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Phenotypic and Proteomic characterization of Clinical *Candida albicans* isolates resistant to Fluconazole

By

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A thesis

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I will dedicate this thesis to my mother who was always been my backbone during this work and to my father in the heavens.

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I would also dedicate this thesis to my 12-year-old self-full of passion to contribute to biomedical research as well as to the legacy of my two favorite scientists, Nobel laureate Linus Pauling, and Rosalind Franklin.

Phenotypic and Proteomic characterization of Clinical *Candida albicans* isolates resistant to Fluconazole.

Geovanni Varoujan Geukgeuzian

Abstract

The opportunistic fungal pathogen *Candida albicans* is a major causative agent of death in immunocompromised individuals. The azole fluconazole is the first line of defense in hospital treatment. Azoles function by inhibiting *Erg11* an enzyme involved in the synthesis of ergosterol, the main sterol found in fungi. Resistance to azoles is on the increase worldwide including in Lebanon. The purpose of the current study is to determine pathogenicity attributes of azole resistant isolates from Lebanese hospital patients. Six isolates-3 resistant and 3 sensitive-underwent a battery of phenotypic tests related to virulence. In addition, the cell wall proteome of all isolates was isolated and underwent profiling by MALDI TOF TOF mass spectrometry to detect different proteins in resistant versus sensitive isolates, and differential presence in response to fluconazole exposure. Our results showed that resistant isolates had increase ergosterol content, and a slight increase in chitin deposition leading to resistance, but were on average attenuated in virulence compared with sensitive isolates. In addition, proteomic profiling revealed the presence of efflux pumps such as Cdr1, and Cdr2 exclusively in resistant isolates, an additional mechanism of resistance, and virulent isolates expressed more virulence genes as opposed to virulence attenuates isolates that did not express any.

In conclusion, our study suggests several mechanisms of antifungal drug resistance in *C. albicans* Lebanese hospital isolates.

Keyowrds: Fluconazole, *C.albicans*, pathogenicity attributes, MALDI, Antifungal resistance.

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Chapter I

Literature Review

1.1 Overview of *Candida albicans*

Members of the *Candida* species, particularly *Candida albicans*, are opportunistic nosocomial fungal pathogens and members of the human mycobiome (Adams & Suhr,2017). *C. albicans* is part of the mucosal microbiota and present in diverse niches within a healthy host. It has evolved as a strict colonizer of warm-blooded animals, (Odds et al,.2001) even though reports of growth on Oak trees in the wild has been recently detected (Bensasson et al,.2017). *Candida spp*. are early colonizers and can also be acquired upon physical contact during child labor. Consequently, these microorganisms colonize the skin, the gastrointestinal tract, the genital tracts, and the oral cavity (Fidel 1998; Barousse et al.,2004) as commensals, without causing major health complications.

However, in immunocompetent or immunocompromised patients, *C. albicans* can switch from a commensal to a pathogen causing superficial infections and invasion of the oral and vaginal epithelial surfaces. This may sometimes lead to more serious lethal systemic infections by breaching the blood/gut barrier and disseminating into the bloodstream, invading internal organs, and causing death through systemic infection. The prognosis for systemic infection is dire with a mortality rate close to 40% (Byrnes et al., 2016). *Candida* species sre the fourth major causative agent of hospital-acquired infections in United states, increasing the financial burden on the health care system up to $1 billion every year (Miller & Johnson, 2002).

1.2 Morphogenesis of *Candida albicans*

One of the key arsenals of *C. albicans* that make it such a successful pathogen is the large amount of diversity it can generate, making it a clinically problematic opportunistic pathogen. Diversity can be achieved not only at the molecular level where myriads of aneuploidies, chromosomal deletions and duplications have been reported (Arbour et al., 2017), but also at the morphological and physiological levels. *C. albicans* is termed a dimorphic organism, since it can switch between two distinct morphological states: the yeast form and the filamentous pseudohyphal and hyphal forms **(Figure 1).** The hyphal forms are found in invasive candidiasis, as hyphae can invade tissues, causing infection. Subsequently, this phenotypic plasticity of *C. albicans* is vital for its pathogenicity and virulence. (Shapiro 2011). As an example, yeast-to-hypha morphogenetic transition helps *C. albicans* species to escape from human macrophages as well as neutrophil phagocytosis (Korting et al., 2003; LO et al.,1997). While the hypha-to-yeast transition is needed for proper cell division and growth. Different morphogenetic states of C. *albicans* show differential susceptibilities and responses to macrophage-derived compounds (Blasi et al., 1995). It is thought that both the yeast and hyphal morphologies are needed for fitness. Mutants locked in either the yeast or hypha form are avirulent. This suggests that the ability to transit reversibly between these morphotypes potentiate the virulence of *C. albicans* (Lo HJ et, al.,1997; Moyes DL et al., 2016; Zheng & Wang, 2004).

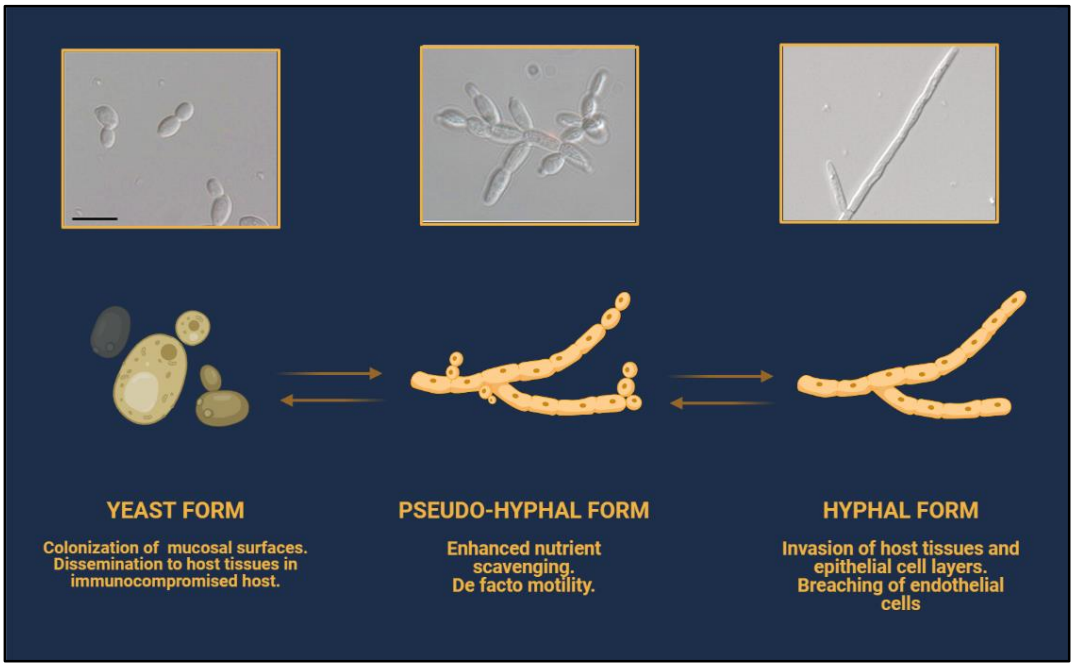

Figure 1. Graphical model of the evolution of morphology and virulence in *C. albicans*. In the mammalian host, the yeast form is adapted for colonization of mucosal cell surfaces. Pseudohyphae were initially most likely important for promoting nutrient scavenging and motility in the host environment. The hyphal form can invade epithelial cell layers by exerting mechanical force, and upon breach and damage endothelial cells. Furthermore, morphological transitions are typically reversible. (Modified from Thomson et al.,2011).

1.3 Morphogenesis and biofilm formation

A biofilm is an organized cluster of microorganisms that live within a self-produced matrix made up of extracellular polymeric substances (EPS) which attach to a biotic or abiotic surface **(Figure 2)**. In natural environments, this aggregation is considered to be a microbial lifestyle. (Huang et al., 2018; Gupta et al., 2016).

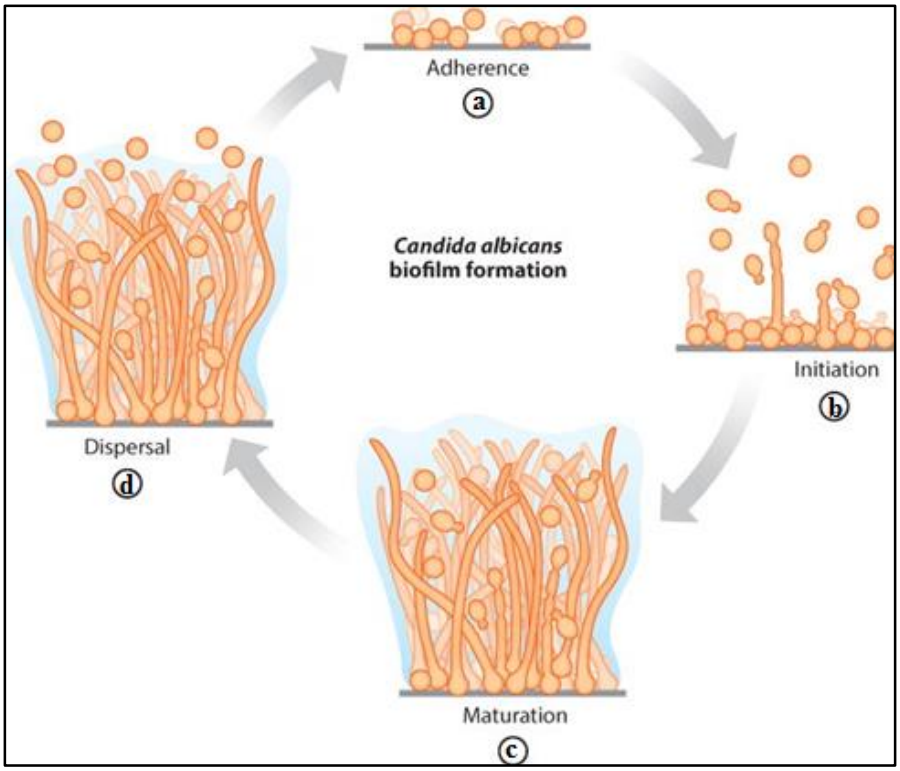

Figure 2. The cycle of *C. albicans* biofilm formation is described in four sequential main stages. **(a)** Adherence, in which the cells in suspension and those circulating (planktonic cells) adhere on a surface layer, 1-3 hrs; **(b)** Initiation of cell proliferation, where the basal layer is formed by anchoring cells,11-14 hrs; **(c)** Maturation, during which the growth of hyphae and the production of extracellular matrix (ECM) is observed; **(d)** dispersal, in which most cells leave the (ECM) and colonize areas surrounding the initial adherence site after 24 hrs (Modified from Nobile et al., 2016).

C. albicans biofilms are generated from different populations of yeast, pseudo hyphae, and hyphae (Blankenship & Mitchell, 2006). It was previously shown that hypha-defective mutants are not capable of colonizing plastic surfaces and build biofilms (Krueger et al., 2004). In addition, analysis of biofilm-defective mutants has revealed that such mutants were defective in hyphal development, highlighting the relationship between hyphal morphogenesis and biofilm formation (Richard et al., 2005).

Moreover, a key feature of biofilms is their resistance, at the "community level", to broad-spectrum antifungal agents. For instance, comparison between *Candida* species in their yeast form and cells in biofilms has revealed that biofilm formation resulted in resistance to some antifungal agents, including azoles and echinocandins. (Zarei et al., 2014).

Ultimately, both, changes in morphology and biofilm formation, play an important role in virulence. *C. albicans* produces biofilms on medical implants, like vascular catheters or surgical instrument, and as such, they are a risk factor for infections and diseases in healthcare facilities (Douglas 2002, 2003).

1.4 Morphogenesis and virulence

Morphogenetic transitions are important for virulence in *C. albicans*. Mutants that cannot undergo a morphological transition often have reduced pathogenicity. These recent findings propose the notion that both yeast and filamentous forms contribute to virulence. Additionally, filaments are vital for tissue invasion and deep-seated infection, whereas yeasts play an important function in early dissemination and infection processes (Saville et al., 2003). Filamentous fungi express virulence factors on their surface such as proteases, adhesins, lipases, and superoxide dismutases that contribute to virulence.

1.5Adhesion (first step of pathogenesis)

The ability of *C. albicans* to perforate mucosal surfaces or penetrate the bloodstream depends on how it "reads" the hosts' microenvironment and carries out three major strategies of pathogenesis which are: adhesion, colonization, and invasion **(Figure 3).** (Hostetter, 1996).

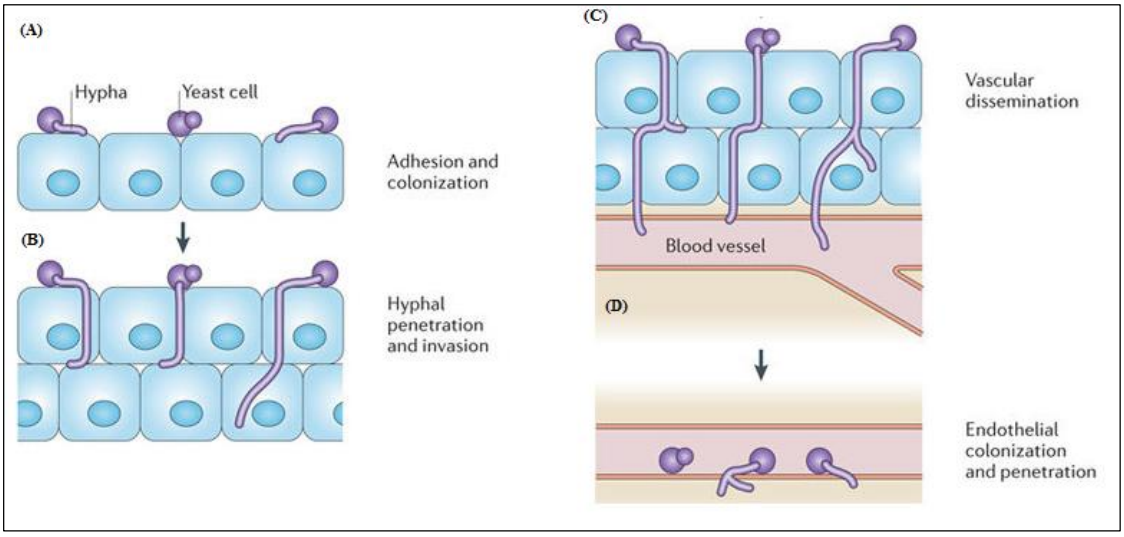

Figure 3. The major steps of *C. albicans* biotic surface adhesion and tissue invasion inside a host. (A) Adhesion to the epithelium; (**B**) epithelial penetration and invasion by hyphae; **(C)** vascular dissemination, where hyphal structures penetrate the blood vessels and seed of yeast cells into the bloodstream; and, finally, **(D)** endothelial colonization and penetration during systemic candidiasis (Modified from Gow et al., 2011).

Adhesins and invasions

*C. albican*s has a unique set of proteins called adhesins, which mediate adherence to other *C. albican*s or other microorganisms, in addition to abiotic surfaces and to human host cells (Garcia

et al., 2011; Verstrepen & Kliss 2006). Adhesins are important virulence factors, as adhesion is the first step towards a successful infection The most studied *C.albicans* adhesins are the agglutinin-like sequence (ALS) proteins that consist of eight members (Als1–7 and Als9). Most adhesins are glycosylated, and they are attached to the cell wall through a glycosylphosphatidylinositol (GPI)-linkage-a modification of the C terminus of the protein. Of all the eight Als2 members, Als3 has a special importance for adhesion (Zordan et al.,2012; Murciano et al., 2012). It mediates the attachment to epithelial cells, endothelial cells, and extracellular matrix proteins. It binds to human cell receptors such as E-cadherin and N-cadherin and, hence, induces host cells to endocytose the organism. Als3 also binds to human cell ferritin and enables *C. albicans* to utilize this protein as a source of iron acquisition. Thus, Als3 is a multifunctional important cell wall protein in *C. albicans* (Liu & Filler et al.,2011). Other CWPs involved in adhesion are Pga1, Big1 and the Hwp family.

1.6 *Candida albicans* cell wall components and proteins (CWPs)

The cell wall of *C albicans* protects it against environmental cues and stress conditions, such as osmotic changes, dehydration, heat, cold or an immune response (Sundstom et al., 2002). It also gives the cells their shape and adhesive properties. In addition, the cell wall in a pathogen is an important antigenic determinant, as it is the first structure that contacts the host. The cell wall of *C. albicans* is known for its plasticity and changes composition between yeast and hyphal forms to suit the environment it is faced with, since hyphal cell walls are thicker, more rigid and have a higher chitin content (Poulain and Jouault, 2004; Galan-Dié et al., 2010; Sem et al., 2016).

The cell wall composition of *C. albicans* consists of an internal chitin layer for rigidity, followed by a beta 1,3 glucan layer and an external beta 1,6 layer to which most glycoproteins, mainly in the form of mannosylated proteins, including the adhesins and proteases, are attached **(Figure 4).**

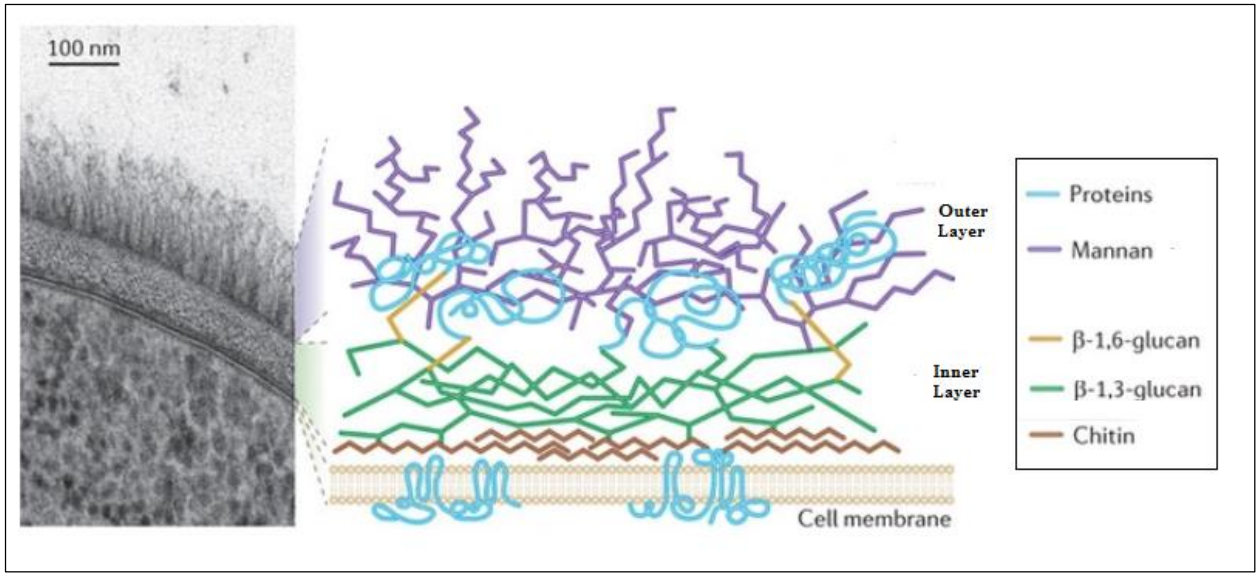

Figure 4 Structure of C. *albicans* cell wall and its components. Two major layers can be observed in the *C. albicans'* cell wall. The outer layer contains the mannose polymers (mannans) that are covalently associated with proteins to form glycoproteins, while the inner layer constitutes the skeletal polysaccharides chitin and β-1,3-glucan. The outer layer cell wall proteins (CWPs) are attached to this inner wall framework predominantly by glycosylphosphatidylinositol (GPI) remnants that are linked to the skeleton by β-1,6-glucan (Modified after Gow et al., 2012).

1.7 Glucans

Glucan is the major molecular component of the yeast cell wall, located adjacent to the cell membrane. It binds to chitin and assembles into a three-dimensional dynamic network to ensure

cell wall integrity. Furthermore, β-1,3 and β-1,6-glucan layers constitute 20% to 40% of the dry weight of the cell wall and are situated directly above the chitin layer (Elizabeth et al.,2019).

β-glucan is detected with ease by the host immune system, producing an effective response for *Candida* infection. Therefore, the masking of β-glucan is an important evolutionary mechanism of *Candida* species. Disturbance at the level of synthesis and organization of the cell wall components, resulting in the unmasking of the glucan layer to the host immune system, makes it capable of recognizing and attacking the fungal pathogen. Thus, *C. albicans* have evolved to go unrecognized by the immune system by masking its glucan layer. Interestingly, *C. albicans*, in their hyphal form, undergo fission without producing bud scars with their inner cell wall layers not exposed to the environment. On the other hand, the yeast forms buds with resulting bud scars exposing mannans to the surface. Consequently, this evolutionary adaptation of hyphal cells might help *Candida* evade or alter the host immune response (Mukaremera et al., 2017).

Ywp1 is a cell wall protein which plays an anti-adhesive role and promotes dispersal of yeast forms, allowing *C. albicans* to seek new sites for colonization. Genetically engineered isolates that express Ywp1 in their hyphal form not only reduce its adhesive characteristics, but also decrease the exposure of its β-1,3-glucan layer, and consequently, promote evasion of the host immune response (Granger, 2018).

1.8 Chitin

Chitin is a monomer of N acetyl glucosamine sugar. In the yeast form, *C. albicans*' chitin content is approximately 1–2% of the dry cell wall weight, but it can increase to 10% in their

hyphal state, supplying the rigidity needed to invade tissues and organs (Chataway et al.,1968). Chitin synthesis is required for growth, development and survival of the fungi, protecting them from lethal cell wall stresses (Merzendorfer, 2006; Munro and Gow, 2001). In particular, *C. albicans* class I chitin synthases enhance the cell wall integrity during early polarized growth in yeast and hyphal cells. Interestingly, it has been reported that certain antifungal drugs, such as azoles, were capable of inducing membrane stress in *C. albicans* and upregulating chitin content, a defense mechanism resulting in resistance (Navaro-Garcia et al.,1995). Moreover, mutations in *FKS1*, involved in beta glucan formation, are compensated for by increased chitin deposition, resulting in decreased virulence but increased resistance to antifungal drugs such as echinocandins (Toutounji et al., 2019).

1.9 Mannans

Mannoproteins are another cell wall constituent of *C. albicans* and play a major role in their virulence and immunogenicity. In fact, most proteins, on the surface of the cell wall, are in the form of mannoproteins. Many of the virulence factors discussed above, such as adhesins and proteases, are mannosylated (Shibata et al., 2012).

Mannoproteins are essential in *C. albicans'* communication with the host, promoting the activation and modulation of the immune response against the fungi (Shibata et al., 2012; Paulovicova et al., 2015). These macromolecules mask the β-glucan layer, and thus, decrease their recognition by the host immune system a process and ultimately reduce phagocytic capacity (Galan-Diéz et al., 2010). Mannans are also known to be pathogen associated molecular patterns, otherwise known as PAMP (Brown et al., 2002).

For example, α 1,2-mannose of N-mannan has been shown to have a vital role for both immune recognition and virulence in *C. albicans*. The *MNN2* gene family, which contains α 1,2-mannose, is comprised of six members *(Mnn2, Mnn21, Mnn22, Mnn23, Mnn24* and *Mnn26).* These proteins play a major role in increasing the pathogenicity attribute of candida species and act as PAMP complexes (A. Hall et al., 2013).

1.10 Cell wall proteins (CWPs)

It is thought that between 5% to 10% of the *Candida albicans* genome codes for cell wall proteins. Expression of cell wall proteins is modulable and depends on the host environment. For example, *C. albicans* cells grown in normal human serum (NS) and heat inactivated serum (HIS), have expressed 372 *C. albicans* unique proteins. 371 proteins have been found in NS, 134 in HIS, and 133 proteins have been identified in both growth conditions. Induction of hyphal form with human serum resulted in the identification of 12 proteins that have not found in any other morphogenesis induction. Furthermore, 147 out of 372 proteins have been categorized as cell wall surface proteins.

There are two classes of covalently bound fungal cell wall proteins (CWPs) as seen in **Figure 5,** the glycosylphosphatidylinositol (GPI)-dependent CWPs, and the Pir-CWPs linked directly to the β-1,3-glucans, that could be isolated from cell walls by treating them with mild alkali agents (alkali-sensitive linkage, ASL). Mannoproteins, in the cell walls of *C. albicans*, are packed with glycosylphosphatidylinositol (GPI) and cross-linked to β-1,6-glucans (Reyna-Beltran et al., 2019).

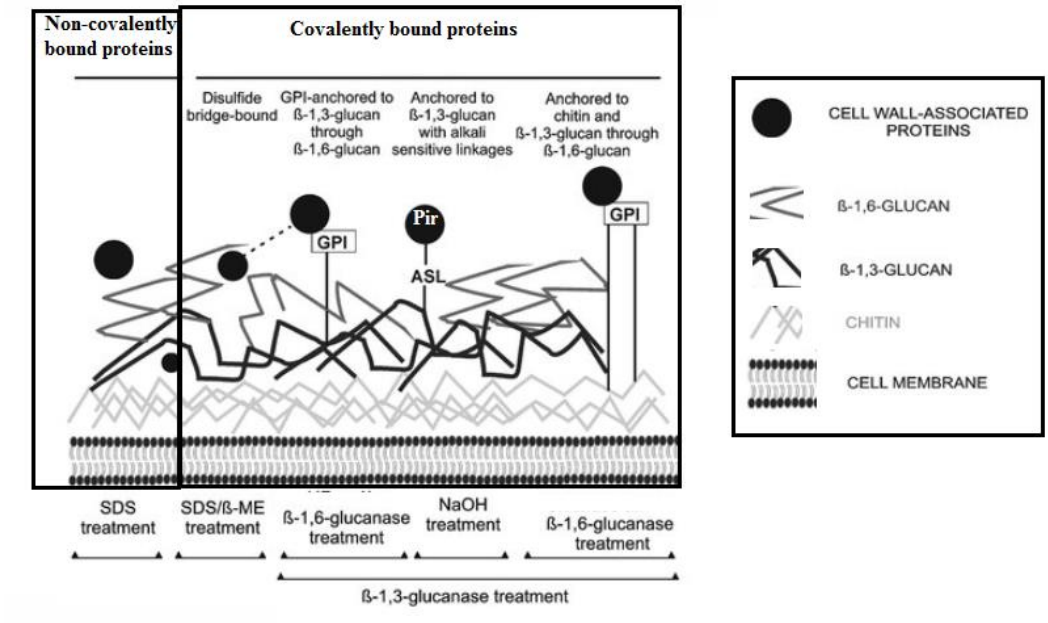

Figure 5. Overview on the *C. albicans* cell wall. Figure shows the dominant types of linkages between polysaccharides and proteins and their respective methods of protein extraction (Modified after Pitarch et al., 2002; Pitarch et al., 2008).

Function of CWPs

CWPs of *C. albicans* have a variety of functions. First, they limit the permeability of the wall, protect the skeletal layer against foreign substances or cells, degrade other enzymes, and reduce entry of toxic compounds (LV et al., 2016). They also determine the hydrophobicity (Brown et al., 2012) and antigenicity of the wall. Additionally, CWPs are also involved in cell wall remodeling and may contribute to cellular adhesion and virulence.

1.11 Sterols

Sterols are an essential component of the membranes of the fungal pathogen *C.albicans*. Their presence reduces the membrane's susceptibility to a variety of stresses, such as ionic, osmotic, and oxidative pressures, and treatment with antifungal medications. Overall, sterols in yeast are crucial factors that prevent fluid entry, and maintain the integrity, rigidity, and fluidity of the plasma membrane (Groll et al.,1998). Ultimately, like cholesterol in animals, ergosterol is the major membrane sterol in most fungal species (Zavrel et al., 2013).

1.12 Antifungals and molecular mechanisms of resistance by *Candida albicans*

1.13 Classes of modern antifungals

Nine out of ten reported fungal-associated deaths result from three main genera: *Cryptococcus*, *Candida*, and *Aspergillus* (Brown et al., 2012). Sadly, treatment options are numbered, and only a handful of drug classes are classified as antifungal drug candidates, as opposed to bacterial antibiotics. The main reason for this is the relatively high level of gene orthology and common pathways between fungi and humans, limiting drug specificity (L.E. Cowen et al 2011). The five classes of antifungal drugs are: polyenes, azoles, allylamines, flucytosine, and echinocandins as shown in (**Figure 6)**. (Groll et al., 1998; Kathiravan et al., 2012)

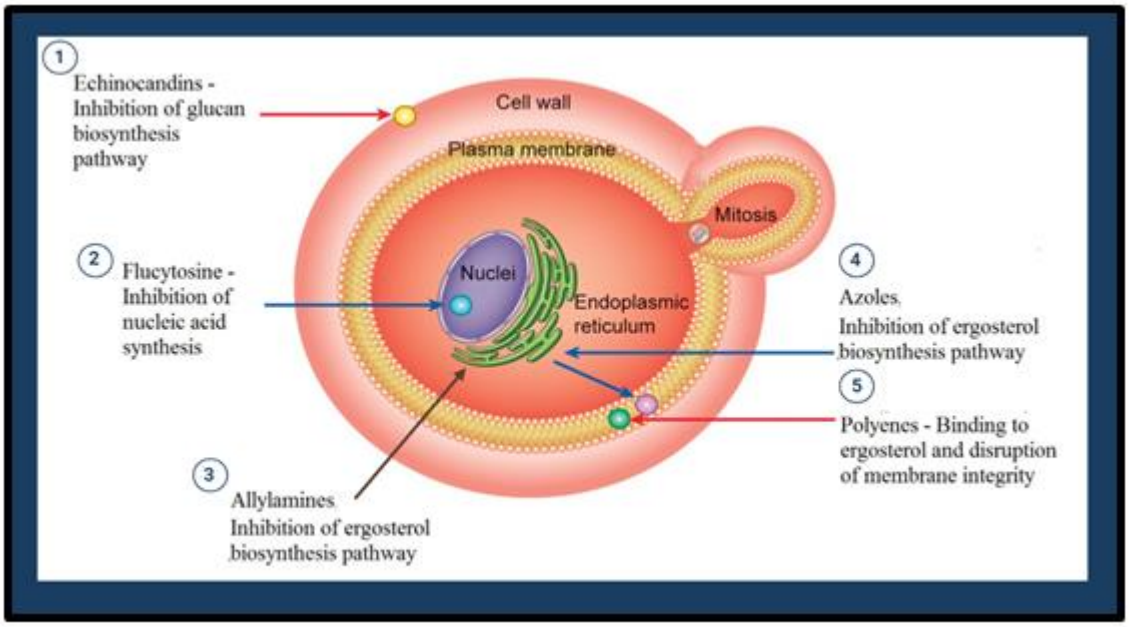

Figure 6 Mechanisms of action of the five main classes of antifungal agents and their cellular targets. (1) Echinocandins inhibit (1,3) β-D-glucan synthase, thereby preventing glucan synthesis, which is present in the cell membrane of *C. albicans*. **(2)** Flucytosine inhibits the thymidylate-synthetase enzyme and hampers DNA synthesis in fungal cells. **(3)** Allylamines impede the action of the enzyme squalene-epoxidase, which in turn, has a role in the synthesis of ergosterol. **(4)** Azoles inhibit the ergosterol synthesis of the *C. albicans* cells. This drug acts by interfering the enzyme lanosterol 14-α-demethylase, which is involved in the transformation of lanosterol into ergosterol. **(5)** Polyenes bind to ergosterol directly and disrupt the membrane structure, promoting extravasation of intracellular constituents, and consequently, cell death (Modified from de Oliveira et al., 2018).

Polyenes

Polyenes bind mainly to ergosterol in the fungal cell membrane. The interaction of the drug with its target results in the formation of pores or channels in the membrane, and this causes leakage of small molecules, resulting in cell death.

Allylamine

Terbinafine, a type of allylamine derivative, inhibits squalene epoxidase which is an enzyme involved in ergosterol synthesis (Johnson & Perfect, 2012).

Echinocandins

One of the pioneering compounds for systemic antifungal therapy that target the cell wall are echinocandins. Examples of echinocandins are: caspofungin, micafungin, and anidulafungin that destabilize the fungal cell wall by depleting glucans through inhibition of glucan synthesis. The latter are necessary to maintain the stability of the *C. albicans* cell wall (Walsh et al., 2008; Sucher et al., 2009).

Flucytosine (Antimetabolite)

Flucytosine (5FC) is an antifungal agent that has been used since the 1970s after being approved by the FDA. Its deamination to 5-fluorouracil (5FU) is an antimetabolite in fungi but not in mammalian cells. Moreover, it is either incorporated into RNA or metabolized to a thymidylate synthase inhibitor, 5-fluorodeoxyuridylic acid, which prevents DNA synthesis in fungal cells. The selection of this drug has some complications in clinical use, since it has shown bone marrow suppression and nephrotoxicity, and its use as monotherapy has been associated with

rapid development of flucytosine resistance. Therefore, flucytosine is nowadays used only in combination with other antifungals (Johnson & Perfect, 2012).

1.14 Azoles

Azoles are heterocyclic compounds that have at least one nitrogen atom as part of their ring. The familiar azoles used as antimycotic agents include the triazoles: fluconazole, voriconazole, and Posaconazole. Azoles target the cytochrome P450 enzyme-lanosterol14-demethylase, which converts lanosterol to ergosterol. In yeast, this enzyme is coded by the gene *ERG 11*.

Upon azole treatment, the C. *albicans* cell membrane is depleted of ergosterol. It accumulates other toxic14-methylated sterols and affects the fluidity of the membrane and inhibits cell growth as revealed in **Figure 7**(Shapiro et al., 2011). As such, fluconazole is a common medication and the first line of defense used for the treatment of *Candida* infections in hospitals and healthcare facilities. It is widely used due to its high bioavailability, high water solubility, and low affinity to related human eukaryotic cell membrane plasma proteins (Lupetti et al., 2002).

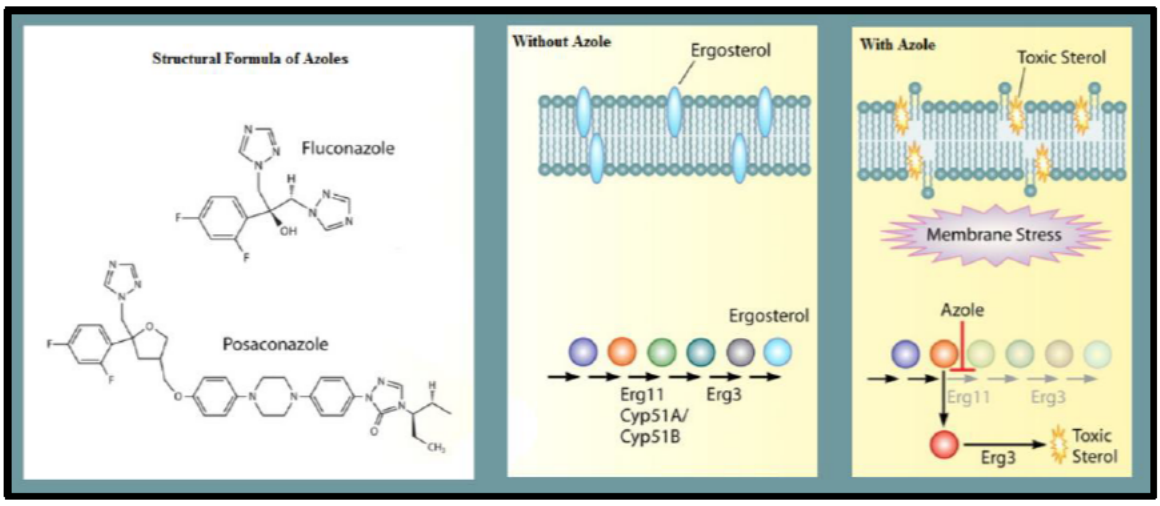

Figure 7. Molecular target of azoles in *Candida albicans*. Azoles target the process of ergosterol biosynthesis by inhibiting the action of lanosterol demethylase which is encoded by *ERG 11* in *C. albicans*. The blockage of ergosterol production causes the accumulation of toxic sterol produced by the *ERG 3* gene in *C. albicans*. This response exerts a severe membrane distress, and consequently, kills the cell (Modified from Shapiro et al., 2011).

1.15 Mechanisms of resistance to azole in *Candida albicans*

C. albicans has evolved multiple mechanisms to generate resistance to azoles **(Figure 8).** One of the main mechanisms of resistance to azoles is the presence of point mutations in *Erg11* (Marichal et al., 1999) . Mutations in *Erg11* change the tertiary structure of the enzyme subsequently, decreasing the affinity of fluconazole to its target protein, lanosterol 14-demethylase. Since the drug cannot bind its target, resistance ensues (Whaley et al., 2017). To support this mechanism, a study involving site-directed mutagenesis of wild-type *Erg11* generated mutants that exhibited increased fluconazole resistance (Xiang et al., 2013).

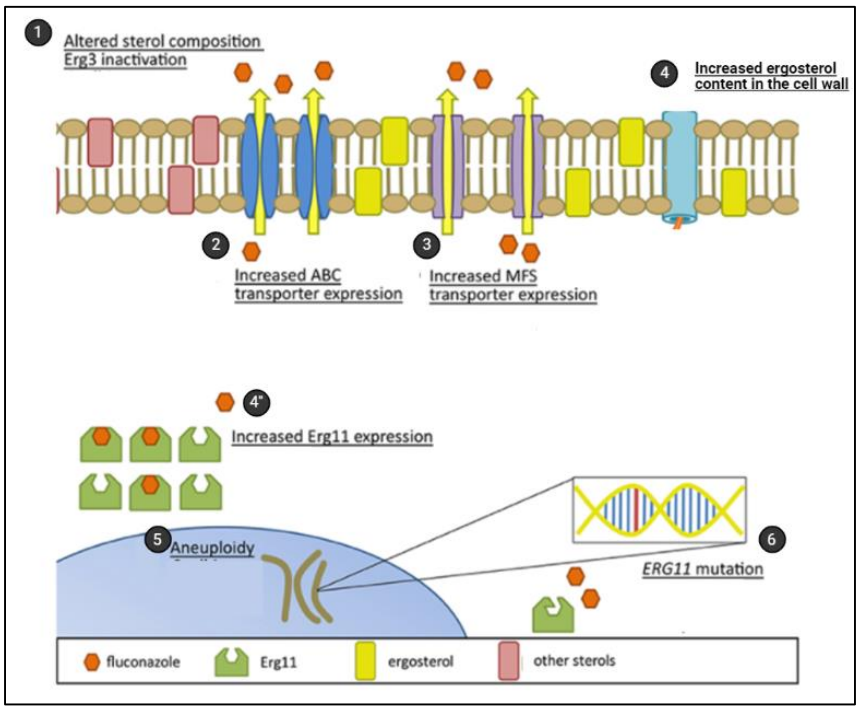

Figure 8. Mechanisms of Fluconazole resistance in *C. albicans*. (1) Inactivation of *ERG3* which results in the expression and presence of alternative toxic sterols in the *C.albicans'* plasma membrane.**(2)** Increased expression of ABC transporters (ATP binding cassette efflux pumps). **(3)** (MFS) major facilitator superfamily transporters on the membrane to reduce the intracellular accumulation of fluconazole. **(4,4")** Increase in ergosterol biosynthesis and the translocation of the sterols to the cell membrane after increased expression of *ERG11*. **(5)** Aneuploidy promoting adaptation to Fluconazole exposure**. (6)** Mutations in *ERG11* gene and reduced affinity of fluconazole to its target, lanosterol 14-demethylase (Modified from Whaley et al., 2017).

Other mechanisms of fluconazole resistance in *C. albicans* involve the overexpression of drug efflux pumps that belong to the two major superfamilies ABC (ATP binding cassette), Cdr1, Cdr2, and MFS (Major facilitator superfamily) such as Mdr1, Mdr2, Nag 3 and Nag 4. ABC transporters in *C. albicans* often consist of multiple subunits of which one or two are transmembrane proteins .

The ATPase subunits utilize the energy of adenosine triphosphate (ATP) hydrolysis to provide the energy needed for the translocation of substrates across membranes, such as influx or efflux of molecules (Prasad et al., 2015). *Cdr1,* a member of the ABC transporters family, is a major player among these transporters that enables *C. albicans* to outplay the battery of antifungals encountered, such as fluconazole. Deletion of *Cdr1,* a member of ABC transporters, has a major effect in reducing resistance to fluconazole (FLC), ketoconazole (KTC), and itraconazole (ITC) by 6-, 4-, and 8-fold, respectively (Tsao et al., 2009).

The increased expression of CdrI and Cdr2 and multidrug transporters of the ABC family has been recently linked to diminished *C. albicans* susceptibility to azole antifungal agents. Furthermore, the expression of Mdr1, responsible for the ATP-dependent expulsion of certain compounds, has also been found to be upregulated in drug-resistant biofilms (Ramage et al., 2002).

A less common mode of resistance to azole in *C. albicans* is inactivation of the *ERG3* gene, which encodes the ergosterol biosynthesis enzyme sterol 5,6 desaturase. Inactivation of this secondary pathway prevents the accumulation of lethal toxic sterols and generates sterol intermediates that do not bind azoles, resulting in resistance. A few *C. albicans* isolates have documented azole resistance due to this inactivation (Kelly et al, 1997; Notle et al., 1997; Morio et al., 2012). In addition, upregulation of ergosterol due to possible upregulation of *ERG11* is yet another mechanism that generates resistance.

Last but not least, aneuploidy plays a role in azole resistance in *C. albicans,* as shown in several comparative genome hybridization studies (Selmecki et al., 2006), where multiple copies of chromosomes with mutations involved in the acquisition of resistance increase resistance

phenotypes. Similarly, loss of heterozygosity (LOH) has been revealed in several azole-resistant *C. albicans* (Coste et al., 2006).

1.16 MALDI TOF/TOF -MS/MS

Protein identification by mass spectrometry (MS) is an important proteomic tool and is achieved by peptide mass fingerprinting (PMF), or analysis of peptide sequences. During MS experiments, mass-to-charge ratios of peptides are established by a matrix assisted laser desorption/ionization time of flight mass spectrometer (MALDI-TOF). After peptide detection, the results will be searched on theoretical matches in database to identify the unknown proteins of interest.

During (PMF), the peptides are subjected to tandem mass spectrometry (MS/MS) and become fragmented into daughter ions. Then, the mass-to-charge ratios from the obtained data provide their respective amino acid sequences. The latter, along with their mass values, are compared to a database for protein identification (Damodaran et al., 2007). Recently, MS laboratory approaches are widely used for their fast identification of infections caused by various microorganisms (Putignani et al., 2011). Microbiologists are using this method to characterize *Candida species* from clinical isolates (Pulcrano et al., 2013), and we have successfully utilized such an approach to determine differentially expressed cell wall proteins in cell wall mutant isolates. In addition, MS/MS has been applied to determine the effect of different antifungal treatments on *C. albicans* cell wall proteome (Heilmann et al., 2012).

1.17 Aim of study

The aim of this study is to undertake a more thorough and holistic approach to determine the mechanisms of drug resistance, and pathogenicity attributes of fluconazole resistant *C. albicans* isolates from Lebanese hospitals. We have previously determined the drug resistance profile of those isolates (Bitar et al., 2014; Basma et al., 2009) to various antifungal drugs and sequenced the *ERG11* gene to determine possible point mutations involved in resistance (Fattouh et al,2021). In this study, 6 isolates-3 sensitive and 3 resistant-were subjected to a battery of phenotypic tests such as ergosterol content, cell wall chitin content, biofilm formation and adhesion capabilities, virulence potential in a mouse model of disseminated candidiasis, and measurement of resistance to cell wall disrupting agents to determine phenotypic attributes specific to resistance. In addition, we sought to determine the cell wall proteomic profile of resistance versus sensitive isolates in response to azole exposure to determine possible novel mechanisms involved in resistance, and to identify proteins that explain the phenotypes attributes observed. As most of the phenotypes assayed involve changes in cell surface protein expression, we decided to extract the cell surface proteome instead of the total cellular proteome.

Chapter II

Materials and Methods

2.1 Isolates utilized

Six *C. albicans* isolates that were previously recovered from clinical settings and stored in cryobanks at -80°C in 15% glycerol were used for this study. The isolates were obtained from hospitalized patients in tertiary care centers in Lebanon (Basma et al., 2009; Bitar et al., 2014). Three out of the six isolates were susceptible to fluconazole, designated as isolates S1, S2, S3, and 3 were resistant, designated as isolated R1, R2, R3, to fluconazole. The susceptibility to fluconazole was assessed by the E-test method (Basma et al., 2009; Bitar et al., 2014). *C. albicans* SC5314 (ATCC® MYA- 2876) reference that is susceptible to all antifungals was used as a control. Isolates were inoculated in 5 mL potato dextrose broth (Conda Laboratories) and incubated at 30°C for 24 h with shaking at 100 rpm. Isolates were then plated on potato dextrose agar (Conda Laboratories) and incubated at 30°C for 48 hours.

2.2 Quantification of ergosterol content

Single colony of each isolate was cultured in 50 mL potato dextrose broth for 17 h at 35°C with shaking at 100 rpm. Ergosterol was extracted and quantified according to the equations in (Arthington-Skaggs et al., 1999) by using the optical density measurements at 230 and 280 nm, taken by a Genesys 10S UV-V is spectrophotometer. The experiment was performed in biological triplicates for each isolate and the average % ergosterol (erg.) was computed then, the % change in ergosterol (erg.) was calculated for each clinical isolate according to the

following formula: % change in erg. content = [(% erg. in clinical isolate - % erg. in SC5314) / % erg. in SC5314] x 100.

2.3 Murine model of disseminated candidiasis

To measure virulence in a disseminated model of systemic candidiasis six to eight-week-old female BALB/c mice were injected in the tail veins with10^7 cells, of each clinical isolate, in addition to the reference SC5314, in 0.2 mL of phosphate buffered saline. A control group composed of six mice were injected with 0.2 mL of 1x phosphate buffered saline. Water and food were administered *ad libitum* (Bahnan et al., 2012; Daher et al., 2011; Hashash et al., 2010; Toutounji et al., 2019). Mice were followed up daily for a period of 30 days and the number of moribund mice was counted. Moribund mice were euthanized. Results were analyzed through the generation of Kaplan Meier curve. Mice manipulation followed all ethical standards of the Lebanese American University's Institutional Animal Care and Use Committee which approved the execution of this experiment on December 3, 2019, under approval number: *LAU.ACUC.SAS.RK1*.

2.4 Biofilm capacity

One colony of each isolate was grown in 5 mL potato dextrose broth overnight at 30°C with shaking at 100 rpm. 107 *C. albicans* cells/mL in a total volume of 0.2 mL were used to inoculate a well of a 96-well plate that was pre-treated overnight with 5% fetal bovine serum at 4°C. The 96-well plate was incubated for 3 h at 37°C with shaking at 75 rpm. The wells were washed once with a 1x phosphate buffered saline solution and 0.2 mL of fresh potato dextrose broth was added. The plate was incubated for 48 h at 37°C with shaking at 75 rpm. Biofilm formation capacity was determined based on a modified protocol described by (Peeters et al., 2008).

All wells were washed once with a 1x phosphate buffered saline solution to remove planktonic *C. albicans.* Biofilm was fixed with 0.2 mL of 99% methanol for 15 min, then methanol was removed and the wells were air-dried for 20 min. 0.2 mL of 0.2% crystal violet was added and after 20 min was washed 5 times with sterile water. To release the crystal violet, 0.2 mL of 33% acetic acid was added and the optical density was measured at 595 nm, using the Multiskan TM FC Microplate Photometer coupled to the SkanIt TM Software for microplate readers. The experiment was performed in biological triplicates.

2.5 Cell surface disruption assay

A spotting assay was performed. Colonies were grown in 5 mL potato dextrose broth at 30°C with shaking at 100rpm. Tenfold serial dilutions were performed, and 10^5 ,10^4 ,10^3 and 10^2 *C albicans* cells were spotted on potato dextrose agar (PDA,), supplemented with 0.025% SDS-- a cell surface disruptant-- (Plaine et al., 2008) and incubated at 30°C for 24 hrs. As a control, serial dilutions were also spotted on PDA media lacking SDS. Colonies were observed for two consecutive days and images were taken using Fiji/ImageJ version 1.48 to track growth. The experiment was performed in biological triplicates.

2.6 Quantification of cell wall chitin

Colonies were incubated overnight in 10 mL potato dextrose broth at 30°C at 100 rpm shaking. To isolate the cell wall and its respective proteins (CWPs), we followed the protocol described by (El Khoury et al.,2018) with minor modifications as such: *C. albicans* were pelleted by

centrifugation for 5 min at 4000 rpm, and the pellet was suspended in 5 mL of 5 mM Tris at pH 7.8 and transferred to a 15 mL Falcon tube containing cold undrilled glass beads of 3 to 5 diameter to have a bead to pellet volume ratio of 1/1. The suspension was vortexed for 30 sec, followed by incubation for 30 sec on ice and the cycle was repeated 30 folds. The suspension was then transferred to previously weighed 50mL Falcons. Glass beads were washed with 5 mL of 1 M cold NaCl and the suspension was transferred to the same 50 mL Falcon tube of known weight and centrifuged for 10 min at 3000 rpm. Later, pellets that contain *C. albicans* cell wall were weighed. Cell wall proteins were removed by suspending cell wall in 0.5 mL of protein extraction buffer at pH 7.8/100 mg of wet weight cell wall and transferred to a 1.5 mL Eppendorf tube. Moreover, the buffer for protein extraction, was prepared by mixing 150 mM NaCl, 100 mM Na-EDTA, 50 mM Tris, 2% SDS, and 8 μL/mL of buffer of β-mercaptoethanol. The suspension was heated for 10 min at 99°C, cooled down for 5 min, and centrifuged for 5 min at 3000 rpm. This step was repeated twice. The pellet was washed 3 folds by suspension in 1 mL of sterile water, followed by centrifugation for 5 min at 3000 rpm. The pellet was suspended in 1 mL of 6 N HCl, heated for 15 min at 99°C, then collected by centrifugation for 5 min at 3000 rpm and suspended in 1 mL of sterile water. Assessment of cell wall chitin content was performed following the protocol described by (Kapteyn et al., 2000). The experiment was performed as biological triplicates.

2.7 Growth kinetics

All six isolates and the control were grown overnight in 2 mL of potato dextrose broth at 30°C with shaking at 100 rpm. To refresh the overnight cultures, 8 mL of potato dextrose broth was

added, followed by an incubation step for another 2 h at 30°C with shaking at 100 rpm. 10 mL of potato dextrose broth was inoculated with 10^4 refreshed *C. albicans* cells/mL and incubated at 30°C with shaking at 100 rpm. The optical density of the cultures was measured at 600 nm every 2 h for a duration of 10 hr. The measurements of optical densities were carried out using a Genesys 10S UV-V spectrophotometer. For each isolate, the experiment was performed in biological triplicates .The average OD600 at each time point was computed and the % change in doubling time (dt) was calculated according to the following equations with the time cultured being 10h. (1) Doubling time = time cultured in h / [log10(OD600 at start/OD600 at 10h) / 0.3].(2) % change dt = [dt in clinical isolate - dt in SC5314) / dt in SC5314] x 100.

2.8 Adhesion assay

Six clinical isolate, 3 susceptible and 3 resistant to fluconazole, were spotted on PDA plates and incubated for 14 days at 37 °C, after which cells were washed vigorously with tap water for 30″. Isolates that adhered to agar underwent invasive washing. Resistant filamentous growth were photographed and compared with prewashing conditions (Younes & Khalaf, 2013).

2.9 Proteomic profiling of isolates

For cell wall protein extraction, all six isolates were grown in PDA media for 48 hours at 37^0 C with 100 rpm shaking mode. The cells were cultured either in the presence or absence of fluconazole during this time interval. The concentration of fluconazole added was equivalent to half the MIC for each of the azole susceptible or resistant isolate.

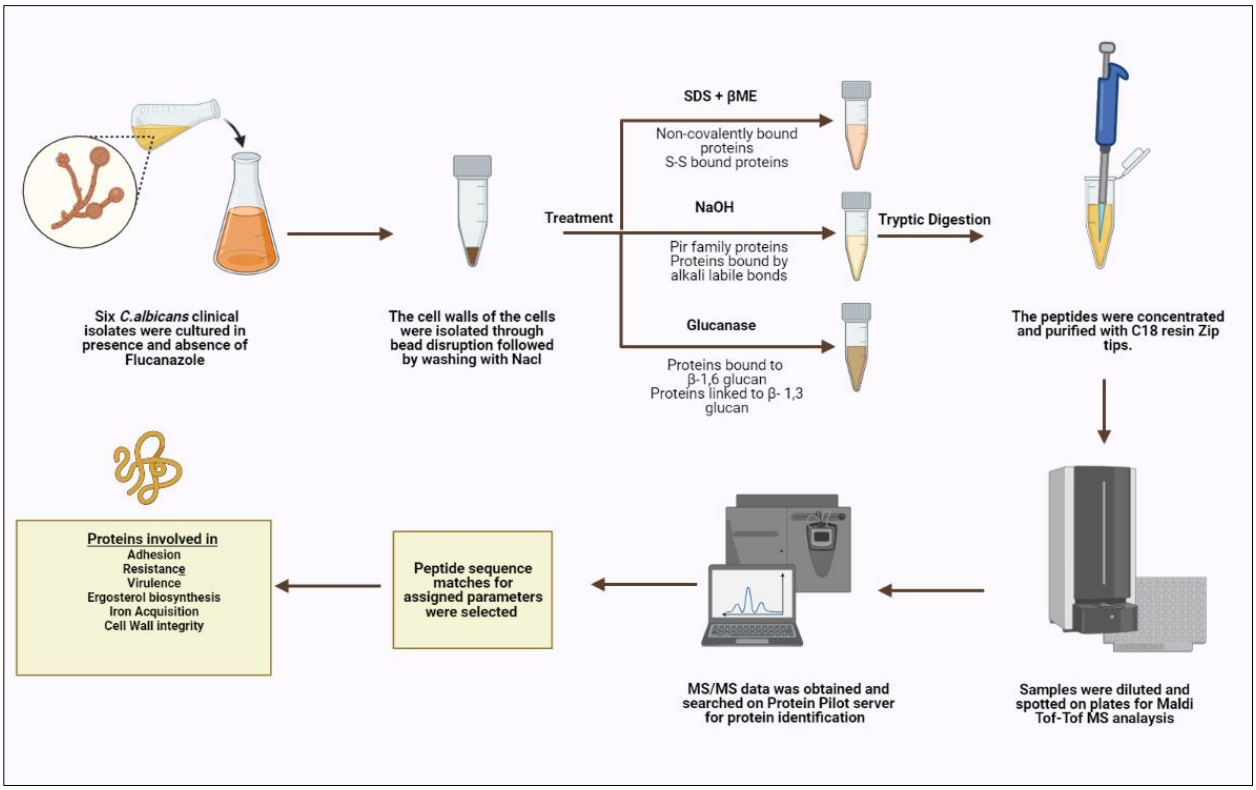

Figure 9. Overview of the of the proteomic workflow followed in this study. Isolates were cultured in the presence or absence of fluconazole. The cell wall was isolated through bead disruption followed by washing with NaCl. SDS +βME, alkaline (NaOH) and glucanase protein extraction treatments were used to separate the cell wall proteins based on their linkage characteristics at the level of the cell wall. Upon extraction, the peptides were concentrated and purified with C18 resin tips and diluted to be spotted on plates for MALDI-TOF/TOF MS analysis. MS/MS data was retrieved and searched on the Protein Pilot server for protein identification. Obtained matches based on the assigned parameters were selected. CWPs involved in adhesion, resistance, virulence, ergosterol biosynthesis, iron acquisition and cell wall integrity were identified.

2.10 Cell wall isolation and protein extraction

Cell wall extractions for each isolate were performed as follows. *C. albicans* cells were first centrifuged at 4,000 rpm for 5 min, then re-suspended in 5 mL Tris (5 mM, pH=7.8). Protease inhibitor cocktail (6 µL, abcam ab65621), along with cold glass beads, was added. 30 cycles of vortexing were applied to ensure breakage as follows: 30 sec on vortex, followed by 30 sec on ice rack. Samples turned orange, reflecting a reaction between the acidic cytosol and the protease inhibitor. Beads were then removed, and the efficiency of breakage was later examined under the microscope. The samples were spun and the supernatants containing intracellular proteins were poured out, while pellets were re-suspended in NaCl (40 mL, 1 M) and spun accordingly. This NaCl washing step was repeated 3-4 folds. Protein extraction buffer (50 mM Tris, 2% SDS, 100 mM Na-EDTA, 150 mM NaCl, pH 7.8) with β-ME (8 µL per 1 mL SDS extraction buffer) was added (0.5 mL buffer per 100 mg wet weight walls) and the pellets were re-suspended. The 12 samples were boiled for 10 min and spun for 5 min at 3,000 rpm. The supernatants containing SDS extractable proteins were collected for later analysis and extraction buffer and β-ME was added again to re-suspend pellet. Furthermore, the 12 samples were boiled, cooled, centrifuged for 5 min at 3,000 rpm, and re-suspended in water. Wash steps with Type 2+ water were performed to remove the excess SDS contaminant. The final pellets were frozen in liquid N2 and freeze-dried. Lyophilized cell walls were finally stored at -20 ℃ until use (El Khoury et al., 2018).

2.11 Extraction of alkali labile CWPs

Cell wall pellets were incubated overnight with NaOH (30 mM) at 4 ℃. They were then neutralized with aqueous acetic acid (30 mM) (Sorgo et al., 2010). Samples were spun, and supernatants collected and treated for tryptic digestion with trypsin.

2.12 Glucanase treatment of cell wall pellets

Cells were cultured in PDB broths at 37 ℃ with 1 mg of glucanase in sodium acetate buffer (1 mL, 150 mM, pH=5) overnight (Cabezon et al., 2009). Spectrophotometric analysis was used to estimate the cell numbers. Supernatants were collected and treated for tryptic digestion with trypsin.

2.13 Tryptic digestion

The cell wall extracts were incubated in a reducing buffer (10 mM DTT, 100 mM NH_4HCO_3) at 55 ℃ for 1 hr. The 12 samples were cooled to room temperature and spun. An alkylating buffer (65 mM iodoacetamide, 100mM NH_4HCO_3) was added to the pellets that were kept at room temperature in the dark for 45 min. Subsequently, the quenching solution (55 mM DTT, 100 mM NH_4HCO_3) was added to the samples for 5 min at room temperature. Ammonium bicarbonate buffer (50 mM) was used to wash the samples 5 times. Pellets were re-suspended in solution containing ammonium bicarbonate (50 mM8) and trypsin (1 µg/µL). Samples were left at 37 ℃ for 16 hrs. They were then spun, and the supernatants were collected and prepared for Zip Tipping by adding TFA 0.1% volume-to-volume ration.

2.14 Peptide concentration ZipTip

Zip tips (Millipore® Ziptips, Sigma-Aldrich, 0.6 μL C18 resin, volume 10 μl) were wetted in acetonitrile solution and then equilibrated with 0.1% TFA HPLC water solution. The binding of the sample was ensured by fully pressing the pipette a minimum of 10 folds in the digest tube. The membrane was then washed in a 0.1% TFA HPLC water solution. Sample elution was performed using 10 μL of elution buffer 0.1% TFA volume to volume ration in HPLC water/acetonitrile one-to-one ration.

Digested CWPs were spotted on a stainless-steel target plate (Opti-TOF TM 384 Well Insert, 128x81 mm RevA, Applied Biosystems). BSA digests were as well spotted and utilized as a standard solution. The sample and BSA digest spots were laid over with α-cyano-4-hydroxy-cinnamic acid matrix solution (10 mg CHCA matrix in 50% acetonitrile with 0.1% TFA) and air-dried. MALDI-TOF-TOF MS spectra were 27 acquired on 4800 MALDI-TOF-TOF analyzer (operated by the 4000 Series Explorer software version 3.7). The instrument was externally calibrated using TOF/TOF Calibration Mixture (Mass Standards Kit for Calibration of AB SCIEX TOF/TOF™ Instruments). MS reflector positive mode at a laser intensity of 2500 was set at acquisition method. The selected mass range was 499 Da-2500 Da with a focus mass of 1500 Da. Reflector positive default was used as a processing method with a minimum signal-to-noise ratio of 5. The resulting mass lists were manually scanned for known contaminant mass peaks, including keratin, matrix, and trypsin autolysis. The identified contaminant mass peaks were used to create an exclusion list that was applied in the interpretation method for the MS/MS data acquisition. The minimum signal-to-noise filter for the monoisotopic precursor selection for MS/MS was also set at 5. “Strongest precursors first” option was selected for precursor sorting order per spot, and “weakest precursors first” option was selected for MS/MS acquisition order

per spot with a maximum of 30 precursors per spot for each. MS/MS 1kV positive was utilized as an MS/MS acquisition method with a fixed laser intensity of 3500 and a precursor mass of 1570.677 Da. CID was set “on” with specifying medium gas pressure and air gas type. Metastable suppressor was also assigned “on”. MS/MS positive default was utilized as an MS/MS processing method with a signal-to-noise threshold of 5 for monoisotopic peaks.

2.15 Protein identification

MS/MS Ion Search was performed using the contaminants and CRAP databases to remove any additional contaminants whose peaks were not included within the exclusion list. The peptide and fragment tolerance values were set at ±2 Da. Conversely, the default settings of the 4800 MALDI-TOF-TOF analyzer were used where the resolution per mass peak as displayed by the machine is on average 4000, which is lower than the preferred acceptable values, even though this may seem too tolerant. These settings were considered as another limitation of the machine. Thus, we had to select a slightly more lenient tolerance level. Carbamidomethyl C was chosen as a fixed modification, whereas Oxidation at M was selected as a variable modification. Up to two missed cleavages were permitted for trypsin. A peptide charge of 1+ was assigned and MALDI-TOF-TOF was picked in the instrument type option. After these parameters were set, the data file was selected and searched (Khoury et al., 2018).

Peptide identification was performed by searching on Protein Pilot 4.5 software Revision 1656 (AB SCIEX) using the Paragon database search algorithm (4.5.0.0.1654). Furthermore, the MS/MS spectra obtained were searched by using the following parameters: trypsin specificity,

Cys-carbamidomethylation, and the search effort set to thorough; the obtained data was blasted against a protein sequence database from UniProtKB (Truong et al., 2016).

2.16 Statistical analysis

Statistical analysis was performed using the GraphPad Prism version 9.1 software. For the ergosterol content and chitin quantification experiments, Brown-Forsythe test comparison tests were performed to compare the data obtained for each category (sensitive or resistant to fluconazole) with that of the data obtained from the reference SC5314. An ordinary two-way ANOVA comparison tests were performed to compare the datasets of fluconazole-sensitive versus fluconazole-resistant isolates. Furthermore, P values below 0.05 were considered statistically significant. For murine study of disseminated candidiasis, a Kaplan Meier curve was generated and logrank test for trend was used to assess the significance of the survival curves.

2.17 Protein-Protein interactions

The STRING database (version 11) was used to generate a protein–protein interaction prediction map of detected proteins (Szklarczyk et al., 2019). The confidence score and false discovery rate (FDR) stringency were set at 0.400 and 5%, respectively, and the chosen organism was *Candida albicans*. The STRING database was also used to highlight proteins with the following biological processes: cellular response to drug, response to fungicide, and/or ergosterol biosynthetic pathway (GO: 0006696).

Chapter III

Results

3.1 Fluconazole-Resistant isolates exhibiting an increase in ergosterol content compared with fluconazole-sensitive isolates and the control

The ergosterol content of the plasma membrane of *C. albicans* was measured, since its increase was previously observed in many fluconazole resistant isolates in the literature. We observed that two out of the three *C. albicans isolates*, R2 and R3 isolates exhibited close to a two-fold increase in membrane ergosterol content while R1 showed a slight decrease compared to the reference isolate SC5314. Within the fluconazole sensitive group of isolates, one (S3) out of three *C. albicans* showed a significant 230% increase in cell wall ergosterol content while S1 and S2 showed a 13% and 45% decrease respectively **(Figure 10. A)** when compared to the reference SC5314**.** These results suggest that the S3 sensitive isolate is behaving as would be expected from a heteroresistant isolate. In this experiment, this isolate revealed an increase in ergosterol content , a common property observed among fluconazole resistant isolates.

Figure 10.B illustrates the average % change in ergosterol content for both fluconazole-sensitive and resistant groups compared to the reference SC5314. Analysis of the boxplot graph revealed a significant 53% increase in ergosterol content at the level of the plasma membranes of fluconazole-resistant isolates when compared to the susceptible isolates.

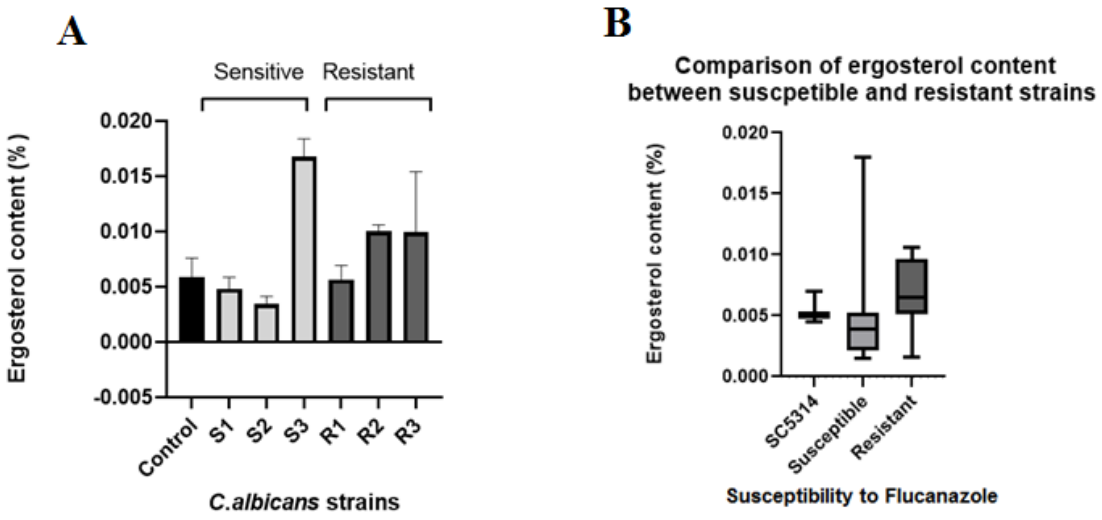

Figure 10. Ergosterol content.(A) The black bar graph represents the *C. albicans* reference SC5314, control, while the light grey bar graphs represent the fluconazole-sensitive *C. albicans* isolates, and the dark grey bar graphs represent the fluconazole-resistant *C. albicans* isolates. For all isolates and the control, quantification of ergosterol amounts were performed in biological triplicates. **(B)** Boxplot graph was generated to represent the % change in ergosterol content in fluconazole-sensitive and fluconazole-resistant clinical *C. albicans* isolate groups and upon comparison with the control, SC5314. For all isolates, the experiment was performed in biological triplicates.

3.2 Fluconazole-Resistant isolates exhibiting less virulence when compared with susceptible isolates and the control

The virulence potential of the six clinical isolates and the control, SC5314, as well as their progression of infection was evaluated to investigate a possible correlation between fluconazole resistance and systemic candidiasis. A Kaplan Meier curve was generated and presented in **(Figure 11. A),** showing mice survival curves. Ten out of the 18 mice injected with the sensitive isolates were moribund by day 13, where only five out of 18 mice injected with resistant *C. albicans* isolates were moribund during the same time interval. Moreover, during the virulence

assay S3 sensitive isolate had attenuated virulence a property to inherent to fluconazole resistant isolates.

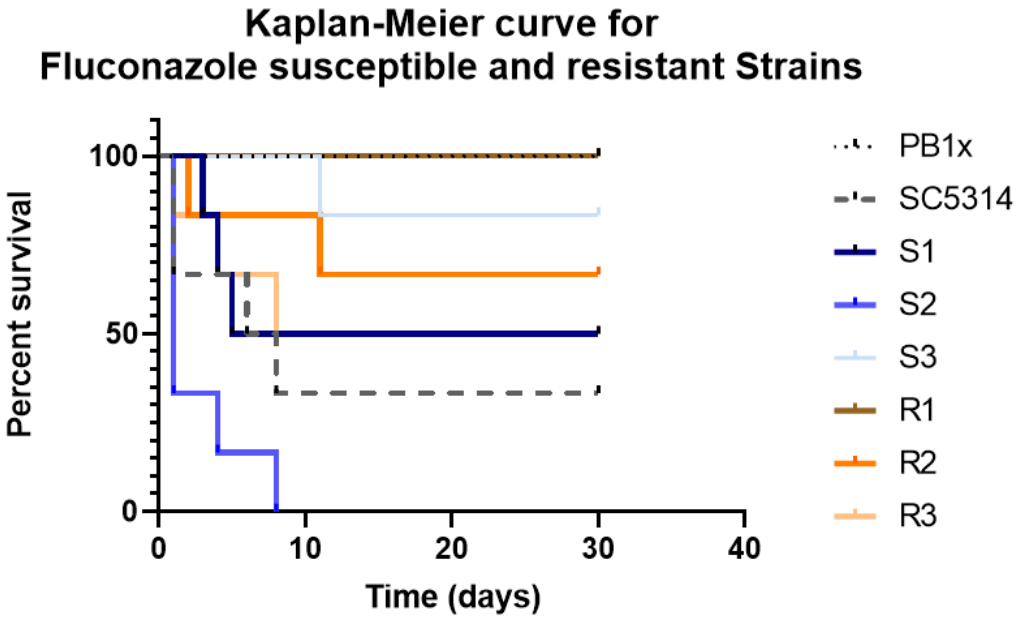

Figure 11. Kaplan Meier curve. Virulence potential in a murine disseminated model of infection. BALB/c mice were injected with 10^7 cells of SC5314 and fluconazole-sensitive and resistant *C. albicans* isolates and monitored for survival over 30 days. The survival rate in most fluconazole-susceptible and all resistant isolates was higher compared to the control. Resistant isolates exhibited attenuated virulence compared with the sensitive isolates.

(Figure 12) showed that the fluconazole-susceptible isolates group are twice as virulent compared to the resistant isolates and to the control, SC 5314, respectively. Overall, our data analysis shows that fluconazole resistance is associated with decrease in virulence.

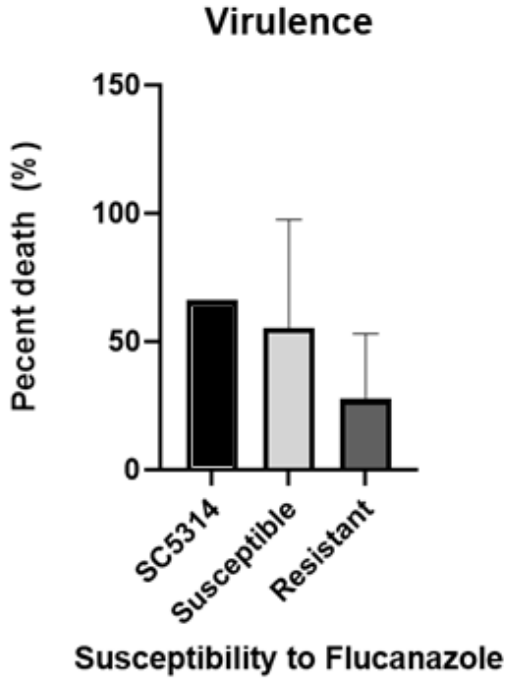

Figure 12. Virulence potential comparison. Bar graph comparing the percent death of mice injected with the control strain, fluconazole-susceptible and resistant isolates. Black bar graph represents the control SC 5314.The light and dark grey bar graphs represent the fluconazole-susceptible and resistant isolates groups, respectively.

3.3 Fluconazole-Resistant isolates showing a decrease in potential of biofilm formation

The biofilm formation potential of all *C. albicans* isolates was assayed to determine the relationship between biofilm formation and fluconazole resistance. All fluconazole-sensitive isolates S1, S2 and S3 exhibited a 7%, 39%, 21% decrease in the potential of biofilm formation when compared to the reference SC5314, respectively. Within the fluconazole-resistant group of isolates, two out of the three (R2 and R3) revealed a 44% and 65% decrease in biofilm formation

potential, respectively, when compared to the reference SC 5314.Yet, R1 fluconazole-resistant isolate showed (x~1.4) folds increase in the potential of biofilm formation when compared to the reference and (x~1.1) increase when compared to the azole-susceptible isolate group **(Figure 13A).**Interestingly, S3 sensitive isolate showed more biofilm formation when compared to the median of the resistant isolates, similar to what you would expect for fluconazole sensitive isolates. Moreover, in the boxplot of (**figure 13.B),** the median of biofilm formation in fluconazole-resistant isolates showed 48% and 39% decrease in the potential of biofilm formation when compared to the reference SC 5314 and the fluconazole-susceptible isolates group, respectively. Thus, fluconazole-resistant isolates have, on average, a decreased potential of biofilm formation.

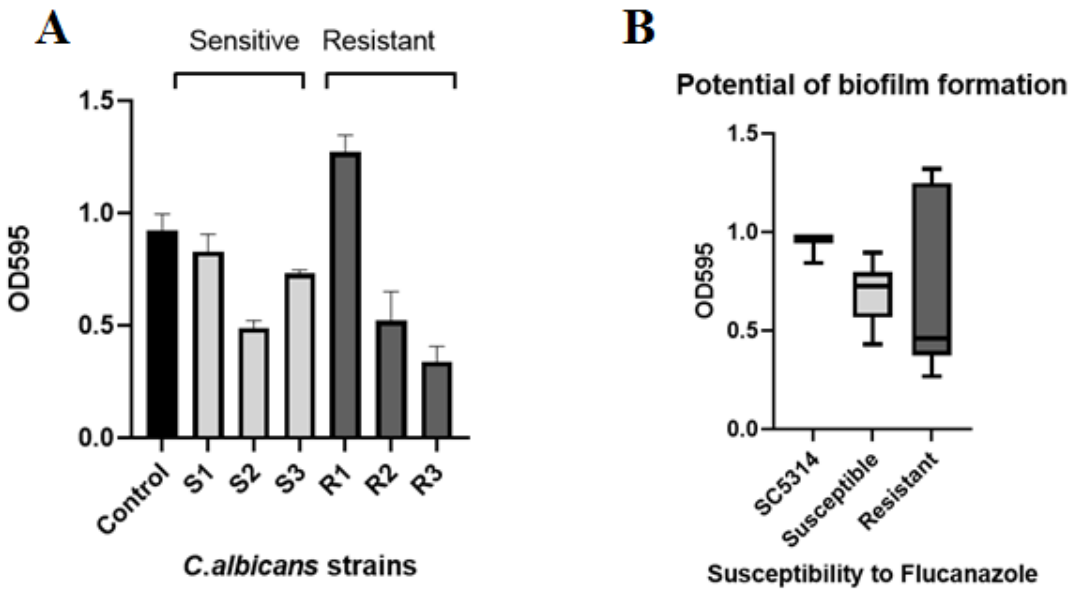

Figure 13. Biofilm formation. (A) Biofilm formation is represented as measurements of optical density at 595 nm (OD595) for all *C. albicans* isolates. The black bar graph represents the *C. albicans* reference SC5314, the light grey bar graph represents the fluconazole-susceptible *C. albicans* isolates assayed in this study, and the dark grey bar graph represents the fluconazole resistant. **(B)** Boxplot graph was generated to compare the potential of biofilm

formation in fluconazole-resistant clinical *C. albicans* isolate groups with that of the reference SC 5314 and the fluconazole-susceptible isolates group. Resistant isolates exhibited a less potential of biofilm formation. For all isolates, the experiment was performed in biological triplicates.

3.4 No correlation found between cell wall disruption and fluconazole resistance

All six clinical isolates in this study and the reference SC 5314 were spotted on potato dextrose agar plates in 10x serial dilutions (10^2 to 10^5 cells) in the presence or absence of SDS (0.025%) a cell surface solubilizing and disrupting agent **(Figure 14 A and 14B),** in order to determine cell surface rigidity measured in terms of cell growth. Both groups exhibited a higher degree of cell wall rigidity compared to the control SC5314. No difference in colony growth was observed between the two groups, however, as growth was present at all concentrations. Increasing the SDS concentration to 0.05% resulted in no growth for all isolates (data not shown). Our results do not show any specific relationship between fluconazole resistance and loss of cell wall integrity.

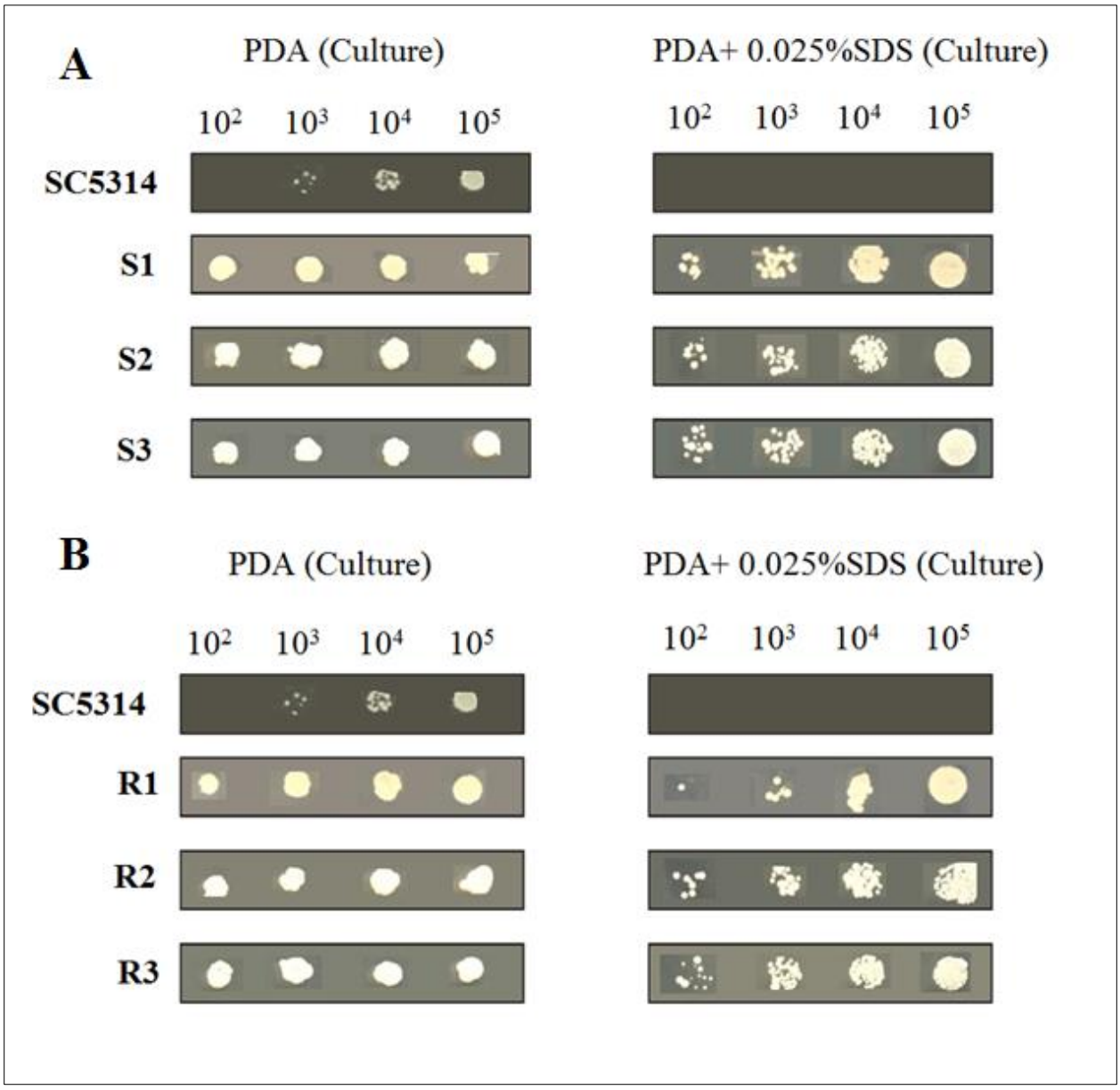

Figure 14. Cell surface integrity assay. **(A)** Fluconazole-sensitive isolates were spotted on PDA plate with or without SDS. **(B)** Fluconazole-resistant isolates spotted on control and SDS supplemented plates. No discrepancy was observed between the two groups. For all isolates, the experiment was performed in biological triplicates.

3.5. Fluconazole-Resistant isolates exhibited a small increase in cell wall chitin content

Cell wall chitin content was quantified to investigate a possible link between fluconazole resistance and increased cell wall thickness. Two out of three (S1 and S3) fluconazole-susceptible isolates exhibited an increase of 34% and 32% in cell wall chitin content while (S2) showed a 15% decrease when compared to the reference SC5314. On the other hand, all fluconazole-resistant isolates (R1, R2, and R3) showed 53%, 31% and 42% higher chitin concentration compared to SC5314, respectively **(Figure 15 A)** Moreover, S3 sensitive isolate exhibited a chitin content value similar to the median of cell wall chitin content in fluconazole resistant isolates**.** This isolate thus behaved as a fluconazole resistant isolate. A boxplot representation **(Figure 15 B)** showed that fluconazole-resistant isolates have on average 0.2 mg/ml of cell wall chitin while susceptible isolates revealed 0.18 mg/ml. Thus, resistant isolates exhibited a 10% increase in cell wall chitin content compared to the susceptible group.

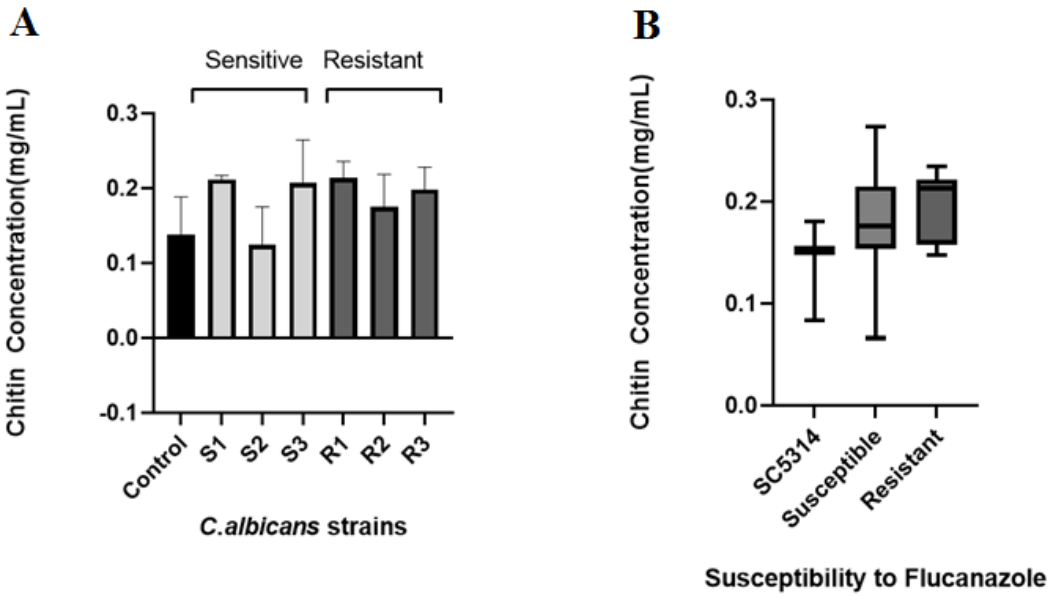

Figure 15. Cell wall chitin content. (A) Chitin content is represented as a concentration in mg/mL for 100 mg of wet weight cell wall for all *C. albicans* isolates. The black bar graph represents the reference *C. albicans* SC5314, the light grey bar graph represents the fluconazole sensitive *C. albicans* isolates, and the grey bar graph represents the fluconazole resistant *C. albicans* isolates. **(B)** Boxplot represents the chitin concentration at the level of the cell wall of fluconazole susceptible and fluconazole resistant clinical *C. albicans* isolate groups when compared to the *C. albicans* reference SC5314. For all isolates, the experiment was performed in biological triplicates.

3.6 Fluconazole resistant isolates are more adhesive than the susceptible isolates

Isolates were grown on PDA and washed with tap water to remove non-adhesive cells. Sensitive isolates S1 and S3 showed mild to no adhesion, respectively, whereas S2 was adherent to the PDA medium when compared to the Control SC5314.Interestingly, S3 sensitive isolate exhibited adhesive properties in line with most fluconazole sensitive isolates. Moreover, all fluconazole resistant isolates R1, R2 and R3 showed a noticeable adhesive attribute when compared to the susceptible isolate group. Hence, we concluded that fluconazole resistance does not impair the adhesive properties of resistant isolates.

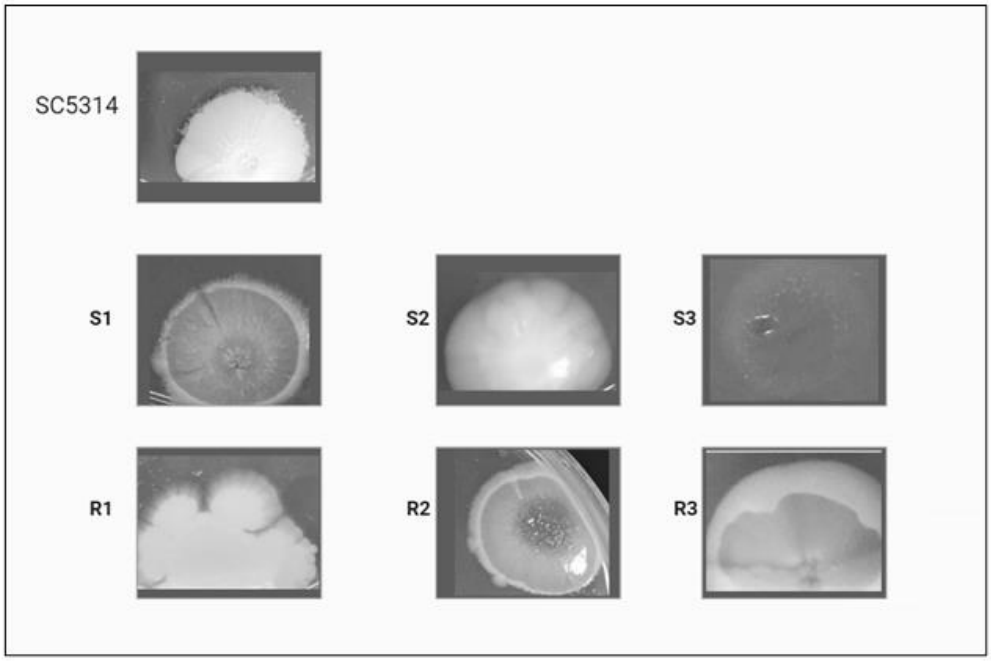

Figure 16. Adhesion assay. All Six *C. albicans* isolates and the reference, SC5314, were assayed for adhesion by growth on PDA media for 14 days at 37 °C followed by washing with water. Adhesive isolates were able to remain adherent to PDA, while non-adhesive isolates were washed away.

3.7 Fluconazole resistance slightly decreasing the growth kinetics of the *C. albicans* resistant isolates.

The growth kinetics of all *C. albicans* isolates was tracked over a period of 10 hrs to observe a possible correlation between growth fitness and fluconazole resistance. Two out of three fluconazole-susceptible isolates (S1 and S2) exhibited a 42% and 65% decrease in doubling time when compared to the reference SC 5314. However, S3 susceptible isolate revealed a 30% increase compared to the same control. On the other hand, the R1 fluconazole-resistant isolate exhibited a slight 2% decrease in its growth when compared to the reference SC 5314 while the other isolates R2 and R3 showed a remarkable decrease up to 57% and 62% respectively **(Figure 17 A).** Moreover, sensitive isolate S3 revealed fast growth kinetics as observed in other fluconazole sensitive isolates when compared to the fluconazole resistant isolate group. On average, whiskers boxplots **(Figure 17 B)** showed that the fluconazole-susceptible isolate group exhibited, on average, a 39% decrease in growth rate while the fluconazole-resistant isolates group exhibited a 58% decrease compared to the same reference. Subsequently, resistance to fluconazole in our isolates resulted in a 19% decrease in growth rate. However, the difference between the two isolate groups was not significant (P-value more than 0.005). Thus, no significant difference between the growth rate of fluconazole-resistant and susceptible isolate groups was determined and azole resistance does not significantly alter the growth kinetics of our resistant isolates.

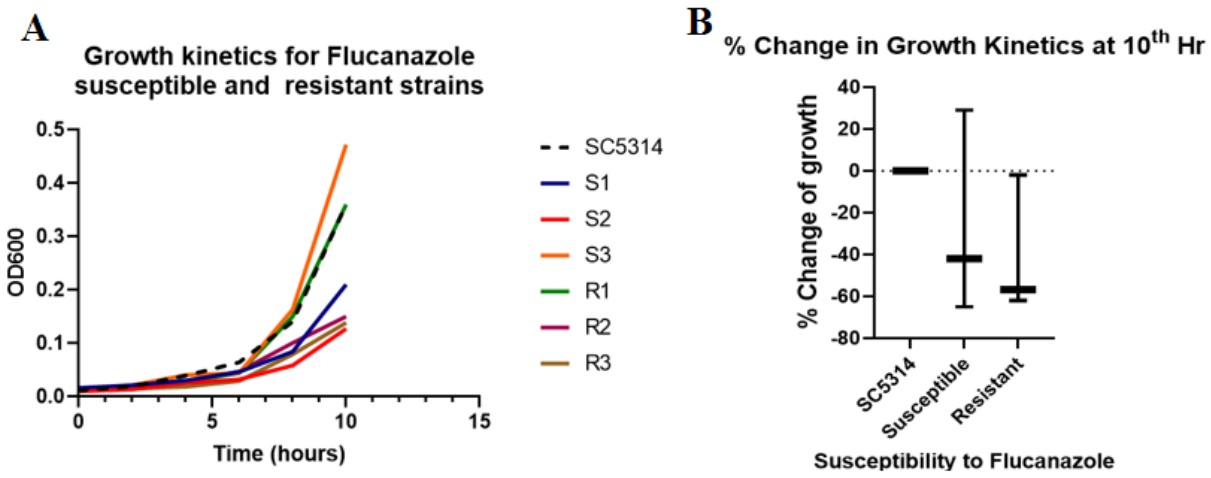

Figure 17. Growth kinetics. (A) Growth is represented as measurements of optical density at 600 nm (OD600) for all *C. albicans* isolates every 2 hours during five intervals (10hrs). **(B)** The % change in growth kinetics at the 10hr of culture for the control and fluconazole-susceptible and resistant isolate groups is represented in the whiskers box plot.

Cell wall proteome profiling of fluconazole susceptible and resistant isolates in the presence or absence of fluconazole

Despite the limitations of the 4800 MALDI TOF/TOF Analyzer (applied biosystems), we identified proteins in both fluconazole-susceptible and resistant *C. albicans* isolates when cultured in PDB media, with or without fluconazole exposure. Proteins were identified using protein pilot search, where matching scores ranged between 28 and 11.

3.8 Proteins involved in adhesion

Two cell wall proteins Pga1 and Big1 were identified with related function to adhesion of *C. albicans.* Two resistant and one sensitive isolate displayed adhesins in the absence of azole. Interestingly, no adhesins were found in the sensitive isolate in the presence of azole, where most of resistant isolates revealed an adhesin. However, the decrease in number of adhesins in the susceptible group mirrors the phenotypic data that suggests that resistant isolates are more adhesive **(Table 1).**

Treatment	Susceptibility to fluconazole	Strain	Protein Accession	Name of Gene	Protein Description	Score
Without Fluconazole	Susceptible	S1	Q5ACL7	***PGA1***	Predicted GPI anchor protein	13
	Resistant	R1	Q5ACL7	***PGA1***	Predicted GPI anchored protein	12
		R2	Q59WG7	***BIG1***	Endoplasmic reticulum protein	19
With Fluconazole	Susceptible	***Absence of hits***				
	Resistant	R2	Q59WG7	***BIG1***	Endoplasmic reticulum protein	20
		R3	Q59WG7	***BIG1***	Endoplasmic reticulum protein	17

Table 1. Proteins involved in adhesion. Protein hits were observed more in fluconazole-resistant *C. albicans* isolates, as opposed to the susceptible isolate group in both the absence and presence of the azole drug. Proteins identified using protein pilot are represented with their matching scores and their accession numbers.

3.9 Proteins involved in cell wall integrity and chitin content

In the absence of fluconazole, one protein involved in ensuring cell wall integrity, Pga1, was deteced in S1 susceptible isolate while the other resistant and susceptible isolates did not exhibit any CWPs with similar characteristics.

When isolates were treated with fluconazole, we observed an increase in the detection of CWPS that plays a vital role in the rigidity of the cell wall in both isolate groups (susceptible and resistant). Four CWPs involved in ensuring the integrity of the wall were identified in the presence of the drug. In S2 susceptible isolate we detected Phr1 and Pga14 while R2 and R3 resistant isolates revealed the presence of Rho1 and Rot1, respectively. Furthermore, the latter isolate revealed the presence of an important protein for chitin synthesis, Chs1 **(Table 2).**

Treatment	Susceptibility to fluconazole	Strain	Protein Accession	Name of Gene	Protein Description	Score
Without Fluconazole	**Susceptible**	**S1**	Q5ACL7	***PGA1***	Putative GPI-anchored protein	13
With Flucanazole	**Susceptible**	**S2**	P43076	***PHR1***	pH-responsive surface glycosidase	16
			Q5A4X8	***PGA14***	Hydrophilin	16
	Resistant	**R2**	Q5A200	***RHO1***	Small GTPase of Rho family	13
		R3	P23316	***CHS1***	Chitin Synthase I	20
			C4YHM2	***ROT1***	Protein rot precursor	19

Table 2. Proteins involved in cell wall integrity and chitin synthesis. Proteins were identified in the fluconazole-susceptible and resistant *C. albicans* isolates. Proteins identified using protein pilot are represented with their matching scores and their accession numbers.

3.10 Efflux pumps and transporters

In the absence of fluconazole, none of the susceptible isolates showed the presence of pumps or exporters at the level of their cell wall proteome. However, in R1 we have detected one major facilitator superfamily transporter Nag 4 within the same growth condition.

In the presence of fluconazole, we were able to detect three important efflux pumps in the resistant group, and from two different superfamilies: ABC (ATP binding cassette), Cdr1 and Cdr2, and major facilitator superfamily, Nag 3. These proteins play significant roles in fluconazole resistance upon the exposure of fluconazole and reduce the intracellular concentration of the azole drug. No efflux pumps were detected in the sensitive isolates including the heteroresistant S3 isolate **(Table 3).**

Treatment	Susceptibility to fluconazole	Strain	Protein Accession	Name of Gene	Protein Description	Score
Without Fluconazole Treatment	Susceptible			*Absence of hits*		
	Resistant	R1	Q59RG0	*NAG4*	Major facilitator superfamily multidrug transporter	14
With Fluconazole Treatment	Susceptible			*Absence of hits*		
	Resistant	R1	Q5ANA3	*CDR1*	Pleiotropic ABC efflux transporter of multiple drugs	19
		R3	Q9P457	*CDR2*	Multidrug resistance protein CDR	18
		R3	A0A1D8PQG0	*NAG3*	Major facilitator superfamily multidrug transporter	14

Table 3. Efflux pumps and transporters. Transporter CWPs were identified mostly upon the presence of fluconazole in most resistant *C. albicans* isolates, but not in sensitive isolates. Proteins identified using protein pilot are represented with their matching scores and their accession numbers.

3.11 Proteins involved in ergosterol biosynthesis and iron acquisition

None of the sensitive isolates, including S3, revealed proteins that are involved in ergosterol biosynthesis in the absence of the azole drug. However, one of the fluconazole-resistant isolates revealed the presence of Erg9 and subsequent increase in the translocation of ergosterol into the plasma membrane in the absence of fluconazole treatment. Interestingly, this isolate also exhibited the presence of a cytosolic protein Atm1, which allows for increased iron acquisition ability and enhances further resistance.

On the other side, upon fluconazole treatment, we detected in both S1 and R1 Erg3 and Erg6 CWPs which play vital roles in ergosterol biosynthesis **(Table 4.)** However, resistant isolates reveal more ergosterol biosynthesis enzymes, a result that mirrors our ergosterol content assay.

Treatment	Susceptibility to fluconazole	Strain	Protein Accession	Name of Gene	Protein Description	Score
Without Fluconazole	**Susceptible**			***Absence of hits***		
	Resistant	**R2**	P78589	***ERG9***	Squalene synthase	21
With Fluconazole	**Susceptible**	**S1**	O93875	***ERG3***	Delta(7)-Sterol 5(6) desaturase	20
	Resistant	**R1**	Q5AJG1	***ERG6***	Sterol-24-C-methyl transferase	20
		R2	Q59R09	***ATM 1***	Iron-Sulfur cluster transporter	27

Table 4. Proteins involved in ergosterol biosynthesis and iron acquisition. We observe an increased detection of *ERG* proteins in the fluconazole-resistant group when compared to the susceptible group in the presence and absence of fluconazole. Proteins identified using protein pilot are represented with their matching scores and their accession numbers.

3.12 Proteins involved in virulence

In the absence of fluconazole treatment, both susceptible and resistant isolates revealed the presence of CWPs that play a major role in immunogenicity and virulence of *C.albicans*. Interestingly, we observed proteins of the mannan family that belong to the *MNN2* gene family that play vital roles in virulence, as was previously discussed. Members of this group were more present in the more virulent isolates such as S2 and R3 .We detected in those isolates Mnn26 Mnn21, and Mnn24 CWPs . On the other hand, R1 and S3, both attenuated in virulence, did not exhibit any CWPs involved in virulence **(Table 5).**

Treatment	Susceptibility to fluconazole	Isolate	Protein Accession	Name of Gene	Protein Description	Score
Without Fluconazole	Susceptible	S1	Q5ANI6	***STP3***	Transcriptional regulator	15
		S2	Q5A9D4	***CSP37***	Hyphal Cell wall protein	18
			Q59R28	***MNN26***	Alpha1,2 Mannosyl transferase	28
			Q5A5U4	***RHD3***	Cell Wall Protein	16
			Q59KJ7	***MNN21***	Alpha=1,2-manoosyltransferase	18
	Resistant	R2	Q5AC08	***SAP6***	Candida pepsin6	23
			Q5A5U4	***RHD3***	Cell wall protein	16
			P0CY27	***MNN22***	Alpha-1,2 mannosyltransferase	19
			Q5A4E3	***OCH1***	Initiation-specific alpha-1,6-mannosyltransferase	16
		R3	Q5AD72	***MNN24***	Alpha-1,2 Mannosyl transferase	17

Table 5. Proteins involved in virulence in the absence of fluconazole. We observe an overall increase in the detection of CWPs, such as proteins of the Mnn family in the most virulent isolates in both fluconazole-resistant and susceptible isolates. Proteins identified using protein pilot are represented with their matching scores and their accession numbers.

Upon fluconazole treatment, CWPs involved in virulence, such as the *MNN2* gene family, were not present in azole-susceptible isolates even in the most pathogenic isolate S2. Only 3 proteins,

Sap6, Phr1 and Sap10 were identified in S1, S2, and S3, respectively. Hence, fluconazole exposure decreases the detection of CWPs involved in virulence in azole-susceptible isolates and subsequently impede their pathogenicity. On the other hand, all resistant isolates revealed proteins involved in virulence. The most virulent fluconazole-resistant isolate R3 revealed enhanced detection of proteins involved in virulence. Moreover, Pga26, Stp3, Kex2, Rsr1 and Ipp1 were identified in this isolate. Kex2 has a secondary vital role in phenotypic switching. In addition, we identified Sec9 in R2 resistant isolate and Tfp1 and Ipp1 in R2. Interestingly, Tfp1 null ioslates caused iron starvation and a decrease in fluconazole resistance (Jia et al., 2015). Overall, fluconazole exposure does not have a major effect on the detection of CWPs involved in virulence in our resistant isolates. Details about the mentioned proteins are shown in **(Table 6).**

Treatment	Susceptibility to fluconazole	Isolate	Protein Accession	Name of Gene	Protein Description	Score
With Fluconazole	Susceptible	S1	Q5AC08	***SAP6***	Candida-pepsin 6	19
		S2	Q94CL7	***PHR1***	PH-responsive protein 1	16
		S3	Q5A651	***SAP10***	Candida-Pepsin 1	22
	Resistant	R1	Q5AJB1	***TFP1***	V-type proteon ATPase catalytic subunit A	17
			P83777	***IPP1***	Inorganic phosphatase	28
		R2	Q59XP0	***SEC9***	Protein transport protein	19
		R3	Q5AA09	***PGA26***	Predicted GPI-anchored protein	12
			A0A1D8PEG3	***KEX2***	Kexin 2	18
			P52498	***RSR1***	Ras-related protein	20
			P83777	***IPP1***	Inorganic phosphatase	15

Table 6. **Proteins involved in virulence in the presence of fluconazole.** We observe an overall decrease in the detection of CWPs, involved in virulence, in susceptible isolates upon the exposure of fluconazole. However, resistant isolates within the same treatment conditions maintain the presence of virulence factors at the level of their cell wall proteome. Proteins identified using protein pilot are represented with their matching scores and their accession numbers.

3.13 Protein-Protein interactions

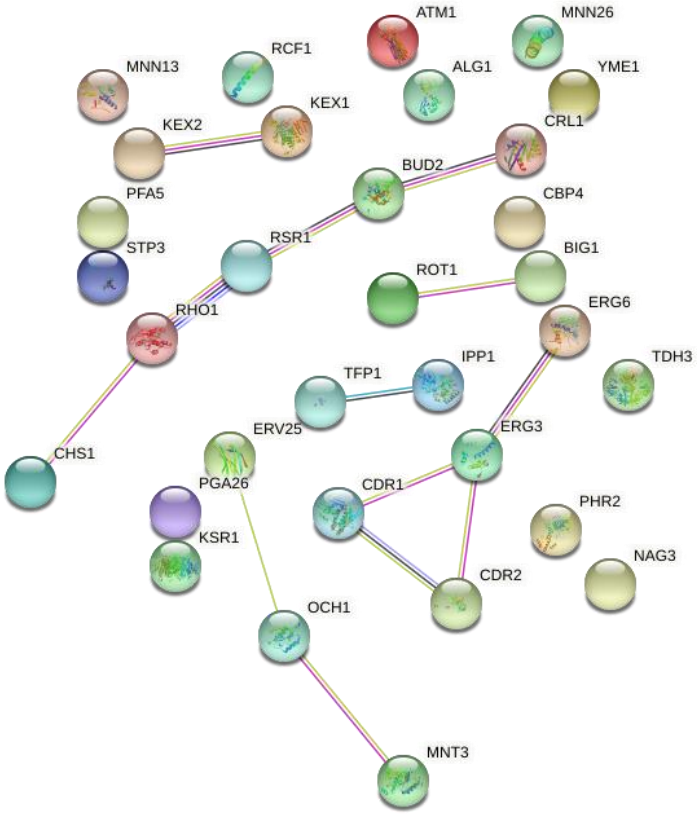

Figure 19. A prediction map of the protein–protein interactions in the fluconazole-resistant isolates group upon fluconazole exposure. The map showed proteins involved in biological processes related to antifungal therapy: cellular response to drug (Mnt3, Och1, pga26, Erg3, Erg3, Cdr1, and Cdr2), response to fungicide (Cdr1 and Cdr2), ergosterol biosynthetic pathway (Erg6 and Erg 3), and cell polarization and cytokinesis (Crl1, Bud2, Rsr1, Rho1 and Chs1).

Upon fluconazole exposure, the resistant isolate group exhibited interconnected pathways between ergosterol biosynthesis (Erg3 and Erg6) and enhanced ABC transporters activity (Cdr1 and Cdr2). Other protein interactions were noted in the pathway of cell wall integrity and mannosylation as a response to the drug (Mnt3, Csh1, and bud2).

Chapter IV

Discussion

In Lebanon, only a few studies have addressed the issue of azole resistance and investigated the public threat of antifungal resistance developed by *C. albicans*. To our knowledge, no study has attempted to correlate pathogenicity attributes with resistance phenotypes, and cell wall proteome remodeling in Lebanon. Therefore, one of our main objectives was to assess the phenotypic traits of fluconazole-resistant isolates, and establish their relationship with the cell wall proteome in the absence of the azole drug. We have successfully utilized mass spectrometry previously, in order to identify differentially expressed cell wall proteins in cell wall mutant isolates (El Khoury et al., 2018). Here, we utilize the same technique to identify genes differentially regulated between sensitive and resistant isolates, in the presence and absence of fluconazole exposure.

4.1 Upregulation of ergosterol content and mutations in *ERG11* that result in resistance

A main mechanism that results in the acquisition of resistance in *C. albicans* is the upregulation of sterol biosynthesis. Such upregulation increases plasma membrane sterol and negates the effect of fluconazole, resulting in resistance. Therefore, we measured the amount of membrane ergosterol in our isolates-- both resistant and sensitive-- and found a significant increase of 53% in resistant isolates. Such a finding explains the increased resistance observed. One sensitive isolate, S3, however, had a very high increase in ergosterol biosynthesis, in stark opposition to

the other two. However, after re-performing the antifungal resistance E-test, the isolate was found to be sensitive with very heavy trailing, an example of heteroresistance. The trailing effect in drug resistance implies colony growth within the inhibition zone-- albeit at a too low a concentration to warrant classification as resistant. This trailing effect could explain why our isolate behaved as a resistant one as far as ergosterol content.

In addition, point and frameshift mutations in *ERG11* result in resistance, since such mutations alter the tertiary structure of the protein preventing azole binding. We have recently sequenced the entire ORF of *ERG11,* and it has revealed many frameshift mutations in resistant isolates (Fattouh et al., 2021). The combination of increased plasma membrane sterol and decreased drug affinity can partially explain the resistance phenotype observed. Our proteomic data also partly supports such a mechanism, since Erg9 has been detected in R2, one of our resistant isolates, with no key Erg proteins detected in the sensitive isolates in the absence of azole exposure. This implies possible upregulation of ergosterol biosynthesis enzymes as a mechanism of resistance.

It has been also suggested that the need for iron increases during azole stress, especially given that iron is required by multiple enzymes acting in the ergosterol biosynthetic pathway (Sorgo et al., 2011). Moreover, iron depletion has induced susceptibility to fluconazole. Our proteomic profiling detected an interesting contaminant, cytosolic protein Atm1, in R2, the isolate which exhibited the presence of Erg9. Atm1 is a mitochondrial ABC transporter which plays an important role in iron metabolism and supply of sterol to the cytosol (Gaur M, et al., 2005). In addition, Pga10, known as Rbt8, a cell protein that aids iron acquisition has also been detected in R2. Hence, overexpression of ergosterol through the upregulation of ERG gene expression could

require an increased need of iron acquisition, explaining the presence of these 2 iron acquisition proteins.

Furthermore, Tfp1, a protein linked primarily to virulence, but which has also shown to be involved in iron homeostasis, is present in the R1 resistant isolate upon fluconazole exposure. Mutation in the *TFP1* gene is known to cause iron depletion (Jia et al., 2015). Expression of *TFP1* ensures that sufficient iron is acquired to act as a cofactor in ergosterol biosynthesis.

4.2 MFS and ABC transporters are more detected in fluconazole-resistant isolates

Another mechanism for acquiring fluconazole resistance is through the upregulation of membrane efflux pumps (Hampe et al., 2017), that pump out the intracellular drug. In our study, we were able to detect three major efflux pumps (Cdr1, Cdr2 and Nag3) in our fluconazole-resistant isolates. Nag3 is a major facilitator superfamily (MFS) efflux pump while Cdr1 is a pleiotropic ATP-binding cassette (ABC) efflux transporter that transport azoles out of the cell, thus, reducing drug accumulation intracellularly and allowing for resistance (Alarco et al.,1999). Moreover, Mdr1 an MFS transporter was detected in our resistant isolate R2 (Awad et al., 2021). Interestingly, no efflux pumps were detected in our sensitive isolate, including the heteroresistant isolate S3, suggesting that efflux pump upregulation could be an additional exclusive mechanism for resistance acquisition in our isolates.

4.3 Fluconazole-Resistant isolates exhibiting attenuated virulence

According to previous results, an inverse association between drug resistance and virulence in response to echinocandins exposure was revealed. Isolates resistant to caspofungin have presented a significant increase in cell wall chitin content, masking the virulence factors of these isolates and subsequently reducing their potential in virulence (Toutounji et al., 2019).

In this study, we observed a similar pattern where resistant isolates had attenuated virulence compared with the sensitive ones, as twice the number of mice were moribund in the sensitive isolate than the resistant one. Interestingly, resistant isolates also exhibit a slight increase in chitin content. Increasing chitin content renders the cell wall thicker and less flexible and hinders the deposition of CWP and virulence factors, resulting in decreased virulence. Increased chitin content also contributes to resistance, as it prevents the drug from breaching the cell surface and entering the cytoplasm. Such a mechanism was previously reported in echinocandin resistant *C. albicans* isolates whereby mutations in *FKS1,* required for beta glucan deposition, upregulated chitin content, resulting in increased resistance (Park et al., 2005). Interestingly, our R1 isolate, exhibited the least amount of ergosterol, yet the highest amount of chitin and was the least virulent among all resistant and susceptible isolates Thus, low sterol content was compensated by an increase in chitin amount. Our data, thus, suggests a correlation between increased cell wall thickness and attenuated virulence. Our proteomic data support such a hypothesis, since no virulence related proteins were detected in our R1 and S3 attenuated isolates, as opposed to S2 and R3 that were virulent and exhibited less chitin deposition, and whereby 5 proteins involved in virulence, such as members of the *MNN2* gene family, were isolated. This family of proteins is responsible for the addition of the initial α1,2-mannose residue onto the α1,6-mannose backbone, forming the *N*-mannan outer chain branches. α1,2-mannose is required for the stabilization of the

α1,6-mannose backbone and the consequent regulation of the mannan fibril length. These elongated microfibril structures are of great importance in immune recognition and virulence in *C. albicans*, where they play a role as PAMP complexes.

Moreover, our resistant isolates also exhibited a slight-- even though statistically insignificant-- decrease in growth kinetics and doubling times. This was also previously observed in echinocandin resistance and could be attributed to cell wall thickness, hindering budding and cytokinesis.

4.4 S3 isolate could be a fluconazole hetero-resistant isolate

Last but not least, throughout our work our S3 sensitive isolate behaved like a heteroresistant isolate in many of our assays. This isolate exhibited common pathogenicity attributes, such as high ergosterol and chitin content accompanied with attenuated virulence and low number of virulence factors, similar to what would be expected from fluconazole resistant isolates. On the other side, this isolate revealed some common phenotypic attributes as in fluconazole sensitive isolates. It showed higher biofilm formation and growth kinetics when compared to the median of the resistant isolate group as well as weak adhesive property. In addition, this isolate did not reveal major proteins involved in fluconazole resistance at the level of the cell wall. No major efflux pumps and ergosterol biosynthesis proteins were detected in this isolate. Hence, we classified this isolate as "heteroresistant" in light of the trailing effect and phenotypic profile.

Overall we suggest that the increased ergosterol biosynthesis, iron acquisition and upregulation of (ABC) transporters, and MFS efflux pumps play major roles in the development of fluconazole resistance in our isolates. A summary of the major molecular mechanisms and cell

wall proteins involved in fluconazole resistance in our resistant isolates are depicted in **(Figure 20).**

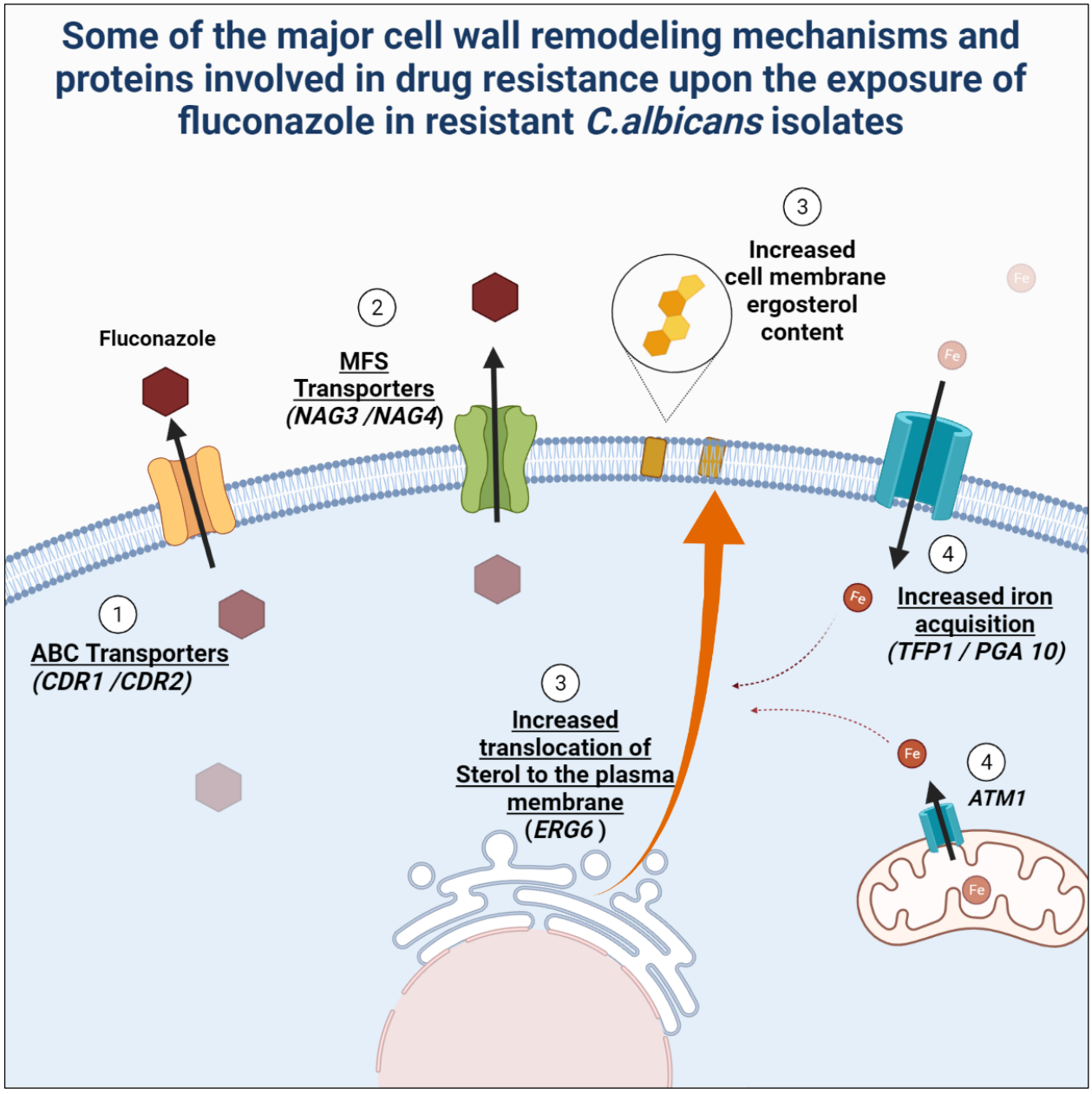

Figure 20. **Summary of the major proteins suggested to be involved in resistance**. Figure depicts detected proteins and molecular mechanisms involved in cell membrane remodeling, and the development of fluconazole resistance upon the exposure of the azole drug discussed in this study.

4.4 Fluconazole-Resistant isolates maintaining their adhesive properties in the absence of fluconazole exposure

Fluconazole-resistant isolates have increased adhesion to agar plates following washing, compared to susceptible ones. The most adhesive-resistant isolate, R1, showed the presence of Pga1, an important protein the role of which in adhesion was elucidated in a previous study A pga1 null isolate exhibited less oxidative stress tolerance and exhibited a 50% reduction in adhesion. Pga1 plays an important role in cell wall rigidity and stability which makes it an important protein needed for adhesion (Hashash et al., 2011).

4.5 Protein-Protein interactions

Our STRING data has shown pathways involved in growth, mannosylation, ergosterol biosynthesis, cell wall integrity and efflux of toxic azole concentrations, as a response to the exposure of fluconazole drug. Interestingly, these results show that increased ergosterol biosynthesis is coupled with the enhanced presence of efflux pumps as a mechanism of resistance. In addition, a link between chitin synthesis and budding was found, where the chitin synthase enzyme Chs1 needed for chitin biosynthesis, is also required for septation, which in

turn is probably needed for budding. The presence of Chs1 here, in our proteomic data, reflects our phenotypic data where a 10% increase in chitin deposition was found in the resistant isolates.

4.6 (ABC transporters) Cdr1 and Cdr2 efflux pumps are promising therapeutic targets to potentiate azole susceptibility in resistant *C. albicans* isolates

The fact that efflux pumps were only detected in resistant isolates is very interesting, as ABC transporters are responsible for high-level resistance of fungi to drugs, such as the fluconazole antifungals. These pumps have potential as secondary sites for chemotherapeutic and combination treatment interventions. ABC transporters contain two major domains: transmembrane domains (TMDs) and nucleotide binding domains (NBD). NBD domains share common orthologues with evolution-related species such as humans, which makes it an unfavorable target. However, TMD domains do have unique parts that are only found in fungi, especially in *Candida* species, making them extremely attractive as potential targets in drug discovery.

Several efflux pumps (Cdr1 and Cdr2) are druggable targets, as many inhibitors have been identified. There are several sites on the ABC efflux pumps that can be targeted. Drug efflux pumps are often localized in the plasma membrane and are extracellular targets. This means that drugs targeting extracellular portions of the pumps will not need to penetrate the cell membrane,

and consequently, will not be subject to outflow by fluconazole-resistant *C. albicans* (Holmes et al., 2016).

As of now, no fungal efflux pump inhibitors have entered advanced clinical trials. ABC inhibitors that target unique extracellular sites in TMD domains of the transmembrane ABC transporter Cdr1 and Cdr2 in fluconazole-resistant isolates, would be a quantum leap in treatment of *C. albicans* infections, and would result in favorable infection prognosis and an improvement in the well-being of hospitalized patients.

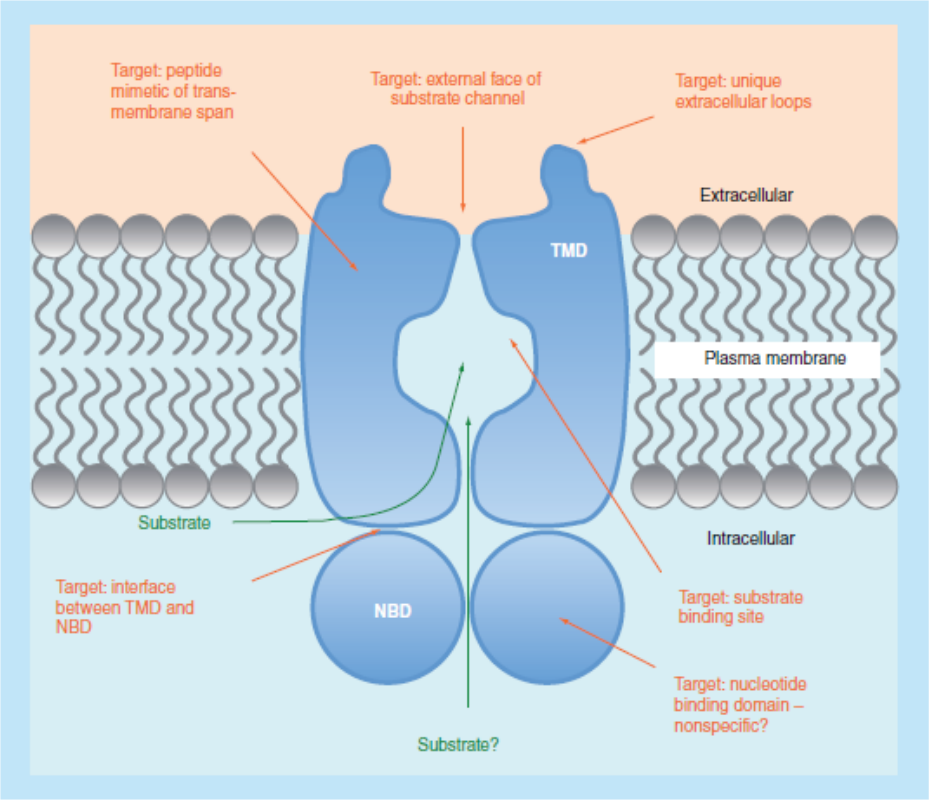

Figure 20. Possible drug targets of *C. albicans* ABC transporters. Transporters are composed of 2 main domains NBD and TMD. TMD domains are possible drug targets since they are unique to fungi and have no human orthologues. (Modified from Holmes et al., 2016).

4.7 Limitations

This study has two major drawbacks. The first is the low number of isolates analyzed with only 3 resistant and 3 sensitive isolates which affects statistical analysis. Future work should increase the number of isolates for statistical significance purposes. The second is the state of our mass spectrometer. Our aging MALDI TOF TOF MS/MS machine has been nonoperational for the past year and has witnessed multiple malfunctions at several intervals including problems with the gauge, followed by a weak vacuum pump and ionization source. These issues prevented the machine from correctly ionizing the concentrated peptides and transferring correctly along the field-free region, leading to inefficient detection. This hindered the efficiency of our work and resulted in low protein scores as well as fewer hits, resulting in a lack of confidence in our data. Moreover, the low efficiency of the MALDI to MS/MS has forced us to search our peptide sequences on protein pilot server. In fact, our protein scores were below the threshold 30. This implies the matches in our study are outside the 95% confidence level and are considered to lack major statistical significance.

Despite this experimental limitation, our data agrees with previous studies that address the molecular mechanisms of resistance displayed by *C. albicans* against fluconazole. The 3 resistant isolates show similar patterns of resistance to most *C. albicans* resistant isolates. These isolates show an increase in ABC transporters and ergosterol biosynthesis and decrease in virulence (Berkow & lockhart., 2017). Such simultaneous coupling of ergosterol biosynthesis and efflux pumps has been reported previously (Ren et al., 2014).

Chapter V

Conclusion and Insights

Our study has identified phenotypic differences between azole-resistant and sensitive isolates. Resistant isolates exhibited increased cell wall ergosterol deposition, chitin content, but attenuated virulence. Cell wall proteomic profiling showed the presence of efflux pumps exclusively in resistant isolates, an additional mechanism of resistance.

Future work should involve whole genome sequencing of resistant and sensitive isolates to reveal novel mutations in genes involved in the ergosterol biosynthetic pathway, which are specific to resistant isolates and correlate such mutations to pathogenicity attributes phenotypes. Furthermore, western blotting or RT-PCR should be performed to confirm the overexpression of detected proteins.

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