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PhD Thesis

Study of the effect of *Candida albicans* recombinant proteins and derived monoclonal antibodies on pro-tumoral processes and as diagnostic and therapeutic tools

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ABSTRACT

Candida albicans is a human commensal yeast that can sometimes cause life-threatening infections, especially in immunocompromised patients. The high mortality rates associated with this fungus make necessary the study of new diagnostic techniques and alternative therapies. In this PhD thesis, five proteins of the yeast (Adh1, Eno1, Ilv5, Kre9 and Qcr2) were produced as recombinant proteins, which showed ability to stimulate the hepatic endothelial cells, leading to an increase in tumor cell adhesion. Monoclonal antibodies against two of those proteins, Adh1 and Kre9, were also generated and tested against *C. albicans*. Those antibodies were able to reduce the *C. albicans* stimulatory effect on hepatic endothelial cells, reducing the tumor cell adhesion. On the other hand, one of the antibodies, anti-Kre9, showed ability to differentiate *C. albicans* from *Candida dubliniensis* and clinically relevant filamentous fungi. The other antibody, anti-Adh1, showed ability to effectively reduce yeast growth *in vitro*, to reduce the necessary antifungal dose to inhibit the fungus, and to increase survival in a *Galleria mellonella* animal infection model. The data presented here point out Adh1 and Kre9 proteins as relevant new candidates to be diagnostic and/or treatment targets to improve the outcome of patients suffering from *C. albicans* infections.

RESUMEN

Candida albicans es un hongo comensal en humanos que a veces puede causar infecciones que ponen en riesgo la vida, sobre todo en el caso de los pacientes inmunodeprimidos. La alta tasa de mortalidad asociada a este hongo hace que sea necesario el estudio de nuevas técnicas diagnósticas y de terapias alternativas. En esta tesis doctoral, cinco proteínas de esta levadura (Adh1, Eno1, Ilv5, Kre9 y Qcr2) fueron producidas de forma recombinante, mostrando ser capaces de estimular las células endoteliales del hígado, lo que indujo un incremento de la adhesión de células tumorales. Además, se generaron anticuerpos monoclonales frente a dos de estas proteínas, Adh1 y Kre9, y su efecto fue probado contra *C. albicans*. Estos anticuerpos fueron capaces de reducir la capacidad estimulatoria de *C. albicans* en las células hepáticas del hígado, reduciendo así la adhesión de células tumorales. Por otro lado, uno de los anticuerpos, el anti-Kre9, mostró ser capaz de diferenciar *C. albicans* de *C. dubliniensis* y de hongos filamentos de importancia clínica. El otro anticuerpo, el anti-Adh1, fue capaz de reducir el crecimiento de la levadura *in vitro*, de reducir la dosis necesaria de antifúngicos que inhibe el hongo, y de incrementar la supervivencia del modelo animal de *Galleria mellonella*. Los resultados presentados en este trabajo señalan a Adh1 y Kre9 como proteínas relevantes para ser evaluadas como nuevas candidatas a dianas diagnósticas y/o de tratamiento, con el objetivo de mejorar los pronósticos de los pacientes que padecen infecciones debidas a *C. albicans*.

LABURPENA

Candida albicans gizakion onddo komentsala da, eta batzuetan bizia arriskuan jartzen duten infekzioak sor ditzake, batez ere gaixo immunogutxituen kasuan. Onddo honekin erlazionatutako heriotza-tasa oso altuak direla eta, beharrezkoa da diagnostiko eta tratamendu teknika alternatibo berrien ikerketa. Doktoretza tesi honetan legamiaren bost proteina (Adh1, Eno1, Ilv5, Kre9 eta Qcr2) ekoiztu egin ziren proteina errekonbinante moduan. Proteina errekonbinante hauek gibel endotelio-zelulak estimulatzeko gai izan ziren, tumore zelulen atxikidura handipena eraginez. Bi proteina hauen, Adh1 eta Kre9aren, kontrako antigorputz monoklonalak ekoitzi ziren eta *C. albicans*en aurka zuten efektua frogatu. Antigorputz horiek *C. albicans*ek gibeleko endotelio-zeluletan duen efektu estimulatzailea murriztu zuten, tumore zelulen atxikidura gutxituz. Bestalde, antigorputz hauetako bat, anti-Kre9a, *C. albicans C. dubliniensis*etik eta garrantzi klinikoa duten onddo haritsuetatik ezberdintzeko kapaza izan zen. Beste antigorputza, anti-Adh1a, gai izan zen *in vitro* legamiaren hazkuntza murrizteko, onddoa inhibitzeko beharrezko antifungiko dosia murrizteko, eta *Galleria mellonella* animalia ereduan biziraupena emendatzeko. Lan honetan aurkeztutako emaitzek Adh1a eta Kre9a diagnostiko edota tratamendurako itu berri interesgarriak direla adierazten dute, *C. albicans* infekzioa pairatzen duten gaixoen pronostikoa hobetzeko asmoz.

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ABBREVIATIONS LIST

1-D	One dimensional
2-D	Two dimensional
Abs	Absorbance
ALCAM	Active leukocyte cell adhesion molecule
AMP	Antimicrobial peptide
APS	Ammonium persulfate
BMGY	Buffered glycerol-complex medium
BMMY	Buffered methanol-complex medium
BSA	Bovine serum albumin
BSI	Bloodstream infection
CBS	Centraalbureau voor Schimmelcultures
CCP	Cell-penetrating peptide
CDR	Complementarity-determining region
CEACAM	Carcinoembryonic antigen-related cell adhesion molecule
CECT	Spanish collection of type culture
CFSE	5-(and-6) carbocyfluorescein diacetate succinimidyl ester
CFU	Colony-forming units
CMC	Chronic mucocutaneous candidiasis
CR3	Complement receptor 3
CR3-RP	Complement receptor 3-related protein
CO_2	Carbon dioxide
CTL	Cytotoxic T lymphocyte
CVC	Central venous catheter
CWP	Cell wall protein
CWRU	Case Wester Reverse University
DC	Dendritic cell
DNA	Deoxyribonucleic acid
(D)PBS	(Dulbecco's) phosphate buffer saline
DTT	Dithiothreitol
EC	Endothelial cell
ECM	Extracelullar matrix

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmatic reticulum
EUCAST	European Committee for Antifungal Susceptibility Testing
FBS	Fetal bovine serum
FICi	Fractional inhibitory concentration index
FITC	Fluorescein isothiocyanate
GPI	Glycosylphosphatidylinositol
GUT	Gastrointestinally induced transition
HRP	Horse radish peroxidase
HUVEC	Human umbilical vein endothelial cells
IARC	International Agency for Research on Cancer
IC	Invasive candidiasis
ICAM-1	Intercellular adhesion molecule-1
ICU	Intensive care unit
IEF	Isoelectric focusing
Ig	Immunoglobulin
IIF	Indirect immunofluorescence
IL	Interleukin
IPTG	Isopropyl β-D-1-thiogalactopyranosidase
LB	Luria-Bertani
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LSLB	Low salt Luria-Bertani
LSEC	Liver sinusoidal endothelial cell
mAb	Monoclonal antibody
MAC	Membrane attack complex
MALDI-TOF/MS	Matrix assisted laser desorption ionization-Time of flight/Mass spectrometry
MCAM	Melanoma cell adhesion molecule
MES	2-(*N*-morpholino)ethanesulfonic acid
MDR	Multidrug resistant
MIC	Minimum inhibitory concentration
MIP	Macrophage inflammatory protein
MR	Mannose receptor

NCPF	National collection of pathogenic fungi
NET	Neutrophil extracellular traps
NO	Nitric oxide
PAMP	Pathogen associated molecular pattern
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PECAM1	Platelet endothelial cell adhesion molecule 1
Pir	Proteins with internal repeats
PMSF	Phenylmethylsulfonyl fluoride
PRR	Pattern recognition receptors
OD	Optical density
RNS	Reactive nitrogen species
RPMI	Roswell Park Memorial Institute
ROS	Reactive oxygen species
SEAL	Protein surface epitopes targeted by monoclonal antibody library
SB	Sabouraud broth
SDA	Sabouraud dextrose agar
SDS	Sodium Dodecyl Sulphate
SOD	Superoxide dismutase
TBSM	Tris buffered saline with milk
TCA	Trichloroacetic acid
TCC	Terminal complement complex
TNF	Tumor necrosis factor
TLR	Toll-like receptor
VCAM-1	Vascular cell adhesion molecule-1
WB	Western blot
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YNB	Yeast nitrogen base
YPD	Yeast Peptone Dextrose

INTRODUCTION

1.1. GENERAL CHARACTERISTICS OF *Candida albicans*

The yeast *Candida albicans* is a normal colonizer of the mucosal surfaces of humans. It can be found in the oral and genital cavities and in the gastrointestinal tract, and its carriage is estimated to vary from 30 to 70% in healthy individuals (Gow & Yadav, 2017). However, when the natural barrier of skin or tissues is damaged, the individuals are immunocompromised, or the microbial balance altered, *C. albicans* can produce infections. The caused diseases range from mucosal infections to invasive candidiasis (IC) depending on the immunosuppressive status and/or the severity of the underlying disease(s) of the patients. IC could be defined as bloodstream infection (BSI, also known as candidemia), and/or deep-seated candidiasis (Clancy & Nguyen, 2013). On the other hand, *C. albicans* is frequently isolated from the vagina of healthy women, and 75% of them will develop at least one episode of vulvovaginal candidiasis during the childbearing years (Sobel, 2005).

The genera *Candida*, which belongs to the *Saccharomycetaceae* family, accounts for more than 150 species, and about 20 of them are human pathogens (Ng *et al.*, 2015; Pfaller & Diekema, 2004). Since the 1980s, an increase of infections due to fungal agents, especially *Candida* spp., has been observed (Bailly *et al*., 2016; Martin *et al*., 2003). This is a consequence of the augmentation of the number of immunocompromised patients due to cancer treatments, transplants, HIV-infection, and to the use of invasive surgical procedures and devices, among others (Pfaller & Diekema, 2007). In fact, although the risk factors for developing IC vary between studies, the severity of underlying illness, extreme of ages (below 1 year old and above 65 years old), use of broad-spectrum antimicrobials, presence of central venous catheter (CVC), neutropenia and total parenteral nutrition are the most commonly observed (Baldesi *et al*., 2017; Cleveland *et al.*, 2015; Paiva *et al.*, 2016; Pfaller & Diekema, 2007; Rajendran *et al.*, 2016).

Candida spp. are the most frequently isolated fungus from healthcare-associated specimens. They are the fourth causative agents of BSIs in the United States (US) (Wisplinghoff *et al.*, 2004), fifth in Australia (Prowle *et al.*, 2011) and the fifth to tenth in Europe (Baldesi *et al.*, 2017; Bouza & Muñoz, 2008; Picazo *et al.*, 2008). The trends and patterns vary across the globe, but in general, a decrease of *C. albicans* isolates has been observed, with a concomitant increase of non-*albicans* species, especially of *Candida glabrata* in US (Ng *et al.*, 2015; Pfaller & Diekema, 2007; Rajendran *et al.*, 2016). This changing epidemiology is worrying as the non-*albicans* species are usually more resistant to the available antifungals (Bassetti *et al.*, 2016; Paramythiotou *et al.*, 2014). However, despite the observed shift towards non-*albicans Candida* species, the majority of the

studies still reported *C. albicans* as the principal causative agent of ICs (Andes *et al.*, 2016; Bailly *et al.*, 2016; Baldesi *et al.*, 2017; Cleveland *et al.*, 2015; Ng *et al.*, 2015; Paiva *et al.*, 2016; Rajendran *et al.*, 2016; Wang *et al.*, 2016).

The mortality related to *Candida* spp. is still very high, between 40 to 60% depending on the study (Baldesi *et al.*, 2017; Paiva *et al.*, 2016; Paramythiotou *et al.*, 2014; Wang *et al.*, 2016), which is higher than the reported for most bacterial infections (Paiva *et al.*, 2016). IC is also associated with a longer stay in the hospital, which in turn augments the cost related to this infection (Pfaller & Diekema, 2007). Although a decrease in the numbers of ICs was noted few years ago in two areas of US (Cleveland *et al.*, 2015), the incidence was very high even in this study (14.4 per 100,000 population), and an increase in candidemia incidence has been reported during the past decade in many non-US countries (Baldesi *et al.*, 2017; Lortholary *et al.*, 2014). Therefore, taking into account the aging of population and the increasing number of individuals at high-risk of IC (cancer, transplanted patients, etc.), there is a need to keep researching *C. albicans*.

1.1.1. MORPHOLOGY

Although commonly known as a dimorphic fungus because of its ability to switch between yeast and hyphae, *C. albicans* is able to form other different morphologies. Recently, Gow and Yadav (2017) have described this fungus as polymorphic (Fig. 1.1). Only the classic four morphologies of this fungus will be review here (yeast, pseudohyphae, hyphae and chlamydospore), but four alternative morphologies have been described; opaque (**a**/α) cells, grey cells, gastrointestinally induced transition (GUT) cells (these three are reviewed in Noble *et al.*, 2016), and Goliath cells (first described by Malavia *et al.*, 2017).

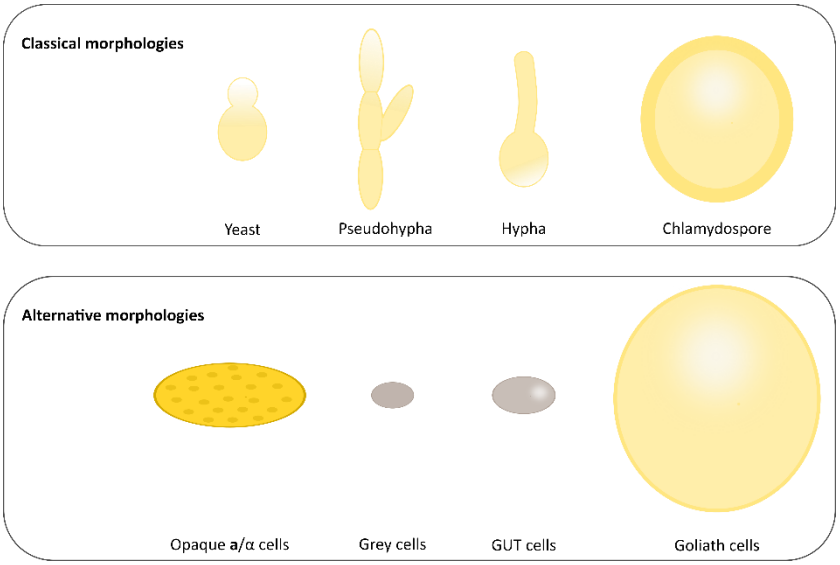


Figure 1.1. Scheme of all the described morphologies of *Candida albicans*. Apart from the classical morphologies - yeast, pseudohypha, hypha and chlamydospore -, others have been described such as the opaque cells or the recently found Goliath cells (Malavia *et al.*, 2017). *GUT= gastrointestinally induced transition.

1.1.1.1. Yeasts

Yeast cells are usually round in shape, although bean-shaped "opaque" morphologies have also been described (see below). They reproduce by budding and nuclear division takes places in the junction between mother and daughter cell (Noble *et al.*, 2016). After nuclear division and cytokinesis, mother and daughter cells are completely detached from each other (Sudbery *et al.*, 2004). Yeast growth switches between polarized and isotropic growth (Sudbery *et al.*, 2004), accounting for its round shape.

It was thought that yeast form was the commensal form of the fungus, and that the filamentous (hyphae and pseudohyphae) forms were the infective ones. However, the three morphologies have been found in disseminated candidiasis and are required for biofilm formation (Noble *et al.*, 2016).

1.1.1.2. Pseudohyphae

This cell morphology consists on elongated, ellipsoid-shaped cells that remain attached to each other after cytokinesis (Noble *et al.*, 2016). Constrictions are formed between mother-daughter cells, where the septa are located. The width of the pseudohyphal cell can vary

considerably, been always greater that the width of hyphal cells (Sudbery *et al.*, 2004). Similar to yeast, the nuclear division occurs in the mother-daughter junction (Noble *et al.*, 2016) and the growth varies between polarized and isotropic. However, in the pseudohyphal cells, the polarized growth period is larger than in yeast cells (Sudbery *et al.*, 2004; Whiteway & Bachewich, 2007). Culture temperatures above 35°C and neutral pH induce pseudohypal growth (Sudbery *et al.*, 2004).

Pseudohyphae have been described as a transition form between yeast cells and true hyphae (Carlisle *et al.*, 2009). However, it is believed to be a genuine cell type sharing more similarities with yeast cells that with hyphae, despite its filamentous shape (Sudbery *et al.*, 2004; Whiteway & Bachewich, 2007).

1.1.1.3. Hyphae

This cell type is a thin tube in shape. In this case, nuclear division takes place within the hyphal cell and one of the nucleus migrates back into the mother cell (Sudbery *et al.*, 2004). After cytokinesis, cells remain attached forming a filamentous structure called mycelia (Noble *et al.*, 2016). Opposite to the observed in pseudohyphal cells, this morphology has no constrictions at the mother-daughter junction, it has parallel sides and the growth is always polarized (Sudbery *et al.*, 2004).

The induction of yeast-to-hypha transition has been extensively studied, due to its role in fungal pathogenesis, and different inductors have been described, generally those encountered inside the host. Presence of *N*-acetylglucosamine (Castilla *et al.*, 1998), body temperature (37°C; Shapiro *et al.*, 2009), nitrogen starvation (Biswas & Morschhäuser, 2005), alkaline pH (Li *et al.*, 2004), blood serum (Feng *et al.*, 1999); phosphate limitation (Romanowski *et al.*, 2012), physiological CO_2 concentration (Klengel *et al.*, 2005), and bacterial peptidoglycan (Xu *et al.*, 2008), all triggered hyphal formation. Interestingly, it has been shown that molecules secreted by bacterial species that belong to the normal flora, and that therefore cohabitate with *Candida* in the human host, are able to suppress germ tube formation without inhibiting fungal growth (Boon *et al.*, 2008; Hogan *et al.*, 2004; Vílchez *et al.*, 2010).

C. albicans, along with *Candida dubliniensis*, are the only two species of *Candida* genera able to form true hyphae (Jackson *et al.*, 2009) and until its definition as a distinct species in 1995 (Sullivan *et al.*, 1995), *C. dubliniensis* was usually referred to as “unusual” *C. albicans*.

1.1.1.4. Chlamydospores

Chlamydospores are usually formed under harsh growth conditions. Their main characteristic is their large size (three to four times that of the yeast cell; Staib & Morschhäuser, 2006). They are spherical cells possessing a thick wall (Noble *et al.*, 2016). The wall is form by two layers, a thin outer layer and a thick inner layer (Jansons & Nickerson, 1970). Chlamydospores contain large lipid droplets and high quantity of RNA (Staib & Morschhäuser, 2006). These cells are formed by budding from the suspensor cells, which form at the tips of the mycelial filaments. As the bud that will become the chlamydospore grows, material is deposited inside (Jansons & Nickerson, 1970). In this case nuclear division takes place inside the suspensor parental cell and one of the nucleus then migrates to the forming chlamydospore (Martin *et al.*, 2005). After cytokinesis, the spore remains attached to the suspensor cell, and the outer layer of the chlamydospore forms a continuum with the suspensor cell's wall (Jansons & Nickerson, 1970). Chlamydospores are usually formed in the absence of a carbon source, and their formation is also influenced by the inoculum size, the age on the inoculum ad the age of the culture (Jansons & Nickerson, 1970).

For long time chlamydospore formation has been used for clinical identification of *C. albicans* (Jansons & Nickerson, 1970). Only *C. albicans* and *C. dubliniensis* are able to form these structures, although the chlamydospore induction conditions differ between the two species, being spore production more abundant in *C. dubliniensis*. Thus, chlamydospore formation is used as discrimination method for these related fungi (Staib & Morschhäuser, 2006). *C. albicans* chlamydospores are best induced in poor-nutrient media containing detergents, at room temperature, under microaerophilic conditions and in the dark (Martin *et al.*, 2005; Staib & Morschhäuser, 2006).

The clinical relevance of these spores is still unknown, as they have rarely been seen in infections (Noble *et al.*, 2016; Staib & Morschhäuser, 2006) and they do not seem to be able to survive for long periods of time (Whiteway & Bachewich, 2007). In fact, *in vitro* germination of chlamydospores has only been achieved with young spores (less than 48 h old; Jansons & Nickerson, 1970).

1.1.2. WHITE-OPAQUE SWITCHING

C. albicans cells are capable of switching back and forth between two phenotypes, white and opaque. This white-opaque transition was first described by Slutsky *et al*. in 1987. They found two types of colonies distinguishable by size, shape and color, due to differences in the cells forming the colonies. The "white" cells are round, similar in size and budding pattern to commonly used laboratory strains, and they form white, hemispheric colonies. On the other hand, "opaque" cells are elongated and have three times the volume of white cells, forming grey-opaque colonies. They found that opaque cells do not form hyphae, whereas white cells are able to form normal hyphae, and that at a low or high temperature (above 35°C) opaque cells switch to white-colony forming cells. In consequence, the difference between these two phenotypes extends to other traits such as adhesion and antigenicity. Since the discovery of these phenotypes in *C. albicans*, genes unique to one or another phenotype have been found (Srikantha & Soll, 1993).

The body temperature induces yeast cells to switch to white phenotype, thus the presence of opaque cells inside human body should be very low. However, certain niches inside the host where the pH is acidic, the level of CO_2 is high or where *N*-acetylglucosamine is present, may favor the switch to opaque state (Huang *et al.*, 2009, 2010; Sun *et al.*, 2015).

1.1.3. PARASEXUAL CYCLE

C. albicans was thought to reproduce only asexually until a mating type locus (MTL), similar to that of *Saccharomyces cerevisiae*, was found in 1999 by Hull & Johnson. However, the sexual cycle of this fungus differs from that of other related yeast such as *S. cerevisiae* (Bennett, 2015). First, *C. albicans* usually exists as diploid and the majority of cells are heterozygous for the mating alleles **a**/α. Therefore, one of the alleles must be lost in order to obtain homozygous **a** or α cells (Nielsen & Heitman, 2007; Zhang *et al.*, 2015). Second, opaque phenotype cells are much more efficient in mating than white cells (Miller & Johnson, 2002); thus, the white-to-opaque switching plays a pivotal role in *C. albicans* parasexual cycle. Third, the fusion between opaque **a** and opaque α cells leads to the formation of **a**/α tetraploids. So far, *Candida* has been shown unable to undergo meiosis. Instead, a concerted, random chromosomal loss takes place in its parasexual cycle in order to rescue the diploid -or nearly to- diploid state (Bennett & Johnson, 2003; Fig. 1.2), some strains being trisomic for one or more chromosomes (Forche *et al.*, 2008). Finally, although not having a conventional sexual cycle, *C. albicans* chromosomes also undergo recombinant events under the

control of Spo11 protein, a conserved protein that takes part in meiosis in other fungi (Forche *et al.*, 2008).

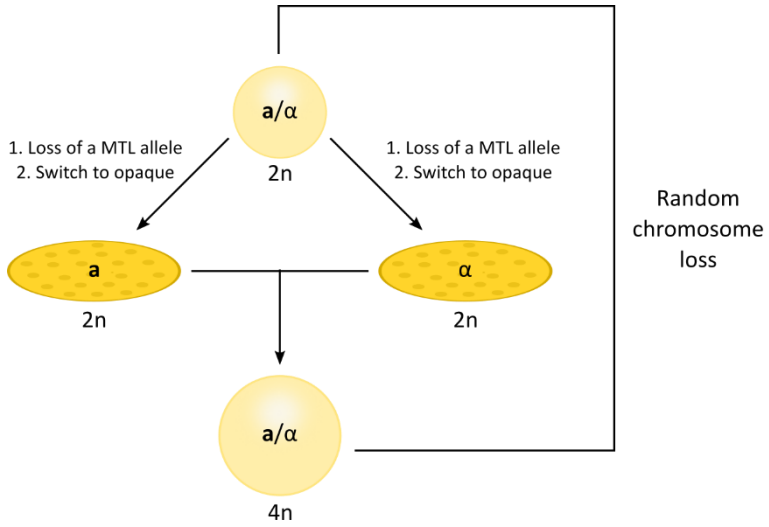


Figure 1.2. Scheme of the parasexual cycle of *Candida albicans*. White heterozygous cells must lost one mating type locus (MTL) allele and switch to opaque in order to mate. The mating of two opaque cells will give raise to an **a**/α tetraploid white cell that will become diploid after random chromosomal loss. Figure based on Bennett & Johnson, 2003.

A few exceptions to this parasexual cycle have been described. First, by the same mechanism of chromosome loss already mentioned, *C. albicans* can form haploid **a** and α cells that can switch to opaque cells, mate and restore in that way the diploid state (Hickman *et al.*, 2013). Second, same sex cells are able to mate under strong pheromone induction (Alby *et al.*, 2009).

Even though capable of mating, some studies have shown that *C. albicans* population is basically clonal (Bougnoux *et al.*, 2008; Tavanti *et al.*, 2004); and therefore, that the most common form of reproduction for this fungus is the asexual cycle. However, Zhang and co-workers (2015) have shown that mating is under selection, and that parasexual cycle can lead to the formation of progeny that is able to grow better than their ancestors. It has been suggested that the parasexual cycle is limited in *Candida* in order to favor the already existing allelic combinations that allow its commensal lifestyle (Nielsen & Heitman, 2007).

1.1.4. SEROTYPE

C. albicans strains are divided into two serogroups, A and B. These two serogroups were first described in 1961 (Hasenclever & Mitchell, 1961). The difference between them is due to differences in mannan structure. Acid-labile β-1,2-linked mannose oligosaccharides are present in both serogroups, whereas serotype A strain mannan contains additional β-linked residues in branching, acid-stable forms (Nelson *et al.*, 1991).

1.2. *Candida albicans* CELL WALL

The cell wall of *C. albicans* is one of the most important fungal organelles, as it is crucial for survival. It provides the physical form of the cell, protection, interacts with the environment and takes part in biofilm formation (Free, 2013). It is also the first part of the fungi that establishes contact with host's cells. In fact, the interaction between this fungus and the host's immune system is mainly mediated by cell wall components (Hall & Gow, 2013). In addition, this organelle is absent in mammalian cells and, therefore, it is a good target for antifungal drug development (De Groot *et al.*, 2004; Free, 2013).

The cell wall of *C. albicans* is a dynamic organelle that changes its composition to adapt to the different environments or host body sites, thus affecting its recognition by the immune system. The composition is different depending on the nutrient availability, growth phase, morphology, temperature, etc. For example, the percentage of chitin is increased and β-glucans are reduced in the mycelium in contrast to yeast cell wall (Ruiz-Herrera *et al.*, 2006).

Several layers have been described in the cell wall of *C. albicans* (Poulain *et al.*, 1978). The inner layer, mainly compose of polysaccharides -chitin and β-glucans-, gives to the yeast its structure, while the outermost layer is mainly compose of mannoproteins (Chaffin, 2008; De Groot, *et al*., 2005; Free, 2013; Ruiz-Herrera *et al.*, 2006). These layers do not differ as much in the nature of their components as in the proportion of each component (Chaffin *et al.*, 1998). For example, although enriched in the outer layer, mannoproteins are also found in the inner layer of the cell wall (Nelson *et al.*, 1991; Ruiz-Herrera *et al.*, 2006).

Lipids are also present in low amount on the cell wall of this yeast, but may have important roles. The phospholipomannan, for example, has some relevance in adhesion, protection and signaling (Ruiz-Herrera *et al.*, 2006) and participates in immune recognition of the fungus (Hall &

Gow, 2013). Finally, although more commonly encountered in other fungi such as *Aspergillus* spp. or *Cryptococcus neoformans*, one study found the presence of melanin in *C. albicans* cell wall during infection (Morris-Jones *et al.*, 2005).

It is also worth noting the presence of fimbriae in the cell wall of *C. albicans*, which are thought to mediate adhesion to host cells (Chaffin *et al.*, 1998). These fibrils are long, between 100 to 300 nm (Hazen & Hazen, 1992), 5 nm in diameter and are mainly formed of mannose and a small percentage of protein (Ruiz-Herrera *et al.*, 2006). This mannose is probably the branched *N*-mannan of the glycoproteins (see below) as the mutants lacking this type of mannan have shortened or not fibrils at all (Hall *et al.*, 2013). They seem to take part in the hydrophobicity of the cell surface, with hydrophilic cells presenting long, compact fibrils and hydrophobic cells short fibrils (Hazen & Hazen, 1992).

Different extracellular stimuli may modulate cell wall, thus modulating the host-pathogen interaction. These stimuli may be environmental signals (pH, temperature, oxygen availability, hormones, CO_2 levels, nutrients, and serum), stresses (osmotic, oxidative, etc.) and microbial molecules such as quorum sensing molecules (Hall, 2015). For example, the carbon source influences the expression of cell wall proteins involved in adhesion, cell wall biogenesis and biofilm formation, as well as the elasticity of the structural components or the mannan composition. Chitin synthesis is influenced by pH, osmolality, micronutrient limitation, etc. Mannan structure is also dependent on pH, presence of blood or serum, and temperature. For a more detailed review on the effect of the environmental conditions in *C. albicans* cell wall, see Hall, 2015. On the other hand, as already mentioned, internal factors of *C. albicans* such as growth phase, cell cycle, and morphogenesis can influence the composition of the cell wall. Not only the abundance of the proteins (Chaffin, 2008) and other components such as chitin vary between yeast and mycelial cell wall, but some proteins are specific for one or another morphotype (Pitarch *et al.*, 2002). The most important components of the *C. albicans* cell wall are discussed below.

1.2.1. CHITIN

This polycarbohydrate is made of *N*-acetylglucosamine units bound by β-1,4-linkages. Chitin accounts for only 2-6% of the cell wall mass of *C. albicans* (Ruiz-Herrera *et al.*, 2006), but is a very important component. This linear polymer's chains are associated through hydrogen bonds to form microfibrils, making it one of the most insoluble natural products (Chaffin, 2008; Ruiz-Herrera *et al.*,

2006). Therefore, it is responsible for providing rigidity and integrity to the cell wall. When other components of the cell wall are disrupted, the amount of chitin is enhanced in order to compensate their lost and maintain the integrity of the cell wall (Degani *et al.*, 2016). Chitin is synthesized at the plasma membrane and extruded into the cell wall (Free, 2013), where the cross-link with other cell wall components occurs.

1.2.2. β-GLUCANS

Two types of β-glucans are present in *C. albicans* cell wall, β-1,3-glucans and β-1,6-glucans. These polysaccharides account for the greatest percentage of cell wall dried weight (30-39% in the case of β-1,3-glucans and 43-53% in that of β-1,6-glucans; Free, 2013). The structure of the β-1,3-glucan gives the cell wall elasticity and tensile strength (Free, 2013). Meanwhile, β-1,6-glucan connects β-1,3-glucans with chitin, other β-1,3-glucans and with mannoproteins (Kollár *et al.*, 1997), although β-1,3-glucan can also directly bind chitin (Chaffin, 2008), and some of the cell wall proteins are thought to bound directly β-1,3-glucan, as explained later. As in the case of chitin, β-1,3-glucan is also synthesized in the plasma membrane and extruded to the cell wall, whereas the synthesis of β-1,6-glucan starts in the endoplasmic reticulum (ER) and the Golgi apparatus, but the elongation of the polymer seems to take place in the cell wall itself (Free, 2013; Ruiz-Herrera *et al.*, 2006).

1.2.3. GLYCOPROTEINS

The cell wall proteins (CWPs) of *C. albicans* are glycoproteins known as mannoproteins. Although other carbohydrates are also present, the sugar moiety bound to the protein is usually mannan (Chaffin *et al.*, 1998), and because of that *C. albicans* CWPs are referred to as mannoproteins. These CWPs make up 40% of the *C. albicans* wall (Ruiz-Herrera *et al.*, 2006). The mannan moiety can be *N*- or *O*-linked to the protein (Fig. 1.3), and sometimes both link types are found in the same protein. The *N*-linked mannosylation is done by addition of carbohydrates to asparagine amino acid in the first asparagine-X-serine/threonine sequence, where X could be any amino acid but proline (Free, 2013). *O*-mannosylation is achieved through binding of the mannoses to a serine or threonine residue (Free, 2013; Hall & Gow, 2013). *O*-mannan is usually short and not very elaborated. On the other hand, *N*-mannan is composed of a core to which highly branched

outer *N*-mannan is attached. The core of the *N*-mannan is processed similarly in all eukaryotic organisms; the elaboration of outer *N*-mannan, however, is fungal specific (Hall & Gow, 2013).

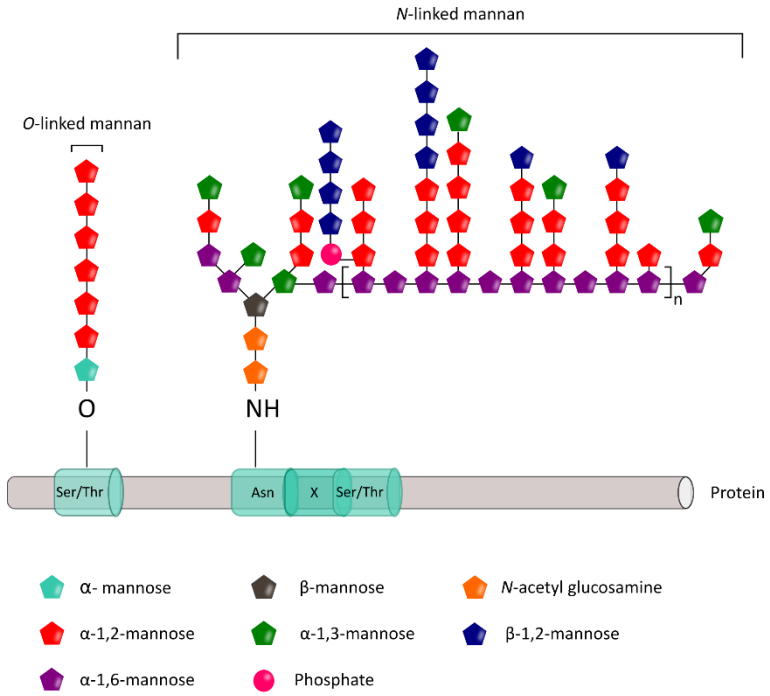


Figure 1.3. Structure of *O*-linked and *N*-linked mannans in *Candida albicans* cell wall. *O*-linked mannans are bound to a serine or threonine residue, and *N*-linked mannans to asparagine in the first asparagine-X-serine/threonine sequence, where X can be any amino acid except proline. Figure based on Hall & Gow, 2013.

Mannoproteins are produced by ER-bound ribosomes and then enter the ER lumen, where the *N*-glycosylation occurs. *O*-glycosylation, on the contrary, takes place as the proteins go through ER and Golgi apparatus (Free, 2013). Both types of mannoproteins are secreted into the cell wall by vesicular trafficking.

CWPs of *C. albicans* are divided it 2 groups depending on the type of binding to the other cell wall components:

1.2.3.1. Covalently bound CWPs

These proteins are divided into two groups according to their structure and composition:

Glycosylphosphatidylinositol (GPI)-CWPs. One-half of the proteins present in the cell wall belong to this category (Free, 2013). GPI-CWPs are rich in serine and threonine residues and highly *O*-glycosylated (Ruiz-Herrera *et al.*, 2006). The GPI anchor of the protein cross-links with β-1,6-

glucans, binding these proteins covalently to the cell wall matrix (De Groot *et al.*, 2005; Free, 2013). GPI proteins are bound to the cell wall through their C-terminus and extent the N-terminus into the outside side of the wall (Kollár *et al.*, 1997). In fact, the functional domain of most GPI-CWPs is located in the N-terminal part of the proteins (De Groot *et al.*, 2005). Proteins of the agglutinin-like sequence (Als) or secreted aspartyl proteinases (Sap) families (see below) belong to this group (Chaffin, 2008).

Pir (proteins with internal repeats)-CWPs. Pir proteins are proteins that have internal repeats. They are bound directly to β-1,3-glucans through mild-alkali-sensitive linkages (Chaffin, 2008; Kapteyn *et al.*, 2000) and some are bound to the cell wall by disulfide bridges (Ruiz-Herrera *et al.*, 2006). It seems that the linkage to β-1,3-glucan may be mediated by the internal repeats present in the proteins. Therefore, the proteins having multiple repeats could interconnect various β-1,3-glucan molecules providing more strength to the cell wall (De Groot *et al.*, 2005). Thus far, few proteins belonging to this category have been found (De Groot *et al.*, 2004; Pitarch *et al.*, 2002).

1.2.3.2. Non-covalently associated CWPs

Several glycolytic and cytosolic proteins have been found associated with *C. albicans* cell wall (Chaffin, 2008; Chaffin *et al.*, 1998). This was unexpected as these proteins lack the signal peptide for the classical trafficking pathway. Their cell wall-associated counterparts are not glycosylated (De Groot *et al.*, 2005; Ruiz-Herrera *et al.*, 2006). However, its presence in the cell wall has been determined by several authors using different techniques (Castillo *et al.*, 2008; Chaffin *et al.*, 1998; Ebanks *et al.*, 2006; Pitarch *et al.*, 2002). The function that these proteins accomplish in the cell wall is controversial and whether they are true components of the cell wall or only transiting into the extracellular medium is still a debate (Ruiz-Herrera *et al.*, 2006). Some of these proteins are enolase (Eno1; Angiolella *et al.*, 1996), alcohol dehydrogenase (Adh1; Pitarch *et al.*, 2002), glyceraldehyde-3-phosphate dehydrogenase (Gap1; Pitarch *et al.*, 2002), phosphoglycerate mutase (Gpm1; Pitarch *et al.*, 2002), phosphoglycerate kinase (Pgk1; Pitarch *et al.*, 2002), etc. Pitarch *et al.* in 2002, found 3 of this proteins, Eno1, Pgk1 and Gap1, to be present in all the CWP fractions, from the chitin bound to the non-covalent cell wall-associated fraction, suggesting that they are truly components of the cell wall, and not only transiting into the extracellular medium.

In fact, some of them have been found to play roles according to their cell wall location. For example Gap1 acts as adhesin, binding to extracellular matrix (ECM) laminin (Chaffin, 2008), and

Gpm1 binds both Factors H and FHL-1 and plasminogen (Poltermann *et al.*, 2007) and vitronectin (Lopez *et al.*, 2014).

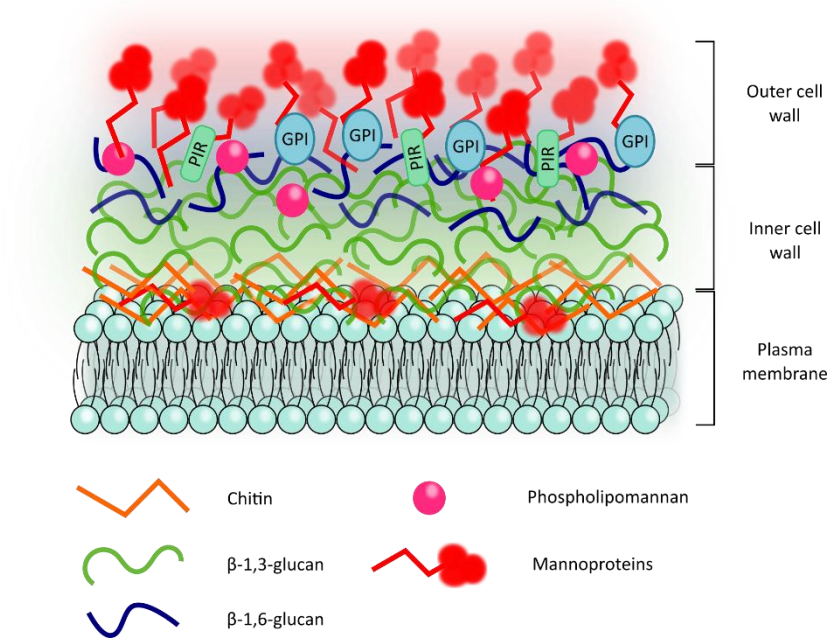


Figure 1.4. Scheme of the composition of *Candida albicans* cell wall. GPI: Glycosylphosphatidylinositol. PIR: Proteins with internal repeats.

Being such a diverse group, CWPs accomplish many different functions in the cell wall. Some are structural proteins, enzymes implicated in cell wall remodeling, sensors, etc. Others are adhesins, participate in iron acquisition or other roles related to virulence that will be discussed in the specific section.

The mannoproteins of the cell wall help in water retention. They also limit the cell wall porosity, preventing soluble cell surface proteins from leaking into the extracellular medium and offering some protection against cell wall degrading enzymes (De Groot *et al.*, 2005). Together with chitin and β-glucans, they participate in cell wall maintenance and protection against cell wall stress, as many of the CWPs are carbohydrate-processing enzymes (glucanases, chitinases, hydrolases, etc.) that may be involved in cell wall remodeling during growth or germ tube formation (Chaffin, 2008; De Groot *et al.*, 2005; Ruiz-Herrera *et al.*, 2006). Other mannoproteins may have structural roles, for example, Ecm33 may be the principal structural cell wall protein (Free, 2013). Degani and coworkers (2016) found an increase in mannoprotein expression in response to hyphal wall stress; therefore, mannoproteins seem to play also a role in cell wall integrity.

As the mannoproteins are located in the outermost part of the cell wall, they are the first molecules that interact with host's immune response. The mannan present in them is the major antigenic component of the cell wall (Nelson *et al.*, 1991) and, therefore, glycosylation may modulate this response (De Groot *et al.*, 2005). In fact, the high antigenicity of some mannoproteins has being associated to their mannan moiety and not the protein part (Ruiz-Herrera *et al.*, 2006). Finally, the CWPs participate in the hydrophobic properties of the yeast, mainly through the acid-labile β-1,2-mannan (Ruiz-Herrera *et al.*, 2006).

1.3. HOST RESPONSE TO *Candida albicans* INFECTION

Immune system is responsible of fighting the pathogens that the host encounters. Innate immunity is the first line of defense, which comprised both physical barriers, such as skin and mucosal tissues, and innate immune cells such as phagocytes. Innate immunity mounts a rapid and conserved response to all pathogens, and it is necessary to optimally activate the adaptive immunity (Romani, 2011), which, in turn, will mount a more specific response against the microorganism and possesses memory, thus acting more efficiently upon a second encounter with the same pathogen. As immunology is a complex subject that is constantly developing, a superficial review of anti-*Candida* immunity will be presented here.

1.3.1. INNATE IMMUNITY

1.3.1.1. Pattern recognition receptors

Host cells are able to recognize structures present in invading pathogens' cell wall known as pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs) present in their surface (Fig. 1.5). The main PAMPs of *C. albicans* are mannan and phospholipomannan, and β-glucans in a deeper layer. PRRs are expressed in immune cells such as dendritic cells (DCs) or macrophages, but also in other type of cells such as epithelial or endothelial cells, although their distribution and abundance varies between cell types.

C. albicans O-linked mannans are recognized mainly through Toll-like receptor (TLR)-2 and -4, whereas *N*-linked α-mannans are recognized by Dectin-2 and mannan receptor (MR) (Netea *et al.*, 2006; Verma *et al.*, 2015). This last receptor may be important in triggering interleukin (IL)-17

production by T_H17 memory cells (Verma *et al.*, 2015). MR has also affinity for chitin (Romani, 2011) and is an important endocytic receptor in DCs, being responsible for 70% of *C. albicans* recognition and internalization (Cambi *et al.*, 2003). Phospholipomannan is sensed mainly through TLR-2, with a partial contribution of TLR-4 and -6 (Jouault *et al.*, 2003). β-glucans are detected through Dectin-1, and this PRR collaborates with TLR-2 in macrophages and DCs to produce inflammatory cytokines upon β-glucan stimuli (Gantner *et al.*, 2003), favoring the development of a T_H17 response (Verma *et al.*, 2015). β-glucans are also bound by complement molecules and recognized through complement-receptor 3 (CR3) (Romani, 2011). Some of these PRRs are intracellular, such as TLR-9, which recognizes fungal DNA (Romani, 2011; Verma *et al.*, 2015).

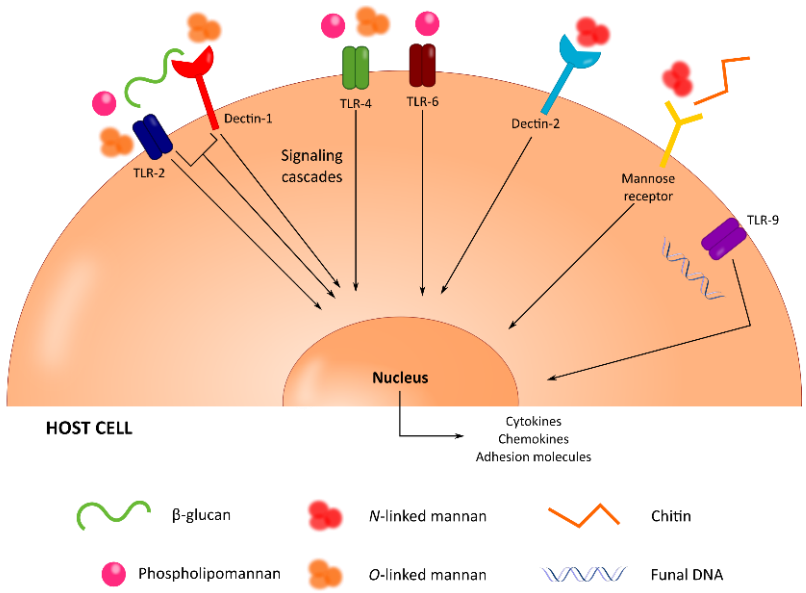


Figure 1.5. The principal pattern recognition receptors involved in *Candida albicans* recognition.

The activation of the PRRs induces a signaling cascade that will end up with the release of cytokines, chemokines and/or the expression of adhesion molecules in the surface of host cells. Cytokines will activate other cells, whereas chemokines attract immune cells to the site of infection, and adhesion molecules help to retain those immune cells in the correct location. Depending on the PRRs activated, the elicited response is different, with different cytokines being released. It is worth highlighting that, as already mentioned, environmental signals can modify cell wall composition, thus influencing the PAMPs displayed by *C. albicans*, and having an impact in innate immune

recognition (Hall, 2015). For example, yeast growth in lactate shift the immune response from the protective T_H17 to a T_H2 response (Ene *et al.*, 2013).

1.3.1.2. Complement response

Complement pathway is composed by molecules that are activated upon encounter with the pathogen. The main complement molecules involved in *C. albicans* infection are C3 and C5, which after cleavage give rise to C3a/C3b and C5a/C5b products. C3b opsonized β-1,6-glucan, enhancing phagocytosis (Rubin-Bejerano *et al.*, 2007) and C3a and C5a recruit innate immune cells to the site of infection (Luo *et al.*, 2013). C5b triggers the lytic terminal complement complex (TCC), leading to membrane attack complex (MAC) formation (Zipfel & Skerka, 2012), that usually destroys the attacking microorganisms. However, MAC deposition of *C. albicans* surface is not fungicidal; therefore, C5a role is probably more important than C5b in *Candida* infections (Luo *et al.*, 2013).

1.3.1.3. Antimicrobial peptides and natural antibodies

Antimicrobial peptides (AMPs) are small molecules able to kill a wide range of pathogens (see below). The AMPs are secreted by epithelial cells in response to microbial infection (Richardson & Moyes, 2015).

Natural antibodies are non-specific antibodies that are naturally present mainly in mucosal sites and help to contain microbial infections (Elluru *et al.*, 2015). Their main functions are the same as the rest of antibodies that will be explained later.

1.3.1.4. Dendritic cells

DCs are professional antigen presenting cells (APCs). Immature DCs are recruited to the site of infection by chemokines and antimicrobial peptides (Richardson & Moyes, 2015). Upon encounter with microorganisms, they phagocyte them and present the molecules (antigens) to immature T cells, thus serving as link between innate and adaptive immune system. The cytokines released during this antigen presentation will determine the type of T cell subset that will be developed from the naïve T cell population (Richardson & Moyes, 2015; Verma *et al.*, 2015).

1.3.1.5. Phagocytes: neutrophils and macrophages

Neutrophils are one of the key immune effector cells that fight *C. albicans* infections. As already seen, one of the risk factors associated with IC is neutropenia. These leukocytes phagocyte fungal cells and, once engulfed, kill them mostly through reactive oxygen species (ROS) production

and lysosomal enzymes (Qin *et al.*, 2016). When fungal cells are too large to be engulfed, i.e. once long hyphae have been produced, neutrophils can form neutrophil extracellular traps (NETs) by releasing their DNA and the content of their lysosomes to the extracellular medium in an attempt to contain the infection. NETs are capable of killing *C. albicans* by the release of calprotectin, an antifungal peptide (Urban *et al.*, 2009).

Macrophages are able to phagocyte fungal cells too, and after being activated, they kill them mainly by production of ROS and reactive nitrogen species (RNS) (Qin *et al.*, 2016). However, not all the *C. albicans* cells are killed and some can germinate and scape macrophages. Macrophages are also able to shape the immune response by cytokine release (Qin *et al.*, 2016). Depending on the cytokines present in the milieu, two kinds of macrophages have been described: the traditionally activated macrophages (M1) and the alternatively activated macrophages (M2). The first one is pro-inflammatory and more capable of killing microorganism, whilst the second one is more tolerogenic and anti-inflammatory, and it helps to avoid inflammation-driven damage to the host and to maintain barrier functions. However, M2 macrophages are also associated with uncontrolled fungal growth (Verma *et al.*, 2015).

1.3.2. ADAPTIVE IMMUNITY

1.3.2.1. T cells

As already mentioned, there are different subsets of T cells involved in fungal immunity; various T helper cells (T_H) and one class of cytotoxic T cells (CTL). Although both play a role in anti-fungal immunity, upon antigen presentation DCs favor T_H response (Richardson & Moyes, 2015). Therefore, this is the predominant cell-mediated response against *C. albicans*.

Several different T_H cells subset have been described; however, only the most relevant for fungi will be presented here. Traditionally T_H1 and 2 responses were described, the first one being pro-inflammatory and the second one anti-inflammatory. Recently another subset, T_H17, has been found to play an important role against *C. albicans* infection. T_H17 are of paramount importance in mucosal *Candida* infections (Trautwein-Weidner *et al.*, 2015), whereas T_H1 responses are still important during systemic candidiasis (Richardson & Moyes, 2015). However, T_H17 response in gastric candidiasis is associated with a deterioration of the disease (Zelante *et al.*, 2007). Nonetheless, T_H17 response, mainly through the action of IL-17A, is probably the most important

immune pathway against *C. albicans* mucosal infection, as indicate by patients with genetic mutations involved in this immune response, which suffer from recurrent infections, specially chronic mucocutaneous candidiasis (CMC) (Conti *et al.*, 2015). On the other hand, although the role of T_H2 is controversial, it is usually regarded as detrimental in the case of *C. albicans* infections (Richardson & Moyes, 2015).

Finally, regulatory T cells (T_{reg}) are more involved in inducing tolerance to colonizing microorganisms, and this response is very important in gastrointestinal settings, where *Candida* acts as a commensal microorganisms, limiting the inflammation in order to maintain the epithelial barrier (Verma *et al.*, 2015).

1.3.2.2. B cells

The main function of B cells is to produce antibodies, although they also present antigens and produce cytokines. There are five classes of antibodies (Immunoglobulin (Ig) A, IgD, IgE, IgG, IgM) and some of them have different subclasses (IgG1, IgG2, IgG3 and IgG4, and IgA1 and IgA2). Antibodies present fungicidal activity, suppress virulence, help phagocytosis through opsonization, activated complement pathway and take part in antibody-directed cell toxicity (Verma *et al.*, 2015). Although monoclonal antibodies (mAbs) have been shown to confer protection against *C. albicans* infection, mice deficient in B cells are not more susceptible to this fungus, suggesting that cell-mediated responses are predominant in *C. albicans* immunity (Richardson & Moyes, 2015).

1.3.3. ENDOTHELIAL CELLS

Due to the importance that the endothelial cells (ECs) have in the present thesis, the development of immune responses induced by these cells upon encounter with *C. albicans* will be more deeply explained.

C. albicans cells that are present in the blood must exit the vascular vessel in order to infect deep-seated organs. Therefore, endothelial cells lining these blood vessels are important in preventing fungal dissemination and play an important role in primary immune responses against this pathogen. It is worth highlighting that the ECs immune response induced by *C. albicans* is unique and different to the one elicited by other microorganism (Barker *et al.*, 2008; Orozco *et al.*, 2000).

Endothelial cells present various PRRs. Expression of TLR-1, -3, -4 and -5 has been detected in ECs, and also a weak TLR-2 expression (Faure *et al.*, 2000). Presence of MR has been detected in different mouse ECs, such as the ones in the liver, bone marrow and lymph nodes (Takahashi *et al.*, 1998), as well as galectin-1, -3 and -9 (Qin *et al.*, 2016). However, ECs from different beds express different receptors; for example, Human Umbilical Vein Endothelial Cells (HUVECs) do not express MR (Grubb *et al.*, 2008; Müller *et al.*, 2007). Therefore, it is likely that *C. albicans* binds different host receptors depending on the endothelial cell type it is invading (Sheppard & Filler, 2015).

The presence of these receptors can also facilitate the adhesion of *C. albicans,* and their activation triggers different responses in ECs (Qin *et al.*, 2016). They secrete prostaglandins (Filler *et al.*, 1994), which regulate inflammatory response and induce T_H17 response (Smeekens *et al.*, 2010). They also secrete cytokines upon *C. albicans* stimulation, such as tumor necrosis factor (TNF)-α, IL-1α, IL-1β, IL-6, IL-8 (Filler *et al.*, 1996; Orozco *et al.*, 2000); chemokines (Müller *et al.*, 2007); and express leukocyte adhesion molecules such as Intercellular Adhesion Molecule (ICAM)-1, Vascular Cell Adhesion Molecule (VCAM)-1, and E-selectin (Barker *et al.*, 2008; Filler *et al.*, 1996; Müller *et al.*, 2007; Orozco *et al.*, 2000).

On the other hand, ECs present other receptors apart from PRRs that are involved in disseminated infection. In order to escape blood vessels to infect the organs, *C. albicans* must traverse the EC lining. The fungus is able to do that by two different mechanisms; active penetration into ECs through hyphae elongation or induction of its own endocytosis. In this last case, the hyphae-expressed invasins Als3 and stress-seventy subfamily A1 (Ssa1) protein (see below) bind to a N-cadherin/Septin 7 complex, inducing pseudopod formation and engulfment of the yeast (Phan *et al.*, 2013). However, more receptors are probably involved in *C. albicans* endocytosis, as knockdown expression of N-cadherin only reduces fungal endocytosis by 40% (Phan *et al.*, 2005).

1.4. PATHOGENICITY

1.4.1. MORPHOGENESIS

The pathogenic role of yeast-to-hyphal transition, although extensively studied, is still a subject of controversy. Traditionally, yeast morphology of *C. albicans* has been related to colonization and dissemination, and hyphal morphology to the infective process. In fact, as shown by inducible mutants, these strains are only virulent when induced to filament. However, it has been

observed that strains locked in hyphal morphology are avirulent too (Sturtevant, 2014). This suggests that both morphologies are necessary for fungal virulence. Surprisingly, Saville *et al*. (2003) produced a tetracycline-regulated strain that, when locked as yeast, was able to penetrate into organs from blood and to propagate inside the host tissues as the wild type strain. Nevertheless, this yeast-locked strain did not kill the host, indicating the need of yeast-to-hyphal transition for optimal pathogenesis.

As noted, yeast cells play important roles in *C. albicans* virulence, among others, colonization, rapid dissemination through the blood, adhesion to host tissues and biofilm formation. On the other hand, hyphal formation is related to other virulence mechanisms, such as active penetration of host tissues, tissue damage, induction of fungal uptake and invasion trough Als3 and Ssa1, and lysis of macrophages and neutrophils after fungal phagocytosis (Thompson *et al.*, 2011). Moreover, hyphae express various specific virulence factors such as some adhesins (Hwp1, Als3, Fav2 and Pga55), tissue-degrading enzymes (Sap4, Sap5, Sap6), antioxidant defense proteins (Sod5), candidalysin (see below), etc. (Noble *et al.*, 2016).

Nevertheless, the expression of these genes is not directly involved in hyphal formation. Therefore, it can be argued that pathogenic role of hyphae is not due to the hyphal morphology itself, but to the virulence factors that are expressed during its formation. However, Zheng *et al*. (2004) produced a mutant strain unable to form hyphae but that still expressed some hypha-related genes (*HWP1*, *ECE1*, *ALS3* and *HYR1*). This strain showed an attenuated virulence in a mouse model of systemic infection, suggesting that the hyphal morphology itself is needed for a fully virulent *C. albicans* fungus.

Finally, it is worth highlighting that several transcription factors simultaneously control the filamentation process and the expression of virulence factors, suggesting a co-evolution of yeast-to-hyphal transition with pathogenesis (Thompson *et al.*, 2011).

1.4.2. ADHESION/INVASION

Adhesion is a key step in *C. albicans* pathogenesis, usually the initial one. This adhesion is mediate by adhesins, which are major CWPs. The binding may take place between proteins or between proteins and sugars (Chaffin, 2008). The fungal adhesins are known to interact with host ligands, the most important ones being ECM, laminin, fibronectin, collagen, entactin and vitronectin.

Moreover, fungal cells can also adhere to serum proteins (Chaffin, 2008) and even to abiotic surfaces.

A large number of *C. albicans* proteins can act as adhesins, but among them, Als family is thought to be the primary *C. albicans* adhesins and it is composed by eight *ALS* genes. Mutants lacking *ALS* genes have altered adhesion (Chaffin, 2008). Although all of them have been found to be expressed in clinical samples and different infection models (Brown *et al.*, 2007), only four of them are highly expressed during infection: *ALS1*, *ALS2*, *ALS3* and *ALS9* (Chaffin, 2008). Als1p and Als3p are involved in biofilm formation and host tissue binding (Liu & Filler, 2011; Tronchin *et al.*, 2008), but it has been suggested that they are not required for virulence (Cleary *et al.*, 2011).

Of all Als proteins, Als3p is the most important for adhesion, and it expression is associated with filamentation of the yeast cells (Mayer *et al.*, 2013). In fact, Als3p also participates in endothelial and oral epithelial cell invasion, acting as an invasin and promoting induced endocytosis of *C. albicans* (Cauchie *et al.*, 2017). This is mediated through Als3p interaction with N- and E-cadherin (Chaffin, 2008).

On the other hand, hyphal wall protein (Hwp) 1 is another protein that has been widely related to adhesion. As its name indicates, this protein is only expressed in hyphal cell surface (Chaffin, 2008). The N-terminal domain of this protein serves as a substrate for mammalian transglutaminases, which cross-link Hwp1 with oral mucosa, thus covalently linking the fungus to host cells (Chaffin, 2008; Mayer *et al.*, 2013). In this way, Hwp1p mediates adhesion to buccal epithelial cells (Tronchin *et al.*, 2008) and, therefore, the mutant strains lacking this protein showed a reduced adherence to those cells and also attenuated virulence (Mayer *et al.*, 2013).

Other proteins implicated in adhesion are enhanced adherence to polystyrene (Eap) 1 protein, yeast-form wall protein (Ywp) 1, pH-regulated antigen (Pra) 1 protein, cell surface hydrophobicity-associated (Csh) 1 protein, Mp65p, heat shock protein (Hsp) 70 protein family, Gap1, Eno1p, Adh1p, integrin-like (Int) 1 protein and glucan 1,3-β-glucosidase (Bgl) 2 protein (Chaffin, 2008; Mayer *et al.*, 2013). It is worth mentioning that the already mentioned Hsp70 family protein Ssa1 acts as an invasin by adhering to E- and N-cadherin (Mayer *et al.*, 2013; Phan *et al.*, 2013). It has been shown to participate in *C. albicans* virulence in hematogenously disseminated and oropharyngeal candidiasis (Sun *et al.*, 2010).

1.4.3. SECRETED ENZYMES

C. albicans is able to secrete several enzymes that degrade the surrounding tissue; for example, phospholipases, lipases and hemolysin. Among phospholipases, only B phospholipases are extracellular (Mayer *et al.*, 2013). These enzymes have been detected in infection samples (Chaffin, 2008) and they are able to disrupt host cell membrane (Naglik *et al.*, 2003). On the other hand, hemolysin is produced by the fungus in order to degrade hemoglobin and acquire iron (Cauchie *et al.*, 2017).

In addition to these, Saps are the most studied secreted *C. albicans* proteins. There are 10 genes in this family, but only Sap1 to 8 proteins are released to the extracellular medium (Mayer *et al.*, 2013). Once in the extracellular environment, Saps could hydrolyze host proteins. Despite being widely studied, their contribution to *C. albicans* pathogenesis is still controversial, as some have suggested that they are not required for epithelial invasion *in vitro* (Lermann & Morschhäuser, 2008; Mayer *et al.*, 2013). It has been proposed that these proteins may also have a role in adhesion (Chaffin, 2008).

1.4.4. TOXINS

The only known *C. albicans* toxin, candidalysin, was discovered recently by Moyes *et al.* in 2016. It is a cytolytic peptide toxin, the first one ever to be described in a human pathogenic fungus, encoded by *ECE1* gene, and produced during germination of the yeast. Candidalysin triggers the immune system and causes cell damage, membrane permeabilization and calcium influx, directly injuring the tissues.

1.4.5. BIOFILM FORMATION

One of the main pathogenic characteristics of *C. albicans* is its ability to form biofilms, both in biotic and abiotic surfaces. *Candida* biofilms have been related to catheter-associated infections or infections of medical devices. It has been suggested that approximately 65% of all the microbial infections are biofilm-related (Nieminen *et al.*, 2014).

These biofilms are formed in different stages (Tsui *et al.*, 2016). In the first step, yeast cells adhere to a surface (adherence step). Previously mentioned adhesins, such as Als3p and Hwp1p

(Nobile & Johnson, 2015), are critical for this step. Then, yeast cells proliferate and begin to germinate (initiation step). Above a yeast cell polylayer, a region of hyphae is produced, which are usually vertically oriented (Soll & Daniels, 2016). Biofilms are not only composed of cells, they are embedded in an ECM formed by proteins, polysaccharides and extracellular DNA as biofilm matures (maturation step). Finally, mature biofilms release non-adherent yeast cells into the milieu, which will find new places to colonize (dispersal step). Hsp90 has been found to be critical for this dispersal step (Mayer *et al.*, 2013; Nobile & Johnson, 2015).

Two types of biofilm have been described for *C. albicans*, a "sexual" and a "pathogenic" biofilm (Park *et al.*, 2013). The sexual biofilms are similar in structure to pathogenic biofilms, but show differences in thickness and function (Soll & Daniels, 2016). They are mainly composed by homozygous white cells that will support the mating of a minority of opaque cells. These biofilms usually do not show virulence traits. On the contrary, pathogenic biofilms are more resistant to fluconazole and impermeable to molecules and to human polymorphonuclear cells (Srikantha *et al.*, 2012; Yi *et al.*, 2011). Yeast cells seeded from these biofilms are more virulent that planktonic yeast cells in animal models (Tsui *et al.*, 2016).

One of the main issues of biofilm formation is their inherent resistance to antifungals. This increased resistance to antifungals is complex and multifactorial, but it is mainly due to a complex architectural structure, to the presence of ECM, which limits the diffusion of the drugs, to an increase expression of efflux pumps and to a higher metabolic plasticity (persister cells) (Fanning & Mitchell, 2012).

1.4.6. IRON UPTAKE

A virulence trait present in almost all human pathogens is the ability to steal iron from the host. Iron, and other metals, are critical for the growth of microorganisms. *C. albicans* is able to acquire iron from host ferritin, transferrin or hemoglobin and heme-proteins. However, it does not synthesize its own siderophores; instead, it steals iron from the siderophores produced by other organisms through an uptake system (Mayer *et al.*, 2013). It has also been shown that Als3 adhesin serves as receptor for host ferritin (Almeida *et al.*, 2008).

1.4.7. IMMUNE EVASION

To finish with *C. albicans* pathogenicity, it is worth mentioning the ability of this yeast to evade immune system, by directly hiding or escaping from effector cells, or by induction of immunodeficiency.

One of the most important ways of escaping the immune cells is by killing them. When *C. albicans* yeasts are ingested by macrophages, the environment inside the immune cells induces fungal morphogenesis. The hyphae grows until it pierces the membrane and mechanically escapes. Besides, hyphal formation produces a number of factors that kill the macrophage (Krysan *et al.*, 2014). The hyphae extension has also been related with a delay in phagosome maturation (Bain *et al.*, 2014), which enhances fungal survival inside the macrophages. Other mechanisms rely on inactivating the molecules that are dangerous for the fungus. For example, two proteins present in *C. albicans* cell wall encode for superoxide dismutase (SOD), which helps to survive macrophage's oxidative burst (De Groot *et al.*, 2005), and chitin inhibits nitric oxide (NO) production, which is part of the RNS produced by macrophages (Wagener *et al.*, 2017). Furthermore, chitin triggers the shift of macrophages to the alternatively activated phenotype M2.

C. albicans can also impede being recognized by host's immune system. Although the β-glucans present in the fungal cell wall trigger a strong pro-inflammatory response (Gow *et al.*, 2007; Williams, 1997), these are normally hidden behind the mannan layer, which is less pro-inflammatory (Sem *et al.*, 2016). Among other advantages, β-glucan masking also delays phagosome maturation (Bain *et al.*, 2014). Moreover, *C. albicans* mannans are able to bind IL-2, thus inhibiting the activation of IL-2-dependent signaling that plays a crucial role in immune system function. In fact, *C. albicans* has an extremely high amount of IL-2 ligands in comparison to other yeasts (Zanetta *et al.*, 1998). Mannan of this yeast binds also IL-1 and TNF-α cytokines (Nelson *et al.*, 1991), hampering an optimal immune system activation.

Proteins play also an important role in immune evasion. For example, Gpm1 and Pra1 bind Factor H and complement component 4b-binding protein (C4bp) in host's plasma, which, once bound to the fungal surface, block opsonization, phagocytosis and complement effector functions (Luo *et al.*, 2015). Pra1 can be secreted to the surrounding medium, where it binds C3 complement and inhibits its activation (Luo *et al.*, 2010). This protein binds also the CR3 receptor present in human immune cells such as macrophages and natural killer cells, which are involved in phagocytosis or cell-mediated killing (Soloviev *et al.*, 2007).

Another way of evading immune recognition is by mimicry of CR3. By binding to the complement molecules, *C. albicans* avoids being opsonized, which reduces the uptake of the fungus by the phagocytes (Nelson *et al.*, 1991).

As mentioned, *C. albicans* can also induce immunosuppression to increase its survival. On one hand, mannan induces immunosuppresion of host leading to the persistence of the infection. It was found that the serum from patients suffering from CMC had immunosuppressive properties, and the immunosuppressive molecule was found to be *C. albicans* mannan (Nelson *et al.*, 1991). On the other hand, although potent immunostimulators, glucans can also suppress monocyte functions directly and T cell functions indirectly, allowing the development of *Candida* infections (Nakagawa *et al.*, 2003).

1.5. DIAGNOSTIC TECHNIQUES

The current gold standard for IC detection is still blood culture (Clancy & Nguyen, 2013; OLeary *et al.*, 2018; Pfaller, 2015). This diagnostic technique misses out 50% of the patients suffering from IC. Even when blood cultures give a positive result, this may take as long as 2-3 days, and sometimes, blood cultures turn positive late in the disease (Clancy & Nguyen, 2013). The delay in detecting and identifying the causative agent supposes a late start of antifungal therapy, while waiting for a confirmation, or the start of antifungal treatment in absence of a conclusive diagnosis, which does not have evidence of clear benefits, may affect antifungal resistance and increases costs (Clancy *et al.*, 2016).

Several studies have addressed the relevance of an early treatment onset, showing a correlation between a delayed start of treatment with an increased mortality rate (Garey *et al.*, 2006; Kollef *et al.*, 2012; Marriott *et al.*, 2009; Morrell *et al.*, 2005; Ostrosky-Zeichner *et al.*, 2011; Rajendran *et al.*, 2016). In one study, only one quarter of patients with IC received appropriate antifungal therapy (Ostrosky-Zeichner *et al.*, 2011), and in another one, only 21% of the treatments were started within the first 24 h of drawing a positive blood culture (Marriott *et al.*, 2009). In the same study, conducted among non-neutropenic ICU patients in Australia, one-quarter of the deaths were related to patients not receiving antifungal treatment at all, as some of the patients died before obtaining a positive blood culture. The study conducted by Paiva *et al.* (2016) has shown that the start of the adequate treatment is delayed in fungemia compared to bacteremia, and that only

23% of the patients suffering for fungal infection received appropriate treatment within the first 24 hours after blood culture collection.

Identification of the *Candida* species is very important also for selecting an adequate antifungal therapy (Pfaller, 2015). As already noted, infections caused by non-*albicans Candida* species are on the raise. Some of these species are intrinsically more resistant to antifungals and resistance patterns change among species. In a multicenter study conducted in 2014 by Bassetti *et al.*, they found a higher mortality rate among patients receiving an inadequate antifungal treatment. The identification to species level through blood cultures and phenotypical characteristics is sometimes difficult and long incubation times are needed. Therefore, there is an urgent need for more accurate and rapid diagnostic techniques. Several non-culture diagnostic techniques have been developed (reviewed in Clancy & Nguyen, 2013 and Pfaller, 2015, among others). Only the most promising ones would be discussed here.

In spite of the concerns in the use of antibody detection in immunosuppressed patients, mannan/anti-mannan antibody detection (Platelia, Bio-Rad) has shown good specificity. On the other hand, β-D-glucan detection has also been studied as diagnostic technique. It has shown promising results, although false-positives are a problem (Bassetti *et al.*, 2018). MALDI-TOF/MS based diagnostic has also proven to be a rapid and useful tool for *Candida* species identification. This method has been shown superior to conventional methods (Clark *et al.*, 2013). Moreover, in addition to identifying the fungal agents to species level, it provides the potential resistance patterns of the analyzed sample (Bassetti *et al.*, 2018), facilitating the choice of the most appropriate antifungal treatment. Finally, polymerase-chain reaction (PCR) protocols have been developed, displaying good sensitivity and specificity in suspected IC, and they present a high discriminatory capacity among *Candida* species.

Of the mentioned non-culture based diagnostic tools, two have been cleared by the USA Food and Drug Administration as supplementary to cultures for diagnosis of ICs (Clancy *et al.*, 2016). The first one is the β-D-glucan assay (Fungitell, Associates of Cape Cod, East Falmouth, MA, USA). The second one is the T2Candida assay (T2 Biosystems, Lexington, MA, USA) based in a PCR combined with T2 magnetic resonance, that is able to amplify and detect *Candida* DNA from whole blood in 4 h. The biggest issue concerning these two methods is the limited data available, especially for several populations at-risk, because most of the studies have been conducted in candidemia patients. On the other hand, more than a new tool for providing conclusive results, these non-culture based diagnostic methods should be regard as biomarkers for the probability of disease.

Even though promising, these non-culture methods are not ready to be widely used in the clinical setting yet, and all of them have some drawbacks. Mannan/anti-mannan detection does not differentiate among *Candida* species, and there is a concern about positive results arising from heavy colonization or subclinical disease. β-D-glucan is also present in other fungi, thus a positive results does not imply *Candida* infection. MALDI-TOF/MS is not completely standardized and different databases for spectra comparison are available. Moreover, not all the studies carried out using this technique were able to identify the isolates to species level (Clark *et al.*, 2013). Finally, PCR protocols are not yet standardized between centers and DNA extraction is sometimes difficult, depending on sample's origin. In general, it could be said that limitations in interpreting the results provided by these methods in clinical setting have hampered their widespread use (Clancy *et al.*, 2016), and that true impact on patients' outcome has yet to be addressed.

1.6. TREATMENTS

Nowadays, only four classes of antifungals are available for clinical treatment of IC: polyenes (Amphotericin B; deoxicolate and three lipid formulations), azoles (fluconazole, voriconazole, itraconazole, posaconazole and the newly developed isavuconazole), echinocandins (micafungin, anidulafungin and caspofungin) and pyrimidine analogs (5-fluorocytosine) (Sanglard, 2016).

Amphotericin B targets membrane ergosterol, leading to the de-stabilization of the membrane and allowing the leakage of cell components (Paramythiotou *et al.*, 2014). It is associated with high toxicity, especially the deoxicolate form. The introduction of its liposomal formulations has reduced the risk of nephrotoxicity, but at increased cost (Pappas *et al.*, 2016). Despite many years of clinical use, resistance to polyenes is very rare, except for some *C. glabrata* and *Candida krusei* isolates (Pfaller *et al.*, 2004).

Azoles target also the ergosterol present in the membrane. Fluconazole is the most used due to its high oral availability, low toxicity, and low cost (Paramythiotou *et al.*, 2014). However, resistances to fluconazole are a concern; especially in *C. glabrata* and *C. krusei* isolates (Pappas *et al.*, 2016), and some physicians prefer the use of other antifungal classes, for example, echinocandins. This last class of antifungal targets the β-1,3-glucan present in the cell wall, an organelle not present in mammalian cells, as already noted. They also present a good safety profile

(Paramythiotou *et al.*, 2014). However, echinocandins are only available by intravenous administration and are more expensive than the azoles.

Finally, 5-fluorocytosine or flucytosine targets the DNA and RNA synthesis (Pfaller, 2012). This antifungal presents two major problems, on one hand, it is toxic for the bone marrow and liver, and, on the other hand, if use as monotherapy resistances are quickly developed (Barchiesi *et al.*, 2000; Bassetti *et al.*, 2016). This is why it is normally used in combination with other drugs, mainly amphotericin B.

The last update of the guidelines for management of candidiasis by the Infectious Diseases Society of America group (Pappas *et al.*, 2016), proposed the echinocandins as the primary antifungal treatment for IC and other *Candida* induced infections, due to their safety profile, better outcomes, early fungicidal activity and the presence of azole-resistances. However, the concentrations achieved by echinocandins in some organs are low (Dodds Ashley *et al.*, 2006) and, therefore, fluconazole and amphotericin B are still used as first line treatments in some cases, such as endocarditis, central nervous system infections or urinary tract infections, among others. Echinocandins are also more expensive than the azoles, so once the patient is stable, and if resistance to fluconazole is not suspected, a de-escalation to this antifungal is usually suggested (Bassetti *et al.*, 2016; Pappas *et al.*, 2016).

In addition to the high toxic potential, antifungal use is changing the epidemiology of IC and resistances have rapidly appear, even against echinocandins, which have been in use for no more than 17 years (Sanglard, 2016). The acquisition of resistance to almost all the antifungals available has been noted for the main fungal pathogens (Perlin *et al.*, 2015). Recently, Bailly *et al.* (2016) have shown that the history of antifungal use influences both *Candida* species distribution and their susceptibility. In fact, echinocandin minimal inhibitory concentrations (MICs) have raised in the most frequently isolated *Candida* species, correlating with the increased use of this class of antifungals. Other study conducted in a Cancer Center in US (Jung *et al.*, 2015) has found an association between the increased echinocandin use and an augmentation of BSIs caused by uncommon *Candida* species (*Candida guilliermondii*, *Candida lusitaniae*, *Candida kefyr*, *Candida famata* and *C. dubliniensis*). Moreover, the appearance of multidrug resistant (MDR) strains has already been documented. MDR is defined as resistance to two or more antifungal classes. Some *C. albicans* strains are resistant to both azoles and amphotericin B (Sanglard, 2016). Other common MDR pattern is resistance to azoles and echinocandins, especially among *C. glabrata* isolates (Perlin *et al.*, 2015). Resistance to three

classes of antifungals have also been documented in *C. albicans* (Jensen *et al.*, 2015) and in the recently identified emerging fungal pathogen *Candida auris* (Lockhart *et al.*, 2017).

Despite the frequent revision of antifungal use guidelines and the study of scores that would predict which patients should be placed on prophylactic or empirical treatment, the mortality rate associated with IC is still very high, up to 60% (Bassetti *et al.*, 2018). It is clear that other treatment approaches would be beneficial for IC patients. In recent years some new antifungal drugs have been developed, although none of them is yet available at clinical settings. They will shortly be mentioned here; for more information see Wiederhold & Patterson (2015) and Bassetti *et al.* (2018). Some of these new drugs belong to already existing antifungal classes. Among new azoles, VT-1161 and VT-1129 (Dorham, NC, USA) are found, which are similar to triazoles. There are also two new drugs that belong to the echinocandin class; ASP9726 (Astellas Pharmaceuticals) and Rezafungin (previously CD101, Cidara Therapeutics). One of the drawbacks of the echinocandins is their requirement for daily intravenous application. The new echinocandins aim to overcome this problem; i.e. Rezafungin is in development for once-weekly intravenous administration. An antifungal belonging to a new antifungal class, SCY-078 (Scynexis, Inc., Research Triangle Park, NC, USA), is in Phase 3 of clinical development. This drug is the first one of the triterpene class, which is also a glucan synthase inhibitor, and is orally available.

On the other hand, antifungals with new mechanism of actions are been developed. E1210 (Eisai Inc., Tokyo) inhibits GPI protein maturation and T-2307 is a new arylamidine that produces a collapse in the mitochondrial membrane of fungi. Finally, MGCD290 (Mirati Therapeutics, San Diego, CA, USA) inhibits histone deacetylation.

Nonetheless, in the last years, alternative treatment options, both prophylactic and therapeutic, have been studied. Three of them will be shortly reviewed here:

1.6.1. VACCINES

Several components of *C. albicans* cell wall have been studied as candidates for vaccine development against candidiasis, mainly against mucosal infections, due to their surface localization and interactions with host's immune system; mannan, β-glucan, Als3, Sap2, Eno1 (discussed later), Hsp90, Hyr1p, Fba1, Hwp1, etc. (for a review see Moragues *et al.*, 2014). Most of them are poorly

immunogenic, needing to be coupled with an adjuvant, and only two of those antigens have been assessed in human clinical trials; we will focus on those.

Two Als family recombinant proteins have been studied as anticandidal vaccines, rAls1p-N and rAls3p-N. Both provided similar protection against disseminated candidiasis in a murine model, but rAls3p-N was proven superior in both oropharyngeal and vaginal candidiasis (Spellberg *et al.*, 2006). Therefore, this one was selected for vaccine development, using aluminum hydroxide as adjuvant (NDV-3 vaccine). In a Phase 1 clinical trial in humans, NDV-3 showed to be safe and well tolerated (Schmidt *et al.*, 2012). A derivative of this vaccine, NDV-3A (NovaDigm Therapeutics) has been tested in a Phase 1b/2a clinical trial, showing ability to statistically reduce the recurrence of vulvovaginal candidiasis, especially in patients under 40 years old (Edwards *et al.*, 2018).

On the other hand, intravaginal immunization using Sap2, more concretely an enzymatically inactive truncated recombinant protein (rSap2t), has shown to confer protection to vulvovaginal candidiasis in a mice model (Sandini *et al.*, 2011). This recombinant protein was expressed in a virosomal vaccine (PEV7, Pevion Biotech, De Bernardis *et al.*, 2012). It has been tested in a Phase 1 trial and proven to be safe and immunogenic (Pericolini *et al.*, 2015). The previously mentioned NovaDigm Therapeutics company has acquired the rights of PEV7, along with other two potential anticandidal vaccine antigens, with the aim of producing a multivalent vaccine that would induce immune responses to different virulence factors (De Bernardis *et al.*, 2015). The other two antigens are Hyr1 protein, which has shown to confer protection against disseminated candidiasis (Luo *et al.*, 2011), and a β-mannan conjugate.

One of the main drawbacks of this approach is that *Candida* is a usual commensal microorganism of humans, and it is unknown whether the vaccination could damage the commensal status of the yeast. Other concern about active immunization is the lack of response induction in immunocompromised patients. In those cases, passive immunization with antibodies is an attractive option, as the majority of the vaccines tested thus far have shown a protective effect based on specific-antibody response.

1.6.2. MONOCLONAL ANTIBODIES

The use of mAbs as a treatment option against *Candida* and other clinically relevant fungi has been under study for several decades, based on the notion that survivals of IC present higher

titers of antibodies that non-survivors against, among others, Hsp90 (Matthews *et al.*, 1987; Matthews & Burnie, 2004). However, there is not always a correlation between antibody titers and protection, because some of the generated Abs could be irrelevant or non-protective.

As in the case of vaccines, *Candida* cell wall surface molecules have been targeted for protective mAb production: mannans (Han, 2010), β-glucans, which can combat different fungal species infections (Torosantucci *et al.*, 2005), Hsp90 (Mycograb, see below), Als3 (Brena *et al.*, 2007), Eno1 (Omaetxebarría *et al.*, 2005), Hyr1 (Torosantucci *et al.*, 2009), etc.

Several antibody isotypes have been studied, mainly IgG and IgM, but specificity of the variable region seems more important than the isotype class (Han *et al.*, 2000). Regarding the mechanisms of protection, various have been proposed, and some of the mAbs present more than one simultaneously. Apart from the already mentioned "classical" mechanisms of antibodies, others have been described. MAbs are able to block virulence factors, inhibit yeast adherence to host cells, biofilm formation, and hyphal growth, and some even present direct candidacidal activity (Bujdáková *et al.*, 2010; Coleman *et al.*, 2009; Elguezabal *et al.*, 2009; Kabir *et al.*, 2011; Manfredi *et al.*, 2005; Mishra *et al.*, 2015; Moragues *et al.*, 2003). Many of them have been shown to be protective in animal models, both as prophylactic or therapeutic treatment (Capodicasa *et al.*, 2011; Chupáčová *et al.*, 2018; Han *et al.*, 2000; Kavishwar & Shukla, 2006; Xin & Cutler, 2011). In some case, they have been used in combination with available antifungals (Han, 2010), allowing a reduction in the drug dose and, thus, potentially decreasing toxicity-related problems. Moreover, the ability of some mAbs to kill *Candida* without involvement of immune cells makes these antibodies an interesting treatment option in immunocompromised patients (Torosantucci *et al.*, 2009; Xin & Cutler, 2011).

Despite the relative success demonstrated by these mAbs, only two have reached human clinical trials. One was developed against *C. neoformans* and will not be discussed here. The other one, named Mycograb, targeted *C. albicans* Hsp90 and showed protection against *C. albicans* infections in animal models and humans in combination with Amphotericin B (Matthews *et al.*, 2003; Pachl *et al.*, 2006). It was produced commercially by Novartis, but in 2006 its use was discouraged in Europe, by a negative opinion of the Committee for Medicinal Products for Human Use, due to some concerns about safety and quality (Bugli *et al.*, 2013). A modified mAb was developed (Mycograb C28Y), but unfortunately, it did not demonstrated to be useful in a murine model (Louie *et al.*, 2011). Novartis ceased the production of Mycograb in 2010 (Elluru *et al.*, 2015).

Finally, some mAbs have shown potential for IC diagnosis, by identification through indirect immunofluorescence (IFF) or specifically binding to *C. albicans* mycelia in tissues, by allowing its distinction from *C. dubliniensis*, and in some cases by concentration of the yeast cells present in blood in order to obtain better DNA extractions for PCR diagnostics or shorter incubation times for cultures (Apaire-Marchais *et al.*, 2008; Bliss *et al.*, 2003; Järvensivu *et al.*, 2006; Kaba *et al.*, 2015; Marot-Leblond *et al.*, 2000).

1.6.3. PEPTIDES

In the last two decades, research into peptides has gain attention. As already mentioned, the main group of peptides with antimicrobial capacity are known as antimicrobial peptides. They are small molecules produced by the immune system that possess a broad-spectrum activity against bacteria, fungi, virus, parasites and even tumor cells. One of the advantages of these peptides is that they have immunomodulatory capacity and, therefore, they can control infections avoiding excessive inflammatory responses. AMPs are usually cationic molecules, positively charged and hydrophobic. They bind to the lipids present in the cell membrane of the microorganism, disturbing it by pore formation or increasing permeability, and leading to leakage of cytoplasmic content. They bind to cell membrane very quickly and their deposition on microbial surface can also help phagocytosis (Pärn *et al.*, 2015).

AMPs are produced by virtually all living beings of all kingdoms. Thus, many studies have focused in the identification and characterization of new AMPs present in plants, insects, amphibians, bacteria, etc. (Dahiya *et al.*, 2016; Gao *et al.*, 2016; Kodedová & Sychrová, 2017; Machado *et al.*, 2016; Proaño-Bolaños *et al.*, 2016; Taniguchi *et al.*, 2016; Wu *et al.*, 2017). So far, about 5,000 peptides have been described, approximately 1,000 present antifungal activity, and by 2013 there were more or less 100 approved peptide drugs (Mukherjee *et al.*, 2016). Although this is a very promising field for new antifungal treatment development, AMPs present some drawbacks that need to be addressed. Some of them possess hemolytic activity, and therefore are cytotoxic for humans. They are not stable, being targets of proteases of gastrointestinal track or even blood (Bruno *et al.*, 2013). However, as they are small molecules they can be modified by amino acid replacement and some studies have shown to be able to ameliorate peptide antimicrobial activity, stability and to reduce cytotoxicity by such bioengineering techniques (Gao *et al.*, 2016; Zhao *et al.*,

2016). Synthetic AMPs have also been produced, and they showed ability to kill microorganisms (Kodedová & Sychrová, 2017).

Other category of peptides with antimicrobial activity are cell-penetrating peptides (CPPs; Pärn *et al.*, 2015). They share some characteristics with AMPs, such as amphiphilicity and cationic structure. They are able to penetrate into cells by minimal membrane disruption. Therefore, they have been studied, above all, as drug-delivery system for virus and cancer cells. However, some CPPs are able to kill microorganism by accumulating in cell nucleus and arresting the cell cycle (Li *et al.*, 2016).

Finally, a third group of peptides with antifungal activity has been described. These peptides are derived from the complementary-determining region (CDR) parts of the variable region of fungicidal antibodies and retain their ability to kill fungi (Kabir *et al.*, 2011; Manfredi *et al.*, 2005; Polonelli *et al.*, 2008). Peptides derived from the constant region of the antibodies also present fungicidal activity (Polonelli *et al.*, 2012). Recently, Polonelli *et al.* (2017) have described fungicidal activity of peptides derived from the sequences of immunoglobulin genes, and ability to protect *Galleria mellonella* insect's larvae from *C. albicans* challenge.

1.7. *Candida albicans* AND CANCER

The relationship between *C. albicans* and cancer has been long studied, as cancer patients are prone to suffer from candidiasis because chemotherapy and radiotherapy suppress their immune system. According to the last study conducted in Europe among adults suffering from cancer, 0.23% of them had endure a fungal infection, being 90% of these infections caused by *Candida* (Cornely *et al.*, 2015). In a study conducted in seven European countries in 2004, it was found that 35% of patients suffering from candidemia were also cancer patients (Tortorano *et al.*, 2004), indicating that cancer is one of the most common underlying conditions associated with candidemia.

However, as in the case of some bacteria and viruses, it has been suggested that *C. albicans* could support the initiation and progression of cancer. This was proposed for the first time by Cawson in 1969, who implied that *Candida* was able to produce leukoplakia, which is a precancerous oral lesion. Since them, more studies have related *C. albicans* with cancer, especially with oral and esophageal cancer in humans (Alnuaimi *et al.*, 2015; Berkovits *et al.*, 2016; Rautemaa *et al.*, 2007).

One of the most important cancer-inducing factor attributed to *C. albicans* is the ability to produce acetaldehyde from glucose or ethanol. Acetaldehyde is classified as group I carcinogen compound by the International Agency for Research on Cancer (IARC, 1985, 2012), and it induces DNA abducts (Hori *et al.*, 2012).

It has been observed that oral cancer lesions are frequently developed in sites where *Candida* is present (Rautemaa *et al.*, 2007), and that the presence of this yeast is more frequent in oral cancer lesions than in the surroundings tissue (Alnuaimi *et al.*, 2015). Recently, Zhu *et al.* (2017) have proposed that chronic fungal infection can lead to esophageal squamous cell carcinoma. Regarding the importance of acetaldehyde in cancer development, it has been found that *C. albicans* isolated from patients suffering from oral lesions that could lead to cancer produce higher levels of acetaldehyde that *Candida* isolated from patients with other kind of oral lesions (Gainza-Cirauqui *et al.*, 2013).

In animal models, *Candida* has been related to oral cancer, when administered together with nitoquinoline1-oxide, a tumor-inductor component (Dwivedi *et al.*, 2009). It has also shown to induce mucosal proliferation in diabetic rats, leading to squamous cell carcinoma in one case (Sano *et al.*, 2014). On the other hand, mice infected with *C. albicans* and challenged with tumor cells presented more and bigger liver metastases that non-infected mice (Rodríguez-Cuesta *et al.*, 2010).

Regarding *C. albicans* mechanisms that could induce cancer development and progression, they were reviewed by Ramirez-Garcia *et al.* (2016). First, the ability to produce carcinogenic compounds, such as nitrosamine or the already mentioned acetaldehyde. Second, the inflammatory process induced by yeast infection. A long-lasting inflammation, such as the one chronic *Candida* infections could sustain, can be detrimental to the host, by damaging epithelial cell barriers. Inflammatory response has also been linked to cancer development (Porta *et al.*, 2011) and progression (Ramirez-Garcia *et al.*, 2011, 2013). Third, the induction of T_H17 response, which has been related with poor cancer prognosis, as sometimes this response favors cancer cell proliferation. The last mechanisms is CR3-related protein (CR3-RP) molecular mimicry. This protein is very similar to the CR3 present in macrophages. When infected with *C. albicans*, the immune system develops antibodies against CR3-RP that could also react against macrophages' CR3, inhibiting their migratory capacity and, therefore, hampering their anti-tumor functions.

Apart from these four, more mechanisms have been proposed. Reales-Calderón *et al.* (2014) observed that upon incubation with yeast, macrophages shift from a M1 to a M2 type. As

mentioned before, M2 macrophages are anti-inflammatory, suppressing the anti-tumor effect of other immune cells and allowing tumor cell progression. In addition, *C. albicans* evades host defense by inducing IL-10 upregulation and T_{reg} generation (Netea *et al.*, 2004), and it is known that T_{regs} can suppress anti-tumor immunity (Beyer & Schultze, 2006). Therefore, yeast induction of T_{regs} increase can help tumors to evade immune system. On the other hand, while researching into the genomic profile changes of ECs incubated with *C. albicans*, various groups have noted that genes related to cancer development (angiogenesis, anti-apoptosis, etc.) were up-regulated in ECs (Barker *et al.*, 2008; Lim *et al.*, 2011).

Taking into account the immune response triggered by ECs infected with *C. albicans* (1.3.3 section), our group proposed a mechanism by which this yeast could induce metastasis development (Fig. 1.6). As already noted, upon *C. albicans* stimulation ECs release cytokines and chemokines, that will recruit innate immune cells to the site of infection, and express adhesion molecules (such as ICAM-1 and VCAM-1) to help adhesion of immune cells in the site. However, in oncological patients undergoing chemotherapy treatment, innate immune cells will be absent or present at very low densities, and therefore adhesion sites will be free. In this setting, cancer cells release from the primary tumor could be found in the bloodstream and adhere to the free adhesion molecules, forming a second tumor focus. As the liver is the main organ responsible for blood filtration and microorganism clearance, this response will be more likely developed in hepatic endothelial cells, thus inducing liver metastases. In fact, cancer patients frequently present liver candidiasis (Walsh *et al.*, 1991).

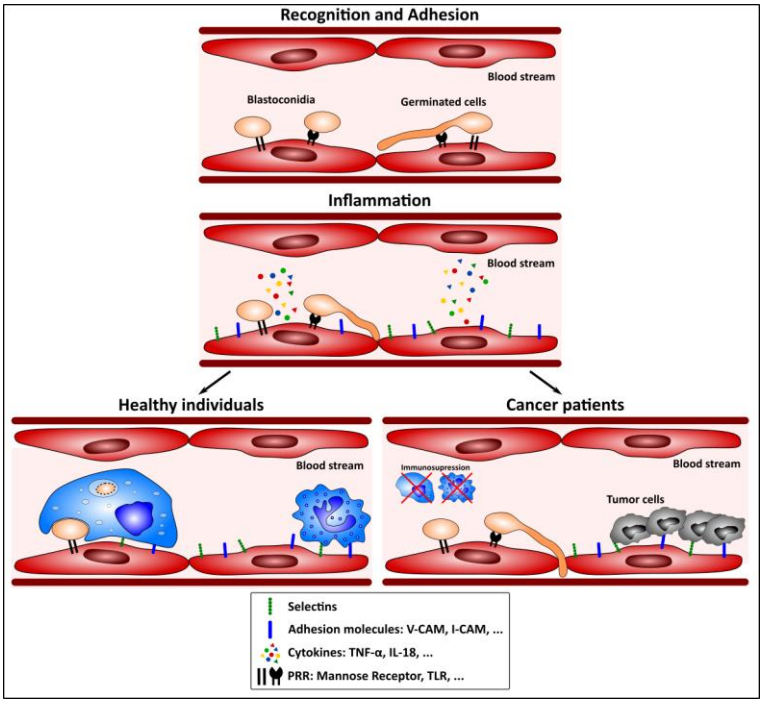


Figure 1.6. Proposed mechanism for *Candida albicans* induced tumor liver metastasis (Ramirez-Garcia *et al.*, 2016). After recognition of *C. albicans*, hepatic endothelial cells secrete cytokines and express adhesion molecules. In healthy individuals, this response will recruit immune cells that will clear the infection. On the contrary, cancer patients under immunosuppressive treatments will present an impaired immune system, allowing the binding of circulating tumor cells to free adhesion molecules.

1.7.1. PROTEINS RELATED TO TUMOR CELL ADHESION INDUCTION

In a previous work, our group found that fractions of *C. albicans*, as well as whole yeasts, could induce an increase of tumor cell adhesion to mouse hepatic endothelial cells (Ramirez-Garcia *et al.*, 2011). We also detected the protein fraction inducing the highest adhesion increase and identified the proteins belonging to that fraction (Table 1.1). Among the 14 identified proteins, four were selected for individual study because they had been previously identified as important antigens of *C. albicans*. These were alcohol dehydrogenase (Adh1), enolase (Eno1), mitochondrial ketol-acid reducto-isomerase (Ilv5) and ubiquinol cytochrome-c reductase (Qcr2). Another protein, killer toxin-resistance protein (Kre9), was detected by western blot using an anti-IL-1β receptor. This cytokine is very important in the inflammatory process and therefore in the ECs stimulatory process explained above. Moreover, although not identified among the 14 mentioned proteins, Kre9 also

belonged to the fraction inducing the highest tumor cell adhesion (unpublished data) and therefore, it was also selected for individual study, making a total of five proteins.

Table 1.1. The 14 proteins identified from the *Candida albicans* fraction inducing the highest increase of tumor cell adhesion to mouse hepatic endothelial cells. Table modified from Ramirez-Garcia *et al*., 2011.

Name	Full name	Accession number/Swiss-prot-Candida DB	
Pdi1p	Protein disulfide isomerase	Q5A5F2	CA1755
Ape3p	Aminopeptidase Y	Q59YB4	CA0871
Ilv5	Mitochondrial ketol-acid reducto-isomerase	Q59WW5	CA1983
Tuf1p	Elongation factor Tu+translation elongation factor Tu	Q4ZMP2+ Q5ABC3	CA4909
Act1p	Actin	Q5AKX0	CA5255
Eno1p	Enolase		
Adh1p	Alcohol dehydrogenase	Q5AKI5/ Q5AK23	CA4765
Dor14p	6-Phosphogluconate dehydrogenase	Q5AKV3	CA5239
Fba1p	Fructose bisphosphate aldolase	Q9URB4	CA5180
Pgk1p	3-Phosphoglycerate kinase	P46273	CA1691
Idh2p	Isocitrate dehydrogenase	Q5A0M1	CA4148
Mpg1p	GDP-mannose pyrophosphorylase	O93827	CA3208
Sfa1p	Glutathione-dependent formaldehyde dehydrogenase	Q5ACP2	CA6000
Qcr2p	Ubiquinol-cytochrome c reductase (core protein 2)	Q59QS9	CA2065

1.7.1.1. Kre9 protein

Among mannoproteins of *C. albicans*, Kre9 deserves special attention. This is a highly *O*-glycosylated protein with a molecular mass of 55 kDa (Brown & Bussey, 1993; Lussier *et al.*, 1998) thought to be the main responsible of β-1,6-glucan biochemical synthesis (Aimanianda *et al.*, 2009). As already mentioned β-1,3-glucan has a structural role, whereas β-1,6-glucan stabilizes the cell wall and serves as link to other cell wall components such as mannoproteins, chitin and β-1,3-glucans (Aimanianda *et al.*, 2009; Iorio *et al.*, 2008; Kollár *et al.*, 1997).

The role of *KRE9* gene has been more studied in *S. cerevisiae* than in other yeasts. In that yeast, disruption of the gene leads to a reduction of 80% in the content of β-glucans (Aimanianda *et al.*, 2009). However, in *S. cerevisiae KRE9* has a homolog gene, *KNH1*, which can compensate the loss of Kre9 protein when overexpressed (Nagahashi *et al.*, 1998). This homolog is also present in *C. glabrata* (Nagahashi *et al.*, 1998), but it has not been found in *C. albicans*. Moreover, *C. albicans* Kre9 is substantially different to both *S. cerevisiae* Kre9 and Knh1, sharing 43% and 32% amino acid identity, respectively (Lussier *et al.*, 1998). When *KRE9* gene is disrupted, *C. albicans* has no detectable levels of β-1,6-glucan and does not form hyphae in presence of galactose or serum (Lussier *et al.*, 1998). In fact, the Kre9 null yeast grows slowly in galactose but it is unable to grow in presence of glucose, at levels present in human serum. This mannoprotein is also interesting because is found only in fungi, and is localized at the cell surface, being secreted to the extracellular medium when overproduced (Brown & Bussey, 1993; Lussier *et al.*, 1998). In consequence, it is accessible to immune system recognition.

The presence of Kre9 in the cell wall of *C. albicans* has been discussed as being transient and residual of a secretion pathway. However, at least in the case of *S. cerevisiae*, Montijn *et al.* (1999) found that the majority of β-1,6-glucan is synthesized at the plasma membrane, suggesting that Kre9 should be present in plasma membrane or cell wall of *Candida*.

1.7.1.2. Adh1 protein

Alcohol dehydrogenase has previously been identified as allergen and antigen by other authors (Swoboda *et al.*, 1993). Although still an issue of conflict because of the metabolic role of the protein, several authors have found it associated with the cell wall of the yeast (Castillo *et al.*, 2008; Ebanks *et al.*, 2006; Pitarch *et al.*, 2002). According to its surface location, it may play a role as adhesin, as it has been suggested to bind host's fibronectin (Chaffin *et al.*, 1998) and serum plasminogen (Crowe *et al.*, 2003).

As already mentioned, Adh1 catalyzes the conversion of ethanol to acetaldehyde and *vice versa*, being the last one a carcinogenic compound (International Agency for Research on Cancer, 1985, 2012). *C. albicans* Adh1 protein, through the production of acetaldehyde, has been related to the ability of the yeast to induce and promote cancer (Ramirez-Garcia *et al.*, 2016; Tillonen *et al.*, 1999; Uittamo *et al.*, 2009). Although not experimentally tested yet, computational analysis has suggested that Adh1p could have other metabolic functions, such as participation on glycolytic processes, response to host's defense, and biofilm formation on biotic and abiotic surfaces.

1.7.1.3. Others

Among the other three proteins studied in this work, enolase is the most researched one. Its main function is metabolic, converting 2-phosphoglycerate to phosphoenolpyruvate in glycolysis and the other way around in gluconeogenesis. It is evolutionary highly conserved and abundantly expressed in many organism (Díaz-Ramos *et al.*, 2012). Apart from its cytosolic location, it has been found in yeast cell wall, associated to β-glucans (Angiolella *et al.*, 1996; Pitarch *et al.*, 2002).

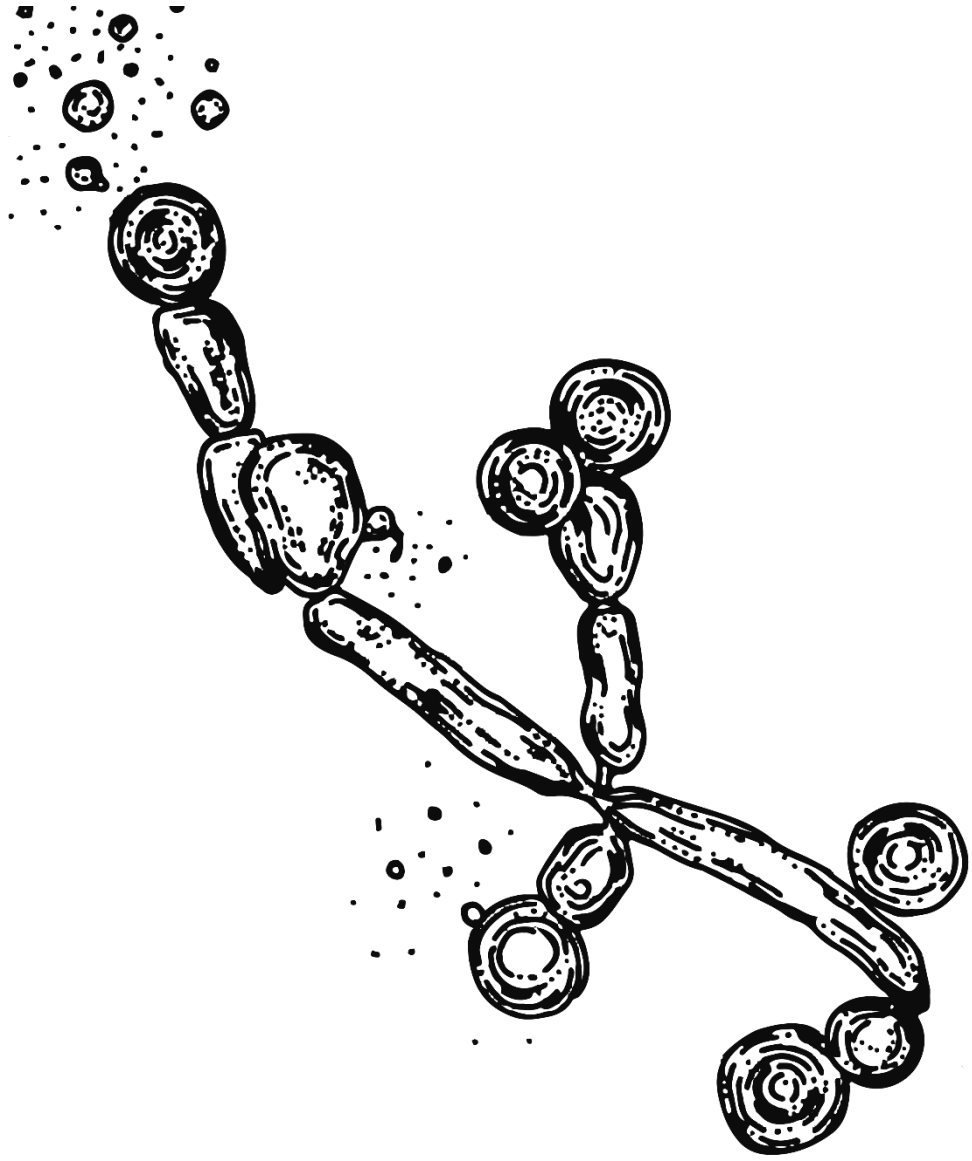
Eno1p was found to be an immunodominant antigen of *C. albicans*, being highly expressed in IC patients (Hernando *et al.*, 2007; Walsh *et al.*, 1991). Therefore, the detection of anti-enolase antibodies in patients, alone or in combination with other antibodies against important antigens such as Hsp90, has been suggested to possess a diagnostic value (He *et al.*, 2015; Li *et al.*, 2013; Pitarch *et al.*, 2008, 2014). In addition, Eno1p has been the target of vaccine development against *C. albicans*, as already noted, and several vaccines have been proved in animal models, although they only conferred partial protection against *C. albicans* challenge (Li *et al.*, 2011; Xin *et al.*, 2008).

Its surface location has been related to an ability to bound plasmin and plasminogen of host cells (Funk *et al.*, 2016). By binding soluble plasminogen, *C. albicans* facilitates its cleavage to plasmin, which degrades ECM proteins (Jong *et al.*, 2003). Thus, *C. albicans* enolase could take part in tissue invasion, and in fact, the binding of Eno1p to plasminogen increased the ability of the yeast to cross the blood-brain barrier *in vitro* (Jong *et al.*, 2003). In addition, Eno1p has also been shown to be responsible, at least in part, for *Candida* adhesion to gastrointestinal epithelium of mice (Silva *et al.*, 2014).

Regarding mitochondrial ketol-acid reductoisomerase protein (Ilv5, also known as acetolhydroxy acid isomeroreductase), which is regulated by *N*-acetylglucosamine, it is involved in the synthesis of branched chain amino acids: valine, leucine and isoleucine (Tyagi *et al.*, 2005). In *S. cerevisiae,* Ilv5 is necessary for the stability and inheritance of mitochondrial DNA (Bateman *et al.*, 2002). More recently, it was found to be associated to *C. albicans* cell wall, and its expression was decreased under oxidative stress conditions, suggesting that this protein can be a part of a group of CWPs that confers protection against oxidative burst of phagocytic cells (Ramírez-Quijas *et al.*, 2015).

Finally, ubiquinol cytochrome-c reductase (Qcr2) is a component of the cytochrome bc_1 complex (also known as Complex III), a central component of the cellular respiratory chain of mitochondria (Xiao *et al.*, 2014), which is highly conserved in other organisms too. Nonetheless, the

C. albicans and human Qcr2 sequences share only 25% of similarity. Despite its cytosolic location, it has been found, together with Ilv5, to be recognized by sera from patients with IC, suggesting that the presence of antibodies against these proteins could have a diagnostic value (Pitarch *et al.*, 2004). Qcr2 has also described to be upregulated in *C. albicans* upon contact with macrophages (Lorenz *et al.*, 2004). On the other hand, an antifungal that targets cytochrome bc_1 reductase has recently been described. Ilicicolin-H had *in vitro* activity against *Candida*, *Aspergillus* and *Cryptococcus*, and the activity of the mammalian enzyme was unaffected (Li & Calderone, 2017). The effectivity of this antifungal compound *in vivo* is currently under study.



OBJECTIVE

Candida albicans is the main fungus related to nosocomial infections, being the fourth causative agent of BSIs in US. The infections caused by this yeast are of special concern in the case of immunocompromised patients, which suffer the most severe forms of infections. Despite the use of antifungal agents, the mortality associated with invasive candidiasis is still very high; above 40%. One of the reasons behind this alarming high mortality is the delay in IC diagnosis and treatment. The current gold standard, blood cultures, take time and are insensitive, leading to a delay in the start of antifungal treatment, which has been related to a worse outcome.

On the other hand, the currently used antifungals are toxic for the host and, depending on the underlying disease of the patients, their administration can be risky. Moreover, resistances to those antifungal are emerging, and some of the non-*albicans Candida* species are intrinsically resistant to the most widely used drugs. There is an urgent need to find new diagnostic and therapeutic approaches against this fungus, especially taking into account the future expansion of the immunocompromised population.

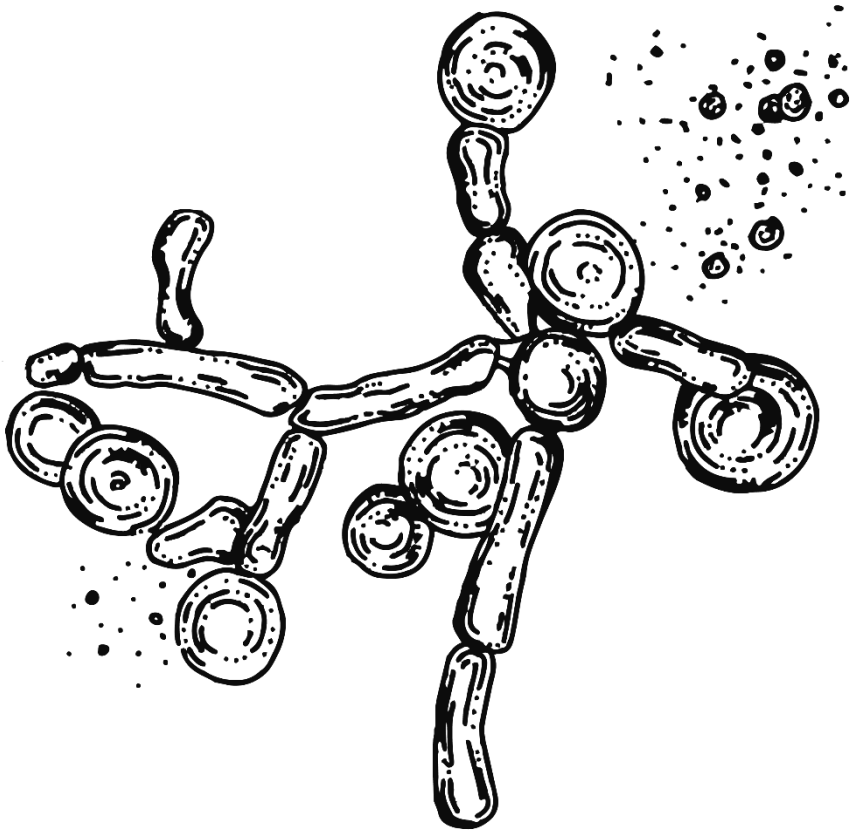
Other aspect of *C. albicans* infection that has not been deeply studied is its role in cancer induction and progression. Several authors have pointed out the relation between *Candida* and cancer in both animals and humans. In the case of our group, we demonstrated that mice infected with *C. albicans* and challenged with tumor cells presented more and bigger metastases in the liver than non-infected mice.

Therefore, the aim of our group is to study and find new diagnostic and/or therapeutic targets that could at the same time, if possible, be useful to inhibit the pro-carcinogen role of *Candida* infections. In order to do that, the fungal fraction inducing the highest tumor adhesion to liver endothelium was selected and the proteins belonging to this fraction were identified.

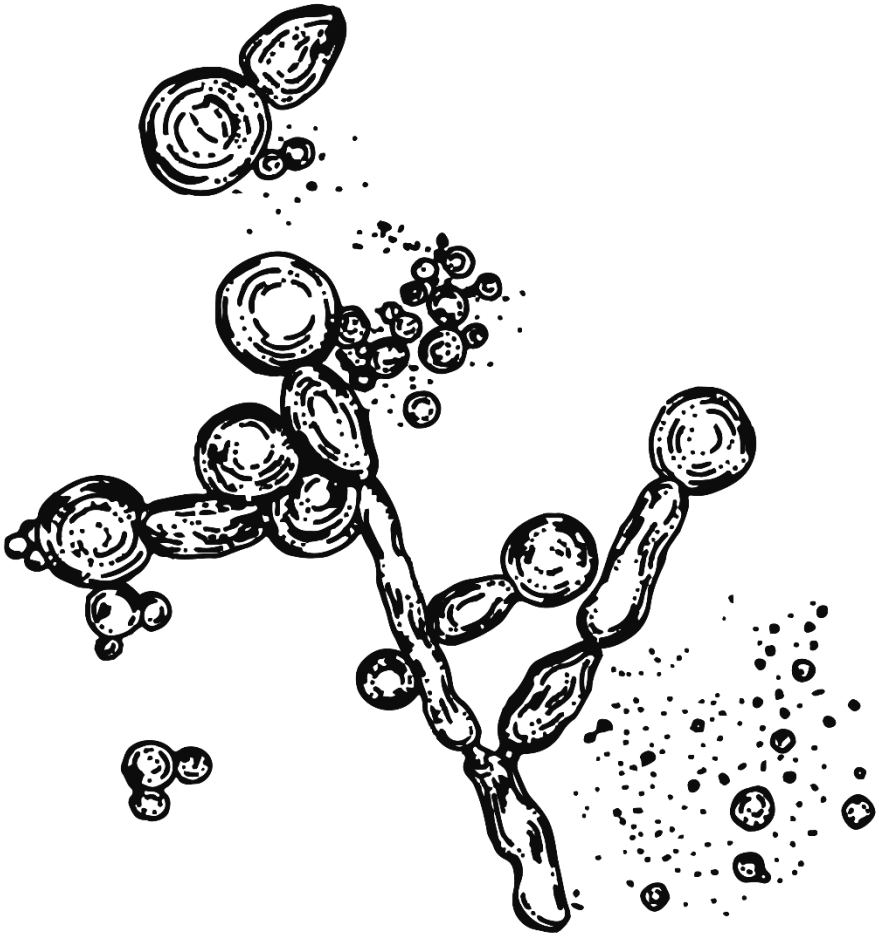
The main objective of this thesis was to study the pro-tumoral role of five of the identified proteins, namely alcohol dehydrogenase (Adh1), enolase (Eno1), mitochondrial ketol-acid reducto-isomerase (Ilv5), killer toxin-resistance protein (Kre9) and ubiquinol cytochrome-c reductase (Qcr2), and their possible role as new diagnostic/therapeutic targets. To do that, three secondary objectives were stablished.

- To study individually the effect of five proteins, Adh1, Eno1, Ilv5, Kre9 and Qcr2, on tumor cell adhesion to liver endothelial cells and on pro-inflammatory cytokines production.

- To analyze the therapeutic effect of Kre9 peptides against different strains of *C. albicans in vitro.*
- To research into the therapeutic and diagnostic value of the monoclonal antibodies Ca9, and Ca37a and C37b, developed against the two proteins of *C. albicans* showing the greatest pro-tumoral effect, Kre9 and Adh1, respectively.



MATERIAL AND METHODS



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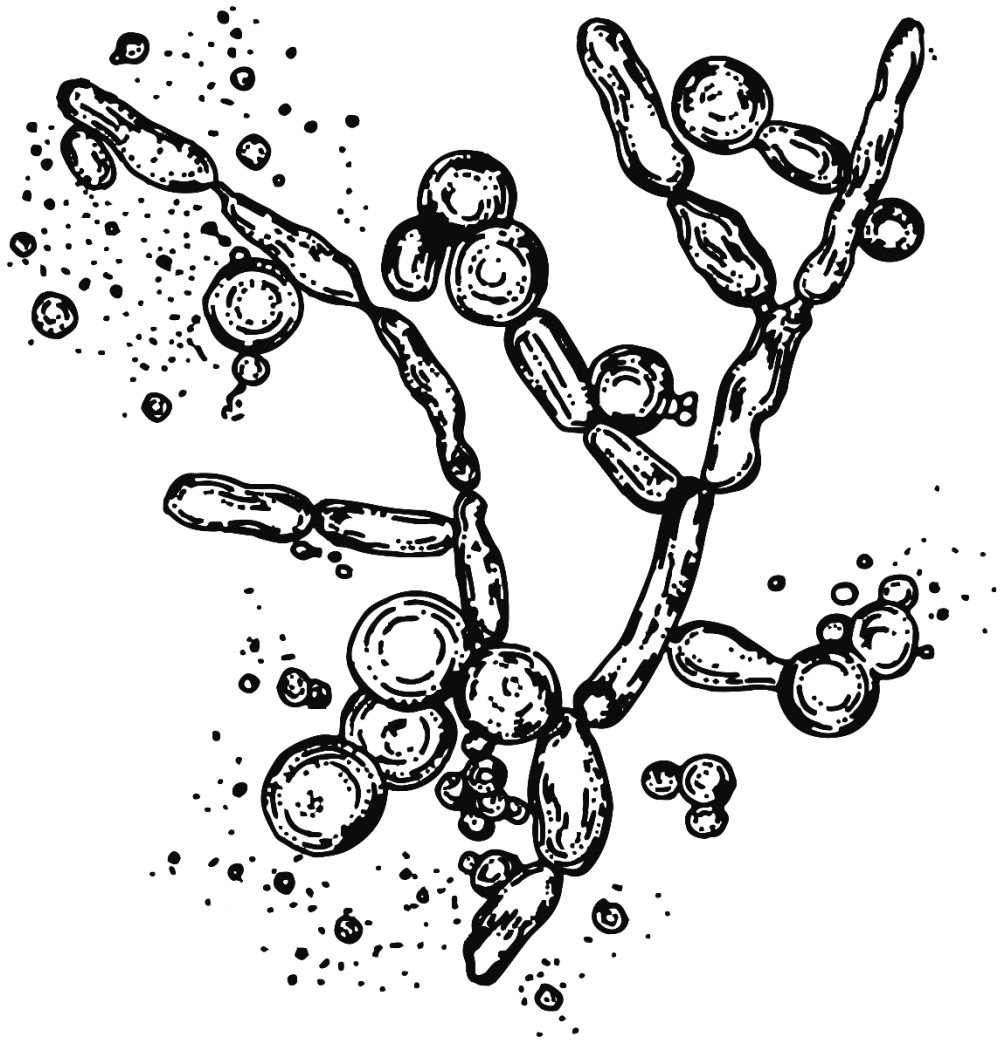

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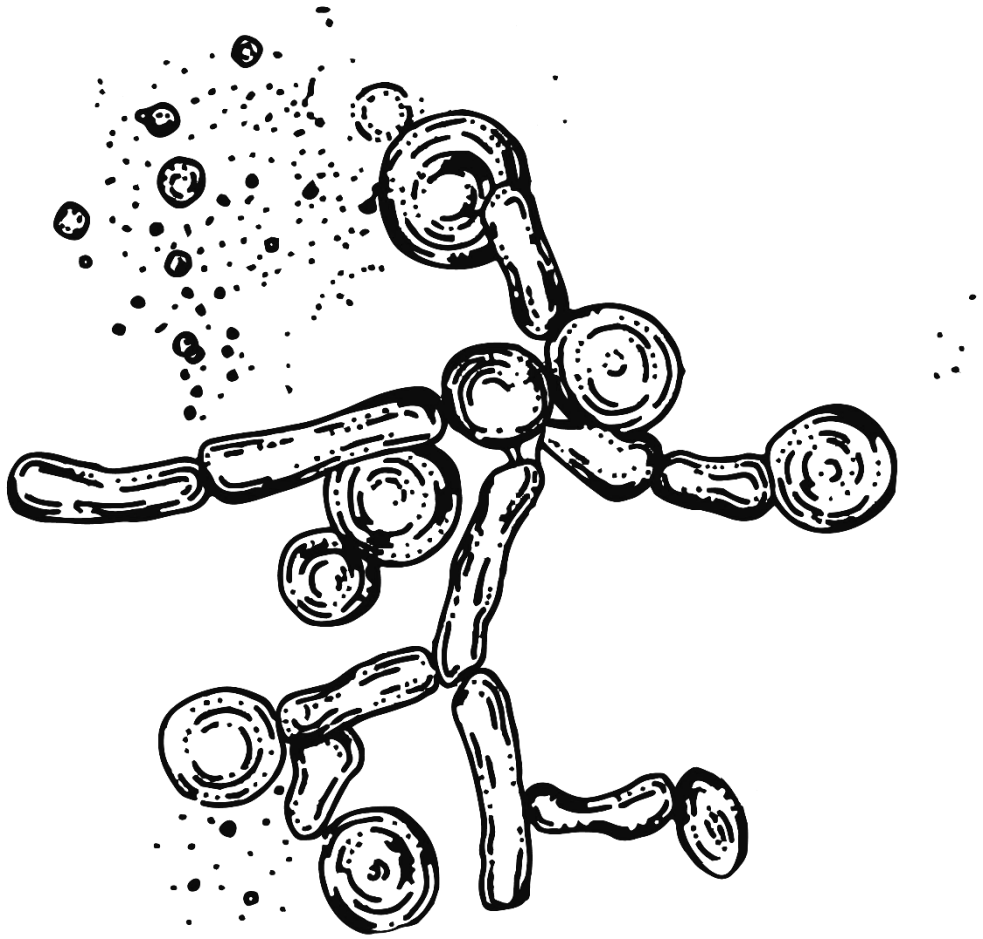

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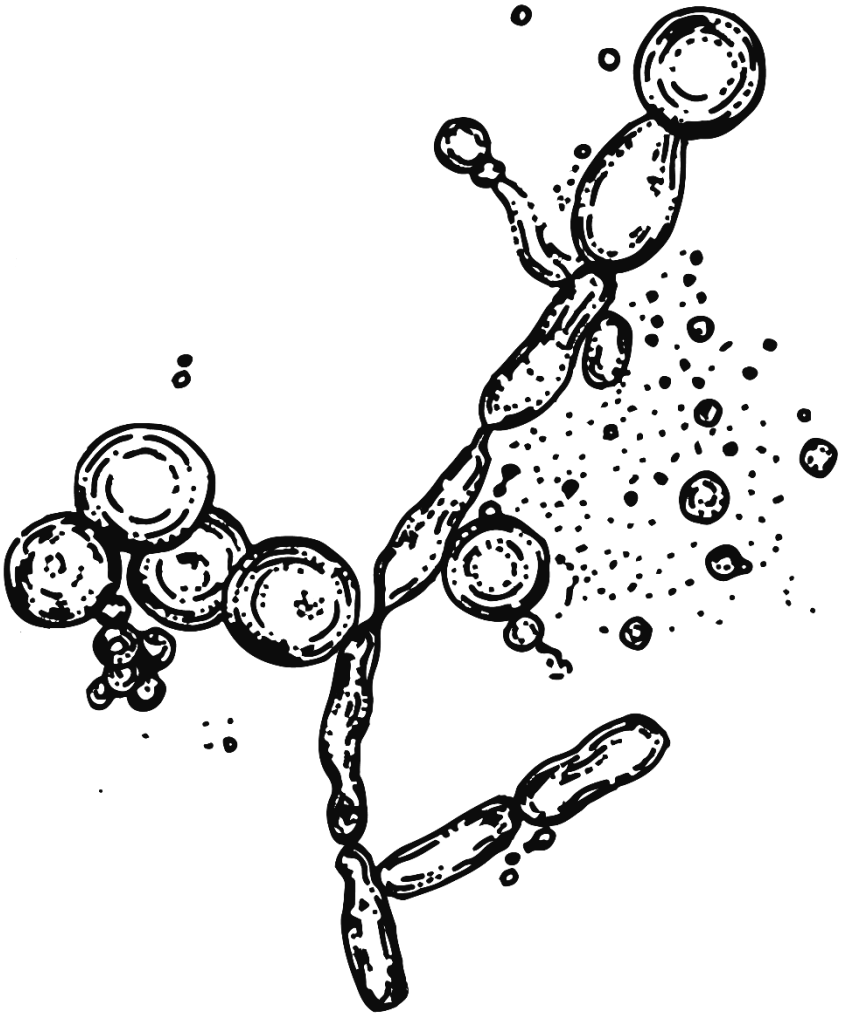

SUPPLEMENTARY MATERIAL

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GCTCAAGGTATTAAACAAGCCAAATCTGTTGCTGAATCAGTTTCTTCTTCTGCTTTATCTGAAGCTGTCAAAGCTG
CTGAATTATCTGTTGCATTACAATCAACTGTCGACTCCCCATTAAACGTCAAAGTTGTCGCTGAAGAAGCCCCAA
TTTCTAAATTCAACTATGTTGCTGTTGGTGATCTTGATGTGTTACCATATGCTGATGAATTGTAAAGAGAAATTGG
AATGATGATATAAACCGAAAAAAAAAAAAGCAAAAAACAGAAAGTTTGAAATCAATTTTTTTAACTATAAACCATC
GATATAAATATTCCCTTTAGCAGTTTATTTAAGTCATATGTGAATAAAATAAACATTCATGTACGTAATAGATTACT

Supplementary material 1. Sequence of the three genes cloned in *Escherichia coli* model. (A) *ADH1* gene, (B) *ILV5* gene and (C) *QCR2* gene. The translated sequence are marked in bold with the primers underlined.

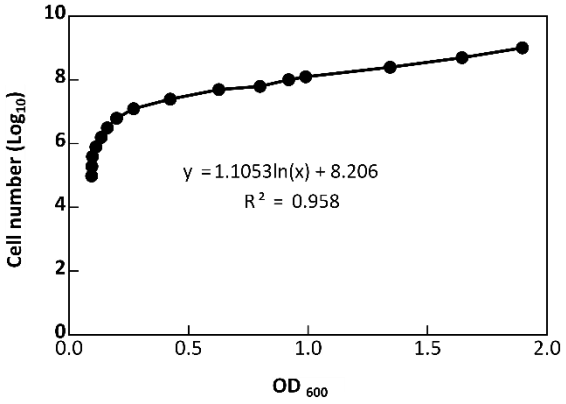
ATCAAACATTCTAAATTAACTATCTTAATAATTTAGGGAATAGTGTATTATTTGCTTTAAAGATATCGGATACTTTTT
TTTCCTTTTGAGTTTTGTTTTGTTTTGTTTTATTTAGATTAGTTTCTATTTTTAATTAATATTCTACATTCGTTATTGAC
GACTACAACATATCAACGACCTAGCACATCCACCCACCAAGATAACACAACTACA**ATCTATCAGACACATCTAGCA**
AAATGAGACAATTTCAAATCATATTAATTTCCCTTGTTGTTTCCATAATAAGATGTGTTGTTGCAGATGTTGACAT
CACATCACCAAAGAGTGGAGAAACTTTTTCTGGTAGTTCTGGATCAGCAAGTATCAAGATTACCTGGGATGATT
CAGACGATTCAGACTCACCGAAATCTTTGGATAATGCCAAAGGGTACACAATTTCTTTATGTACTGGACCTACTT
CAGATGGGGATATCCAGTGTTTGGATCCATTAGTCAAGAACGAAGCTATTTCAGGTAAATCTAAAACAGTTTCC
ATTGCCCAGAACTCAGTACCTAATGGTTATTACTATTTCCAAATTTACGTTACTTTCACTAATGGAGGTACCACTA
TTCATTATTCACCACGTTTCAAATTGACTGGTATGTCTGGTCCAACTGCCACTTTGGATGTCACCGAAACAGGATC
AGTGCCAGCGGATCAAGCTTCAGGATTTGATACTGCAACTACTGCTGACTCCAAATCTTTCACAGTTCCATATAC
CCTACAAACAGGGAAGACTAGATACGCACCAATGCAAATGCAACCAGGTACCAAAGTGACTGCTACAACCTGG
AGTATGAAGTTCCCAACTAGTGCTGTTACTTACTACTCAACAAAGGCTGGCACACCAAATGTGGCCTCTACTATT
ACCCCAGGTTGGAGTTATACTGCTGAATCTGCCGTTAACTATGCTAGTGTTGCGCCATATCCAACATACTGGTAT
CCTGCCAGTGAACGAGTGAGTAAGGCTACAATTAGTGCTGCTACAAAGAGAAGAAGATGGTTGGAT**TGA**ATGA
GGGAAACGGTGCTTATAACTTAGATAATGCTAACATTGGATTTCATTTTTCATTTTTTTTTGTTTGGTTTAGTTTGTT
TATTAGCTTGTTAAACATGTAATACTGTGTTTGGTTTGTATAAGTTGATTGTATTTTTTTTTTTTTTGGCAAAGTAGA

Supplementary material 2. Sequence of the *KRE9* gene cloned in *Pichia pastoris* model. The translated sequence is marked in bold with the primers underlined.

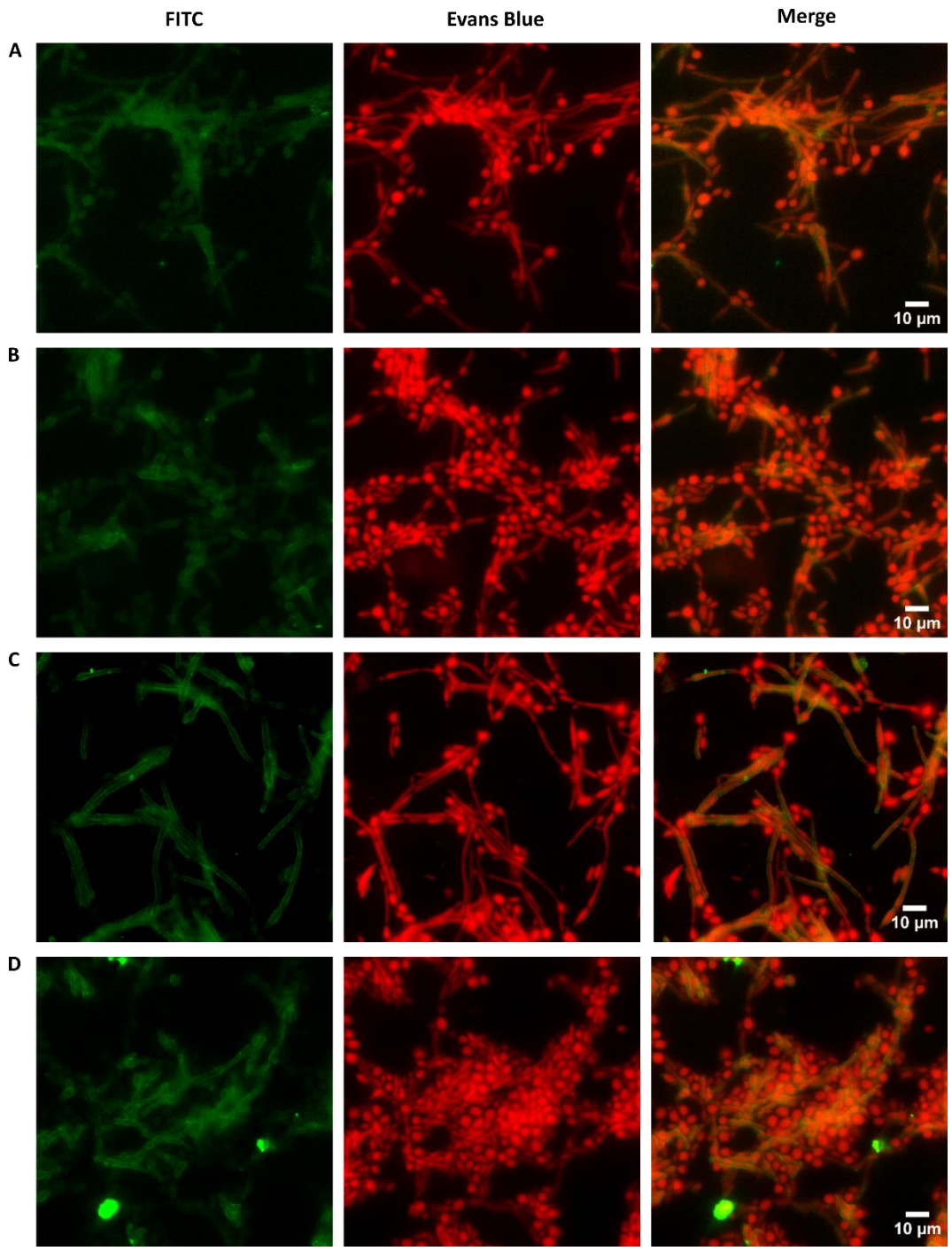
A

Cell number	OD_{600}
1.00E+09	1.90
5.00E+08	1.64
2.50E+08	1.34
1.25E+08	0.99
1.00E+08	0.92
6.25E+07	0.80
5.00E+07	0.63
2.50E+07	0.42
1.25E+07	0.27
6.25E+06	0.20
3.13E+06	0.16
1.56E+06	0.13
7.81E+05	0.11
3.91E+05	0.10
1.95E+05	0.10
9.77E+04	0.09

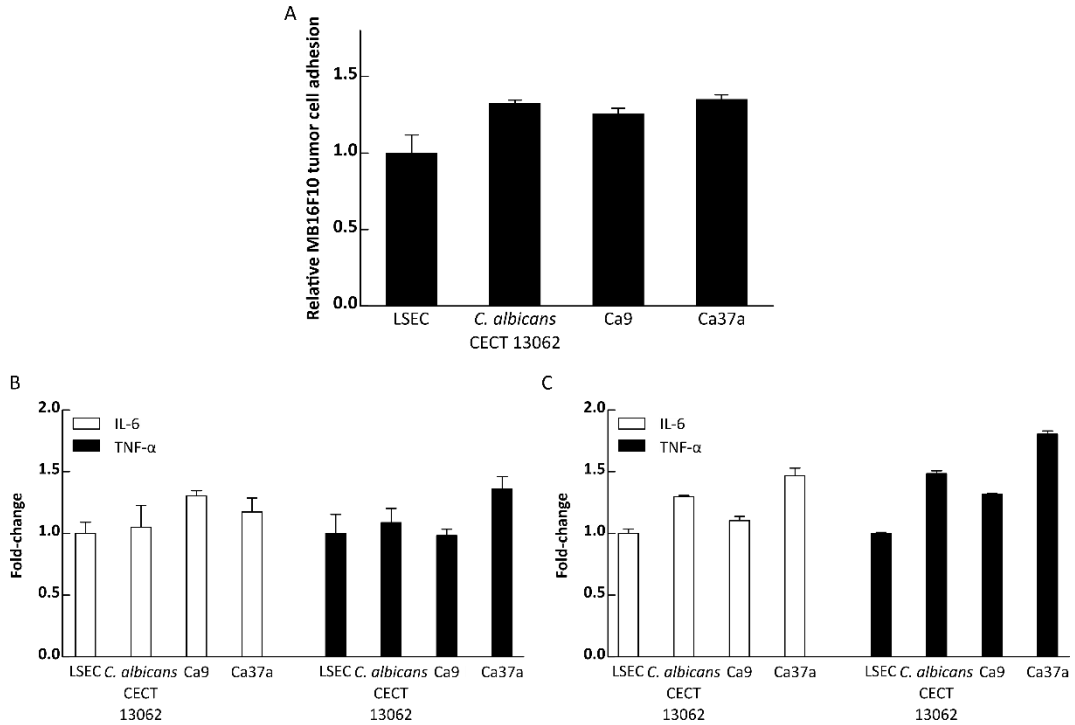
B



Supplementary material 3. Correlation of OD_{600} absorbance values and *Candida albicans* yeast cell numbers.
(A) Table presenting the cell number of yeast and the corresponding absorbance values measured at 600 nm.
(B) Correlation graphic obtained using the data from the table.



Supplementary material 4. Indirect immunofluorescence of different *Candida albicans* strains. (A) CECT 13062, (B) UPV 15-170, (C) UPV 15-171 and (D) UPV15-172. Anti-Kre9 antibody's recognition is visualized in green (FITC) and whole cells are visualized in red (Evans Blue). Merge image shows the confluence of both fluorescence stains.



Supplementary material 5. LSEC stimulation in presence of two different densities of *Candida albicans* alone or treated with 10 µg/ml of Ca9 or 45 µg/ml Ca37a mAbs. (A) Adhesion of MB16F10 tumor cell to LSECs stimulated with 5 x 10^5 cell/ml. (B) Cytokine levels of LSEC stimulated with 10^5 cell/ml. (C) Cytokine levels of LSECs stimulated with 5 x 10^5 cell/ml. MB16F10 cell adhesion was normalized to the adhesion observed in the unstimulated LSEC control. Cytokine data are presented as fold-change relative to the LSEC control.