* species to reside. However, patients with biofilm-associated diseases, such as bronchiectasis, cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) have also shown to have a fungal aetiology (50,51). The most notable of these is *CF*, an autosomal recessive condition that is characterized by excess mucus production plugging the airways, infection, and chronic inflammation. Fungi are frequently cultured, yet bacteria remain the most common causative agent of *CF* infections (52). The most commonly isolated yeast from up to 75% of patients is *C. albicans* (53), and when co-isolated with *Pseudomonas aeruginosa* can worsen clinical outcomes in terms of forced expired volume (FEV1) (54). However, whether *C. albicans* within these biofilm aggregates is considered as a colonizer opposed to active pathogen remains to be ascertained (50). Though it would be prudent not to simply disregard its isolation, and instead perhaps consider the implications of its presence when deciding on antimicrobial management?

Genitourinary tract

Superficial biofilm infections are also frequently reported in woman with recurrent vulvovaginal candidiasis (RVVC). It is estimated that up to 75% of women will suffer from at least one episode of vulvovaginal candidiasis (VVC) during their childbearing years (55), with almost 10% of these women are expected to develop recurrent VVC (RVVC) (56), which is defined as three or more episodes within 1 year (57). Symptoms are on average ~7 years with a definitive diagnosis in 73% of women (58). These women often experience failed azole treatment, as definitive yeast identification is limited, and this impacts the ability to treat azole insensitive yeasts such as *C. glabrata* (59). This is also coupled with the ability of *C. albicans* to form interkingdom biofilms in this environment, which is the causative organism in up to 90% of VVC episodes (60). Some authors argue against the presence of these biofilms in this environment, and state that VVC is a result of polymicrobial invasion of vaginal tissues (61,62). However, there is unequivocal evidence that *C. albicans* biofilm formation on vaginal mucosa in a murine model of VVC, which has been visualized using scanning electron and confocal microscopy (63). This is supported by imaging from the swabbed mucosa of patients with RVVC, where intertwined hyphae are observed as biofilm aggregates (60). Nevertheless, there are no specific large-scale studies analogous to those demonstrating the biofilm capacity of *Gardnerella vaginalis* in bacterial vaginosis, which still creates an element of doubt for clinicians in treating RVVC (64). We are also limited with representative biofilm models of the vaginal environment during VVC to study potential *Candida* biofilm formation, though innovative pre-clinical models are available (65). These approaches are essential in providing important knowledge of the pathogenesis and tolerance of yeasts in RVVC, which could support more effective treatments that simply relying on azoles that will eventually fail. Fluconazole remains the primary treatment for VVC owing to its high cure rates and availability at clinics as well as over the counter (66,67).

Candida spp. biofilm are also important in intrauterine devices (68), where removal of the device is often seen to correlate with improvement of clinical symptoms (69). Experimental studies have shown a wide variety of *Candida* spp. retain the capacity to adhere to intrauterine device, particularly the tail end (70). Other inserted materials, such as urinary catheters have also been shown to support *Candida* colonization, that may lead to urinary tract infections (UTI's) (71). In general, removal of these devices and antifungal therapy is the optimal strategy, as these could lead to candidemia (72).

Skin and wounds

It has become apparent that complex biofilm communities of bacteria and fungi can flourish on the skin and in wounds (19,73,74). One of the first mycobiome studies by Oh et al. (75) investigated the biogeography of the human skin and reported that mycobiome constituents made <10% of the total microbial population. Fungal levels vary between different sites, with the yeast *Malassezia* being the most prevalent fungal species on the skin, making up to 80% of the total skin associated fungi (75,76). Alongside these, *Trichosporon*, *Rhodotorula*, *Cladosporidium* and *Candida* species are also observed (19,73,77). It is noteworthy that dermatophytes, which affect up to 1 billion people (11), are able to form biofilms on keratin substrates such as nails (78).

Given their presence and pathogenic capacity, then it is unsurprising that fungi are important in chronic wound infections. While *Candida* is unlikely to play a significant role in these complex infections, it is frequently identified (19,79). Indeed, in culture-based studies it has frequently been

identified in diabetic foot ulcers (73,77). Over three quarters of the species isolated were *Candida* species (10.6% *C. albicans*, 22.7% *C. tropicalis* and 25% *C. parapsilosis*) (77). These species were also reported by Dowd and colleagues (73), suggesting an unrecognized importance of fungi in these clinical sites. Indeed, it has been shown within a randomized controlled trial that fluconazole treatment reduces the mean healing time of DFUs (80). Pioneering next generation sequencing studies from Kalen and colleagues (2018) has further shown the importance of fungi in wounds, where ITS1 sequencing enabled detection of *C. albicans* from 22% of patients (19). Taken together, these data show that *Candida* spp. play an important accessory role in wound infections, and that by considering them as an important structural element of the complex wound biofilm, and reciprocally using antifungals as an adjuvant alongside antibacterial agents will support successful clinical management. Indeed, we have shown that in an experimental triadic model containing *C. albicans*, *P. aeruginosa*, and *S. aureus* that only triple therapy targeting each component will successfully reduce the overall bioburden (81).

Medical device-related infections

It is reported that approximately 60–70% of all hospital-based infections can be accounted for by direct contact with implanted medical devices (82). Biofilm-related infections are a critical issue for these devices, from which a vast range of indwelling biomaterials that have been associated with fungal biofilm colonization (13,83). Prosthetic joint infection (PJI) is a significant complication to an otherwise ordinarily successful procedure and presents a significant issue for post-clinical management when fungi are present. In a recent review of fungal periprosthetic joint infections comprising of 89 patients, *C. albicans* was the most common clinical isolate (49.4%), followed by *C. parapsilosis* (18%) and *C. glabrata* (12.4%) (84). In another meta-analysis from 2009 to 2019 it was reported that 286 patients had a fungal periprosthetic infection of the knee, hip, shoulder, or elbow. *Candida* spp. were the most identified fungal pathogen (85%), with 30% of these being dual-species interkingdom infections. Notably, the use of antifungal spacers with a two stage revision was required in 65% of cases (85). A critical consideration for PJI and for other wounds, either trauma-induced or otherwise, is that *Candida* spp. and other fungi have access to bone. There is clear evidence, which is subject to an excellent review by Gamaletsou et al. (86), that biofilm infections are key elements in osteoarticular mycoses. These are both difficult to diagnose and treat, and often require surgical intervention.

Within critical care there are a myriad of indwelling lines where adherent candidal biofilm communities can thrive, detach, and cause a fungemia by spreading throughout the human body. Indwelling medical devices, such as intravascular catheters and ventricular-assist devices (VADs) are commonly colonized with *Candida* spp. (87,88). Clinically, unless swift diagnosis to treat a *Candida* infection in the ICU is given in the first 24 h, then this can lead to a 30-fold increased likelihood of mortality (89). Here, the biofilm phenotype is an important determinant in patient outcomes. We and others have highlighted how the presence of a biofilm forming isolates positively correlates with mortality, and that catheter removal or the use of highly active anti-biofilm therapy, *i.e.* liposomal amphotericin B or an echinocandin, can lead to a clinical improvement (90,91). Indeed, a recent meta-analysis of bloodstream infection and biofilms demonstrated that *Candida* spp. were the most associated compared to all other microbes analysed (92).

Candida biofilms are everywhere!

Collectively, it can be summarized that *Candida* spp. form biofilms across a large clinical spectrum, on any available substrate. There is evidence from the emergence of *C. auris* that yeasts can also form resilient populations beyond the host upon hospital surfaces (21,93), such as reusable skin temperature probes that can facilitate spread within a clinical environment (94). The biofilm phenotype of this yeast and others indicates the clinical impact of candidal biofilms is not insignificant (95). Although these are often overlooked and under-diagnosed, it is reassuring that the International Consortium for Osteoarticular Mycoses specifically identified fungal biofilms as an important clinical element for consideration in their review of the subject area (86).

CURRENT ANTIFUNGAL APPROACHES TO MANAGING *CANDIDA* INFECTIONS

Generic treatment of Candida

The management of *Candida* infections is generally driven by applying IDSA guidelines (96), as the protocols for the management of *Candida* infections have not markedly changed. It is relevant to note that these guidelines do not have in account if the infection is biofilm mediated, meaning that the treatment is the same for both planktonic and biofilm cells. Treatment of systemic diseases (invasive candidiasis/candidemia) focus mostly on

echinocandins (first-line drugs) and polyenes (amphotericin B) with step-downs with triazoles (or polyenes); local infections (*e.g.* oral, vaginal) have indications to be first treated with triazoles (*e.g.* fluconazole, voriconazole) and polyenes. In severe cases, polyenes or echinocandins can be first choice (not common and less recommended). The use of antiseptics, as co-adjuvants, in all cases, is also recommended. These protocols are just a generic guideline, and do not consider the individual variation among patients. Their application also depends on the *Candida* species involved in the infection (for example, if it is a *C. glabrata* or a *C. parapsilosis*, the use of an echinocandin should be used cautiously). Table S1 summarizes the protocols most frequently employed to manage *Candida* spp. infection in general.

Azoles, including fluconazole and voriconazole, remain the antifungal of choice for treatment for *Candida* spp. with exception of a few azole resistant species *C. krusei*, *C. auris*, and *C. glabrata*. These compounds are fungistatic through targeting of the ergosterol biosynthetic pathway. They work on the 14-lanosterol demethylase enzyme pathway, depleting the biosynthesis of ergosterol molecules in the cell membrane, and lead to accumulation of sterol precursors (97). Cellular membranes become unstable, leading to impaired growth and a static outcome. Triazoles are the most frequently used, and this can lead to resistance through upregulated efflux pumps, alterations in the ergosterol biosynthesis pathway, and activation of heat shock proteins, is common. Though biofilm mediated tolerance is not strictly induced by azole misuse.

Polyenes, including amphotericin B (AMB), nystatin and liposomal formulations, are an alternative fungicidal option. These insert into the lipid membrane adjacent to ergosterol and form pores, leading to destabilizing the cell membrane enabling cellular lysis (98). Oxidative stress may additionally contribute to its fungicidal activity. Whilst resistance is infrequent due to its membrane-based target, alterations to sterols and anti-oxidative stress mechanisms can protect the cell from polyene, in addition to cell wall changes, *example* enhanced 1,3-alpha- and 1,3-beta-glucans. Liposomal formulations are highly effective against biofilms (99).

Echinocandins, including caspofungin and micafungin, are fungicidal by virtue of inhibiting 1,3-beta-glucan synthase that facilitates cell wall destabilization. They can be considered analogous to penicillin interfering with peptidoglycan in bacteria. They have a wide spectrum of activity, though with an apparent paradoxical effect against *C. albicans* biofilms (97). Their overuse has led to echinocandin resistance through alteration of the glucan synthase enzymes (Fks1-Fks2 complex), changes in chitin composition and stimulation of stress pathways. These were the first class of compounds that were shown to be effective against biofilms and have contributed to the success of caspofungin (100).

The new pipeline of antifungals: Prospects for biofilms?

There is a renewed optimism in the management of fungal infections as new antifungals emerge into clinical use (101). However, a caveat to this is that although there is currently advanced development of novel agents, and a series of clinical trials in progress, the number of antifungal drugs that has been approved by the Federal Drug Administration (FDA) is currently limited to a few. Indeed, the last approval was for oteseconazole in early 2022, which is an azole indicated to reduce the incidence of RVVC (females not of reproductive potential) (102). Ibrexafungerp, a first-in-class oral triterpenoid (101,103,104), has been used for the treatment of adult and post-menarchal paediatric females with VVC (and RVVC – FDA label revision expected soon) (105). Also, the novel echinocandin resafungin (designed to be dosed once weekly) (101), has recently been designated a qualified infectious disease product (2022) by the FDA (106). Rezafungin was also granted the "orphan drug" title for the treatment of invasive candidiasis and candidemia in both the USA and EU (106). Ibrexafungerp and rezafungin target beta-glucan synthase pathways. Importantly, they have shown to be effective alternatives in controlling *C. auris* biofilm formation (*in vitro* and *in vivo*) (26,101,104). Finally, in experimental phase, there is fosmanogepix (PF-07842805) for the treatment of candidemia and/or invasive candidiasis, acting as a prodrug mangopix to target Gwt1 (glycosylphosphatidylinositol anchored wall protein transfer 1), an essential enzyme in cell wall (101,107,108). Together, these new agents offer promise for managing candidal disease, though there is limited data on how these behave against biofilms.

It goes without saying that *Candida* spp. biofilms have high levels of tolerance to the most used antiseptics or antifungal agents (109–111), so finding alternative strategies for managing them are as equally attractive to augment new FDA approved antifungal drugs. Recent approaches include photodynamic therapy (112,113), naturals from plant essential oils and extracts (114–116) and honey (117,118), the use of probiotics (111,119,120) and prebiotics (121,122), marine compounds (123) and the development of novel compounds as antifungal drugs or immunotherapies (124–126) or the search

for possible new drug targets (127,128). Drug repurposing (drug reprofiling, repositioning, or re-tasking) libraries, is an additional strategy we can employ. Studies of antifungal library screens were the first to identify the antidepressant sertraline (129) and antibiotic polymyxin B (130) with antifungal properties. Moreover, a screen of the FDA-approved Prestwick chemical library identified suloctidil and Ebselen as effective compounds against *C. auris* (131). Most recently it was shown that Toyocamycin and Darapladib showed promising activity against *C. albicans* and *C. auris* biofilms (132). However, to date, none of the compounds proved to have antifungal activity in libraries screening reached clinical settings (133).

ANTIFUNGAL CONSIDERATIONS FOR THE MANAGEMENT OF BIOFILM INFECTIONS

Oropharyngeal candidiasis

Topical azoles and polyenes in the form of oral suspensions, gels, creams, lozenges and ointment are usually used to manage oral candidiasis. Miconazole and AMB (nystatin) can also be used, both of which are fungicidal (134). Recurrent or refractory infections are not uncommon and usually require the use of systemic antifungals, such as fluconazole, itraconazole, ketoconazole, and AMB in conjunction with topical agents to control the infection (135). Antifungal resistance remains a serious concern with classical azole therapy, so drug combinations may overcome drug resistance. With β-1,3-D-glucan of fungi being an ideal drug target, combining drugs that act on this essential cell wall component will potentially help in resolving antifungal resistance. Oral ibrexafungerp (SCY-078) is a semi-synthetic potent β-1,3-D-glucan synthases inhibitor, shown to be effective against *C. albicans*, C. *parapsilosis*, *C. tropicalis* (136).

Respiratory tract

Distinguishing between colonization and active infection make it difficult to unequivocally advocate the treatment of *C. albicans* in the airways (50), even though there are reported associations between *Candida* colonization and declining FEV1 in CF patients (53,54,137). It is though that bacterial-fungal interactions may be one reason for this, with the lungs being collaterally damaged (54,138,139). Therefore, should *Candida* colonization be addressed in order to improve patient outcomes despite there being not a generally accepted treatment option for *C. albicans* in CF? Azole intolerance from *Candida* biofilms is a significant issue, therefore the use of polyenes or echinocandins may be a consideration (140). Beyond this, the new echinocandin rezafungin may be a viable option, where promising effects have been shown against *Candida* spp. and *Pneumocystis* spp. in animal experiments (141).

Another pre-clinical compound worth consideration is aureobasadin A, which inhibits inositol phosphorylceramide synthase, an enzyme involved in spingolipid synthesis. This has activity against both planktonic and biofilm *Candida* species (142). Moreover, T-2307 is a novel arylamidine in phase 1 clinical trials, which causes mitochondrial membrane collapse, has been tested against *Candida* spp. and was shown to be more effective than fluconazole, micafungin and AMB (143,144).

Recurrent vulvovaginal infection

Treatment for RVVC generally requires prolonged azole therapy, which is often unsuccessful. We know that fluconazole treatments are ineffective against *C. albicans* biofilms (26), suggesting their formation could contribute to failed clinical treatment. Treatment for RVVC caused by azole-resistant *C. glabrata* involves a 2-week daily treatment with nystatin pessaries or boric acid (145,146). Alternative treatments include a 2-week daily topical 17% flucytosine administered alone or in combination with 3% AMB (147). Although these suppressive therapies are often sufficient to relieve symptoms and a re-emergence of the infection whilst undergoing treatment, RVVC can flare up and patients may require long term treatment (26,148,149). Long-term use of azoles can drive antifungal resistance in *Candida* (58); however, if treatment options remain limited for women with persistent RVVC, this is inevitable.

Ibrexafungerp has potential for the treatment of RVVC in the presence or absence of biofilms (150). This orally administered triterpenoid glucan synthase inhibitor, with tolerability and low toxicity (151), has shown efficacy against a range of *Candida* species, including azole and echinocandin-resistant isolates (152). There is added value in that it can inhibit *C. albicans* and *C. glabrata* biofilms, albeit that to date it has only demonstrated *in vitro* (153). The topical echinocandin CD101 has also significant promise against azole-resistant fungal species in RVVC (154). Probiotics may also be a desirable approach to management of RVVC (155,156).

Wound management

The standard of care for wounds is initial physical debridement of the tissue, which facilitates removal

of the biofilm from the infected area. Much of clinical practice is focussed on empirical antibiotic therapy to manage polymicrobial bacterial infection (157). Guidance published by the International Working Group on the Diabetic Foot (IWGDF) advocates initial treatment based on "likely or proven causative pathogens" (158). In antibiotic naïve patients, depending on severity, early therapy often consists of flucloxacillin treatment, and combined with metronidazole for more moderate to severe infections. Ciprofloxacin is recommended in severe cases particularly when DFUs are accompanied by osteomyelitis (159). Follow up targeted therapies may be required based on culture and susceptibility results and depending on how the patient's clinical response (157). Notably, antifungal therapeutics are not commonly recommended for DFU despite our knowledge that fungi can be a key part of these biofilm.

Despite the fungi being disregarded in wound care, there are a few clinical and preclinical studies worthy of a mention. It was shown that antifungal treatment from a combination of fluconazole, flucytosine, itraconazole and terbinafine, resulted in an improvement in wound healing in antibiotic unresponsive DFU patients (160). Similarly, inclusion of oral fluconazole alongside a standard package of care in 38 patients with DFUs resulted in faster healing than those that received standard care alone (80). Preclinically it has been demonstrated that antifungals incorporated into polymer microparticles or calcium sulphate beads can be used to effectively control fungal growth within an *in vivo* murine model of cutaneous aspergillosis (161) and against a wide range of fungal isolates, including *C. auris* (162).

Medical devices

The clinical management of medical devices is a vast constitutes a full review in itself. However, in general, and where possible, the removal of devices is the mainstay of treatment. Devices that are easily removed include catheters, lines, and oral prostheses. Whereas implanted materials such as those associated with bony interfaces (hip and knee prosthesis), heart valves, artificial breasts, etc, require removal and can be problematic and costly (13). The management of these infections maybe supported with antifungal agents, which for echinocandins and liposomal polyene formulations may preclude the need for surgery (86). Azoles may provide the opportunity to slow the progression of infection, though are unlikely to lead to the resolution of infection without additional surgical debridement or augmentative antifungal strategies. Additionally, in PJI there is a need for moisture stability and void-filling within the surgical site (163). Here there is potential for the localized release of antimicrobial agents to areas of compromised vasculature using drug-loaded calcium sulphate (162). Higher effective doses of antimicrobials can be achieved than would ordinarily only be possible through a systemic route. This approach supports the prevention of biofilm formation at the biomaterial surface, which could be enhanced by changes to surface topography and electrostatic charge (164), which may significantly diminish adhesion and colonization (165,166). Nanotopographical alterations to surface structure have been demonstrated that could significantly decrease yeast adhesion, paving out a promising strategy for implanted biomaterials (167). Other innovative strategies include the use of probiotically produced biosurfactants for treatment of *Candida* driven infection of prostheses (168,169).

As stated above, in bloodstream infection there is unequivocal evidence for the use of echinocandins and liposomal formulations of amphotericin B for managing central line-associated candidaemia through clinical and preclinical studies (90,91,99). Moreover, it has been shown from a series of case studies that liposomal amphotericin B could be used a line lock solution in the prevention and management of fungal line infections (170,171). These approaches remain limited is due to clinical apprehensiveness of fungal line infection management, apart from line removal. Through the continued exploration of different approaches using animal models may offer scope for improving clinical management.

INNOVATIVE AND ACCESSIBLE ANIMAL MODELS FOR THE STUDY OF *CANDIDA* BIOFILMS

Animal models are widely used in the research of biofilm-associated infections and contribute significantly to understanding the pathogenesis of medical biofilms and investigating control strategies. Clinically relevant models are indeed crucial to study aggregates and/or biofilm-like structures in animal tissues or the interplay between the fungal persistence and host immune response. Both vertebrate (*e.g.* Zebrafish, rodents) and invertebrate models (*e.g. Drosophila melanogaster*, *Caenorhabditis elegans*, and *Galleria mellonella)* have been applied to candidal biofilm studies, each of them having advantages and disadvantages (172). There are a significant number of studies using mammalian animal models, such as the rat indwelling catheter

model (8) and a rabbit catheter model (173), in addition to porcine wound models (174). While these are all immensely useful, they are costly and can prohibit progress in evaluating new innovative therapies. Therefore, other more practically amenable models are available and will be briefly discussed.

Invertebrates lack the adaptive immune response but display a fully developed innate immunity that shares several features with the mammalian one (175,176), including physical barriers (cuticle/skin and midgut/intestinal microvilli) and two closely interconnected components, namely the cellular and humoral responses (177). Besides common traits, each invertebrate model has specific characteristics, such as infection susceptibility and route, maintenance conditions, and standardization tools. Choosing the most appropriate is key to having consistent results, as none can fully recapitulate the mammal host. Nevertheless, several authors reported a good concordance in pathogenicity between mouse and invertebrate models (178,179), suggesting such mini-hosts can bridge the gap between *in vitro* assays and *in vivo* vertebrate studies, in agreement with the three Rs principle (Replacement, Reduction, and Refinement) to reduce animal infection experiments with vertebrates.

The worm, *Caenorhabditis elegans*

C. elegans are hermaphroditic nematodes hugely reported as a useful model for studying host–pathogen interactions because of their completely sequenced genome. According to planned experiments, a variety of *C. elegans* strains can be obtained from the C*aenorhabditis* Genetics Center (CGC) at the University of Minnesota (MN, USA), and can be easily maintained in the lab. As they have a rapid generation time and a transparent cuticle fungal colonization, filamentation and biofilm formation can be easily appreciated and investigated (180). *C. elegans* has been also optimized for the high-throughput screening of strain mutants and new antifungals (122), and has been recently used to study cross-kingdom, *that is Candida albicans-Pseudomonas aeruginosa* interactions in polymicrobial biofilms (181). Major limitations to *C. elegans* use are its growing temperature, ranging from 15 to 25 °C, not allowing microbes to fully express temperature-dependent virulence traits, the route of infection (by ingesting pathogens) and the inability to recover infected tissues for histology and fungal load determination (182).

The fruit fly, *Drosophila melanogaster*

Toll signalling is crucial in fungal infections, and studies in *D. melanogaster* were cornerstones for its discovery (183,184). *Drosophila* are insect belonging to the Diptera order, with separate sex and a short generation time (10–12 days). The fruit fly, sharing pros (*i.e.*, a fully sequenced genome) and cons with *C. elegans* as an animal model for fungal biofilms, is a reliable tool for studying treatment options and for elucidating genes involved in biofilm-formation and pathogenesis (185,186). Although most applied to bacterial biofilms, *D. melanogaster* has recently proposed as a convenient model for the emergent yeast *Candida auris* (186). This model has proved useful in demonstrating the importance of antigen I/II in mediating the interaction between *Streptococcus mutans* and *C. albicans* to facilitate colonization in this model (187). It has also been used in another co-infection model to show how the phytochemical plumbago could effectively improve survival in a classic co-aggregate biofilm model of *Staphylococcus aureus* and *C. albicans* (188).

The greater wax moth, *Galleria mellonella*

The use of *G. mellonella* larvae for the study of fungal pathogenesis (reviewed in (189)) has been introduced by Kavanagh and co-workers in 2000 (190). Since then, many researchers explored this insect as a surrogate *in vivo* model. Compared with the better-known *C. elegans* and *D. melanogaster*, *G. mellonella* can survive at a temperature ranging from 25 to 37 °C and can be systemically infected by syringe-injecting pathogens into the hemocoel. The direct injection of pathogens allows for a controlled inoculum and a better standardization of the infection conditions. Being 2–3 cm long, last-instar larvae are easy to manipulate, and infected tissues can be collected for further analyses (191).

Different cellular and humoral immune responses to planktonic and sessile fungi and tissue invasion could be highlighted in recovered larval tissues (192,193), and by administering the drug after the pathogen injection, newer anti-biofilm strategies can be tested (194,195). We have used this model to evaluate acetylcholine as a potent biocontrol agent of *C. albicans*, where it was shown to successfully protect and improve survival (196). It has also been used to assess and screen biofilm mortality potential from stratified groups of biofilm formers (59,197,198). Recently, a *G. mellonella* model for studying foreign body infections has been established (199), broadening the use of invertebrate

models in biofilm-associated infections. The major caveat for this invertebrate model is the still ongoing genome sequencing and thus of genetic tools (5), despite some authors performed *G. mellonella* transcriptomics and proteomics focused on the immune-response to infections (200–202).

Vertebrate models

Zebrafish (*Danio rerio*)
Zebrafish is the most used non-mammalian vertebrate model for studying host-pathogen interactions. It combines some invertebrate characteristics with some mammalian ones. Indeed, *D. rerio* displays high fecundity and rapid development, low maintenance cost (similar to insects and worms) and offers the possibility of multi-routes of infections (similar to mammalian models) (203).

D. rerio larvae are transparent and allow direct visualization of the infection process progressing. It has been successfully used for investigating immune responses to both yeast and mould systemic infections (204). In a recent study, *C. albicans* isolates, with a high or low propensity to form biofilms, were injected in zebrafish larvae to assess *in vivo* virulence (205). Although fish survival after strong biofilm-former isolates was significantly reduced, fungal burden was similar after tissue recovery. These models have also been used to assess the antimicrobial activity of silver nanoparticles (206), proving that it would be beneficial for biofilm treatment studies. Major caveats for zebrafish as an experimental infection model are the growth temperature optimum (26–28.5 °C), the need for a dedicated facility for husbandry, and ethical obligations for animal welfare (207).

CONCLUDING REMARKS

Candida spp., and in particular *C. albicans*, is a tenacious biofilm forming yeast. We have demonstrated that it can be found across a broad range of clinical environments and will continue to burden the at-risk patient populations. The difficulty in managing these infections is primarily their tolerance to antifungals and penetrations issues, particularly where access to device removal is restricted. Despite these hurdles we have reason for hope in the form of new classes of antifungals, combined with exploration into innovative anti-biofilm strategies. The utility of simple animal models will enhance the capacity to speed up this innovation and support our clinical colleagues. Where we will be in a further two decades remains to be seen.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

All authors contributed sections to this review. **Gordon Ramage** collated, edited and proofed the final manuscript. All authors have read and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Current protocols for the treatment of several Candida infections, according to the guidelines from Pappas 2016.

